# Mechanisms underlying innate immunesenescence

by

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# Abstract

Although it is evident that physiological ageing is accompanied by marked alterations in the function of innate immune cells, little is known regarding the underlying mechanism(s). Furthermore, the effect of age on many novel aspects of innate immunity is unknown. This thesis has identified the mechanism(s) behind the well-documented age-related decline in natural killer (NK) cytotoxicity (NKCC) and demonstrated for the first time that human ageing is accompanied by a significant reduction in the generation of neutrophil extracellular traps (NETs).

Following target cell recognition, it was found that NK cells from older adults secreted into the immunological synapse (IS) significantly lower levels of perforin, a pore-forming protein that plays a non-redundant role in NKCC. This impairment led to reduced perforin binding to the target cell surface, an event that correlated strongly with NKCC. Underlying the reduction in perforin secretion was defective polarisation of lytic granules to the IS, which was associated with delayed activation of extracellular signal-regulated kinase 1/2.

Whilst no age-related difference was observed in NET production triggered by phorbol 12-myristate 13-acetate (PMA), neutrophils from older adults generated significantly fewer NETs when challenged with interleukin-8 or lipopolysaccharide, which was accompanied by a reduction in reactive oxygen species generation. As PMA activates cells independent of membrane receptors, aberrant intracellular signalling proximal to the neutrophil membrane may underlie the age-related impairment in NET production.

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# For Mom and Dad

Thank you

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## Abbreviations

| | |
|---|---|
| α-GalCer | α–galactosylceramide |
| ADCC | Antibody dependent cell cytotoxicity |
| Ag | Antigen |
| AFU | Arbitrary fluorescence units |
| ANOVA | Analysis of variance |
| ASMase | Acid sphingomyelinase |
| ATCC | American type culture collection |
| AUC | Area under the curve |
| BM | Bone marrow |
| BSA | Bovine serum albumin |
| C5a | Complement 5a |
| *C.albicans* | *Candida albicans* |
| CD | Cluster of differentiation |
| Cdc42 | Cell division control protein 42 |
| cDCs | Conventional dendritic cells |
| CGD | Chronic granulomatous disease |
| CIP4 | Cdc42-interacting protein |
| CM | Complete media |
| CMA | Concanamycin A |
| CMV | Cytomegalovirus |
| CRP | C reactive protein |
| cSMAC | Central supramolecular activation cluster |

| | |
|---|---|
| DAG | Diacylglycerol |
| DAP10 | DNAX-activation protein-10 |
| DAP12 | DNAX-activation protein-12 |
| DCs | Dendritic cells |
| DLN | Draining lymph node |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| DNAM | DNAX accessory molecule |
| E:T | Effector:target cell ratio |
| EBV | Epstein Barr virus |
| *E.coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK1/2 | Extracellular signal-regulated kinase 1/2 |
| F-actin | Filamentous actin |
| FasL | Fas ligand |
| FCS | Fetal calf serum |
| FDC | Follicular dendritic cell |
| FITC | Fluorescein Isothiocyanate |
| fMLP | Formyl-methionyl-leucyl-phenylalanine |
| FS/SS | Forward scatter/Sideward scatter |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |

| | |
|---|---|
| GPS | Glutamine, penicillin, streptomycin |
| $H_2O_2$ | Hydrogen peroxide |
| $H_2SO_4$ | Sulphuric acid |
| HAP | Healthy ageing phenotype |
| HBSS | Hanks balanced salt solution |
| HI | Heat inactivated |
| HLA | Human leukocyte antigen |
| HMG-CoA | 3-hydroxy 3-methylglutaryl coenzyme A |
| HRP | Horseradish peroxidase |
| iDCs | Immature dendritic cells |
| IFN-α | Interferon alpha |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobulin |
| IL-1β | Interleukin 1 beta |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL | Interleukin |
| iNKT | Invariant natural killer T cells |
| $IP_3$ | Inositol 1,4,5-triphosphate |
| IRP | Immune risk profile |
| IS | Immunological synapse |
| ITAM | Immunoreceptor tyrosine-based activatory motif |
| ITIM | Immunoreceptor tyrosine-based inhibitory motif |

| | |
|---|---|
| JNK | c-Jun N-terminal kinase |
| KIR | Killer cell immunoglobulin-like receptor |
| KLRG1 | Killer cell lectin-like receptor subfamily G member 1 |
| LAK | Lymphokine activated killer |
| LAMP-1 | Lysosomal-associated membrane protein 1 |
| LAT | Linker for activation of T cells |
| LCs | Langerhans cells |
| LE | Life expectancy |
| LPS | Lipopolysaccharide |
| MACS | Magnetic assisted cell sorting |
| MAPKs | Mitogen-activated protein kinases |
| MCMV | Murine cytomegalovirus |
| mDC | Myeloid dendritic cell |
| MedFI | Median fluorescent intensity |
| MFI | Mean fluorescent intensity |
| MHC I | Major histocompatability complex class I |
| MHC II | Major histocompatability complex class II |
| MICA | MHC-class-I-chain-related molecule A |
| MICB | MHC-class-I-chain-related molecule B |
| MIP1-α | Macrophage Inflammatory Protein 1 alpha |
| MIP1-β | Macrophage Inflammatory Protein 1 beta |
| MNase | Micrococcal nuclease |
| MPO | Myeloperoxidase |

| | |
|---|---|
| MTs | Microtubules |
| MTOC | Microtubule organising centre |
| NaCl | Sodium Chloride |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCR | Natural cytotoxicity receptor |
| NE | Neutrophil elastase |
| NETs | Neutrophil extracellular traps |
| NK | Natural killer |
| NKCC | Natural killer cell cytotoxicity |
| NKIS | Natural killer cell immunological synapse |
| $O_2^{\cdot -}$ | Superoxide anion |
| PAD4 | Peptidylarginine deiminase 4 |
| PARP | Poly ADPribose polymerase |
| PBLs | Peripheral blood lymphocytes |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PcB | Pacific blue |
| pDCs | Plasmacytoid dendritic cells |
| PE | Phycoerythrin |
| PI-3,4-$P_2$ | Phosphatidylinositol-3,4-bisphosphate |
| PI-3,4,5-$P_3$ | Phosphatidylinositol-3,4,5-triphosphate |
| PI3K | Phosphoinositide 3-kinase |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |

| | |
|---|---|
| PKC | Protein Kinase C |
| PLCγ | Phospholipase C gamma |
| PMA | Phorbol 12-Myristate 13-Acetate |
| RANTES | Regulated on activation normal T cells expressed and secreted |
| RLU | Relative light units |
| ROS | Reactive oxygen species |
| rpm | revolutions per minute |
| RPMI | Roswell Park Memorial Institute |
| RT | Room temperature |
| *S.aureus* | *Staphylococcus aureus* |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SH2 | Src homology 2 |
| SHIP-1 | SH2-domain-containing inositol polyphosphate 5-phosphatase |
| SHM | Somatic hypermutation |
| SHP1/2 | Src homology region 2-containing protein tyrosine phosphatase 1/2 |
| SLE | Systemic lupus erythematosus |
| SLP-76 | SH2 domain-containing leukocyte protein of 76 kDa |
| SLO | Secondary lymphoid organ |
| TCR | T-cell receptor |
| TLR | Toll-like receptor |
| TMB | 3,3',5,5'-Teramethylbenzidine |

| | |
|---|---|
| TNF-α | Tumor necrosis factor alpha |
| TRAIL | Tumor necrosis factor related apoptotic-inducing ligand |
| Tregs | T regulatory cells |
| v/v | volume/volume |
| VZV | Varicella zoster virus |
| w/v | weight/volume |
| Wasp | Wiskott-Aldrich syndrome protein |
| WIP | WASp-interacting protein |
| ZAP70 | Zeta-chain-associated protein kinase 70 |

# 1.0 Introduction

## 1.1 Population ageing and the "healthy ageing phenotype"

Recent advancements in medical care and public health policies, as well as improvements in socio-economic status mean we are now living longer than ever before. Couple this increase in lifespan to the decline in birth rate that has occurred over the last fifty years [1], and it is not surprising that we are in the midst of a "demographic transition".

Based on current mortality rates, it is estimated that the proportion of individuals aged sixty years and over, who accounted for eight and ten percent of the global population in 1950 and 2000 respectively, will constitute twenty-one percent of the total world's population by 2050, a figure, which according to current projections will equate to approximately two billion people [2]. Should this forecast become a reality then in 2050 older adults will outnumber individuals aged 15 years and under for the first time in history [2]. This change in population demographics will be most evident in developed countries. As early as 2015, subjects aged 65 years and over will account for approximately fourteen percent of the population residing in Canada, Australia and Japan [3], whilst by 2030, twenty percent of the total population of the United States will fall into this age category [3]. Closer to home, recent estimates from the Office for National Statistics suggest that in 2033, twenty-three percent of the UK population will be represented by individuals aged 65 years and over, exceeding those aged ≤16 years by five percent [4].

The fact that we are living longer can on one hand be perceived as positive news, a testament to the improvements made in amongst other things hygiene, medicine and living conditions. However, these "extra years" can only be considered beneficial if they are experienced in relatively good health. In a recent UK government report, data was presented which showed

that whilst life expectancy (LE) over the last twenty years had increased at a fast rate, "healthy life expectancy" had not kept pace [5]. Of concern is the fact that the difference between these two measures widened during this period. Earlier detection and improved management of chronic diseases, together with health becoming a more sensitive issue are two possible reasons why people are now living longer whilst experiencing ill-health [5].

Healthy ageing is a concept that encompasses a broad range of factors, and can be viewed from a physiological, psychological, medical or sociological perspective. Thus, what aspects of life are considered integral for healthy ageing varies significantly between the different fields of gerontology. For instance, whilst psychologists and sociologists view mental stability, social interaction and autonomy as key components of healthy ageing, physiologists consider physical conditioning, nutritional status and lifestyle to be more important [6]. In contrast, the medical profession views the absence of and ability to overcome illness as the defining feature of healthy ageing [6].

Given the differences that exist in what people consider to be the main attributes of healthy ageing, establishing a universally accepted definition for this phenomenon has proven difficult, and hampered the attempts of gerontologists to fully understand how differences in LE and health can arise. Indeed, in a recent article reviewing the findings of studies in which twenty-nine different definitions of healthy ageing had been used, Depp and co-workers noted the percentage of subjects who considered themselves to have aged healthily ranged from 0.4% to 95%, variation that was attributed in part to the lack of a single and concise definition of healthy/successful ageing [7]. In an attempt to tackle this issue, specialists within the ageing field have recently devised the "healthy ageing phenotype" (HAP). Defined as *"the condition of being alive, while having highly preserved functioning metabolic, hormonal and neuro-endocrine control systems at the organ, tissue and molecular levels"*

and further characterised by *"biological resilience to environmental stressors and the maintenance of cognitive and physical function"* [8], the HAP brings together concepts from all fields of ageing research, and currently represents the most comprehensive definition of healthy ageing.

### 1.2 Ageing and infection

Rather than developing strategies that will further extend human lifespan, the current goal of ageing research is to improve the health status of older adults by increasing the duration of healthy ageing. To achieve this aim, a greater understanding of what factors drive unhealthy ageing and how they arise is required.

Infectious diseases are a common occurrence in the elderly population. Compared to younger adults, individuals aged 65 years and over report increased incidences of influenza infection [9], community-acquired pneumonia [10;11], and urinary tract infections [12;13], whilst age is a major risk factor for the development of nosocomial infections [12;14]. As well as being more frequent, infections in the elderly are more severe. For example, mortality arising from community-acquired pneumonia has been reported to be as high as thirty percent in hospitalised older adults [10;15], whilst ninety percent of influenza-related deaths occur in people aged ≥65 years [16]. Strikingly, in the United States, both these infectious diseases rank in the top ten leading causes of death for people aged 65 years and over [17], highlighting the serious clinical consequences that can arise from infection in older adults. However, the severity of infection should not be judged solely in the context of mortality, as elderly individuals who survive infectious episodes experience greater rates of morbidity. For instance, Fein et al reported the incidence of hospital admissions for community-acquired pneumonia rose from <2/1000 for people aged ≤60 years to 12/1000 in subjects aged ≥65 years [18]. Similarly, hospitalisation rates and the number of days spent in hospital are

markedly higher for individuals aged 65 years and over who succumb to influenza infection [19;20].

In addition to reporting increased incidences of infection caused by novel pathogens, older adults experience more frequent episodes of latent virus reactivation. In a randomised population based retrospective study, Horsburgh and colleagues found age to be the strongest risk factor for the reactivation of latent tuberculosis amongst human-immunodeficiency virus negative subjects [21]. Similarly, outbreaks of shingles, caused by reactivation of varicella-zoster virus (VZV) occur more frequently with increasing age [22;23].

### 1.3 Immunesenescence

Multiple factors are thought to underlie the increased incidence and severity of infection in older adults. These include but are not limited to: malnutrition, social environment, use of immunosuppressive medicines, co-morbidities and age-associated alterations in immune function [24]. Collectively referred to as immunesenescence, age-related changes in host immunity are observed as early as one year post birth [25], and include alterations in the architecture and mechanics of innate barriers, as well as changes in leukocyte number, phenotype and function [26-28]. From an evolutionary standpoint, these changes were unlikely to have been of significance when average life expectancy was short (~40 years). However, the dramatic increase that has occurred in human life span now means the immune system has to protect individuals for significantly longer periods of time. Consequently, immunesenescence is considered by many to be the major factor that predisposes older adults to infection-related morbidity and mortality.

Given the interest that exists in immunesenescence, the field of immune gerontology is vast, and as a result conflicting observations are frequently reported. Contributing to these

differences is variation in study protocol. As well as differing in subject inclusion criteria, studies often vary in methodological approach, particularly with respect to cell status (e.g. primed Vs. unprimed), culture conditions (e.g. cells may be cultured in presence or absence of exogenous stimuli), incubation times and assay technique. Thus, when comparing the findings of immunogerontological studies, it is important to consider the effect that differences in experimental set-up may have had on their outcome.

### 1.3.1 Adaptive immunesenescence

Comprised of highly specialised antigen specific B and T lymphocytes, the adaptive arm of the immune system performs two important roles in host protection. In addition to assisting in the elimination of pathogen-infected cells, adaptive immunity confers lifelong protection against re-infection with the same antigen through the generation of immunological memory. Thus, the more frequent episodes of latent virus reactivation reported by elderly subjects [21-23], coupled to the age-associated decline in vaccine efficacy [29;30] suggests that the protective nature of adaptive immunity wanes with age.

#### 1.3.1.1 Age-associated changes in the composition and phenotype of the circulating lymphocyte pool

**T lymphocytes**

One of the most notable features of adaptive immunesenescence is involution of the thymus gland. Characterised by expansion of the thymic perivascular space, and the gradual replacement of functional epithelial cells with fat and fibrous tissue [31;32], thymic involution begins as early as one year post birth, and is complete by around the seventh decade of life, by which time thymopoietic space constitutes less than 10% of the thymus [25;31].

As the thymus is the primary site of T cell development, its involution leads to a marked decline in naïve T cell output [33]. However, in spite of a fall in naïve T cell numbers, the overall size of the peripheral T cell pool is maintained with age. This is because the age-related reduction in the generation of naïve T cells is offset by expansion of the memory T cell pool. Driving this inflation is homeostatic proliferation of pre-existing memory T cells and infection with latent viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), whose persistent stimulation of the immune system leads to the generation and accumulation of antigen specific memory T cell clones [34-36]. This combination of reduced thymic output and an increased number of antigen experienced T cells has a profound impact upon the diversity of the circulating T cell receptor (TCR) repertoire, which undergoes a marked age-associated contraction [37] that is thought to underlie the inadequate immune response elicited by older adults towards novel antigens [38;39].

Physiological ageing is accompanied by distinct changes in T cell phenotype. For instance, when compared to younger subjects, a greater proportion of circulating T cells in older adults exhibit a surface phenotype that is characterised by the absence of the co-stimulatory molecules CD27 and CD28, and increased expression of the terminally sulphated glycan carbohydrate CD57 and the inhibitory receptor Killer cell lectin-like receptor subfamily G member 1 (KLRG1) [40-42]. This phenotype, which is observed primarily on $CD8^+$ T cells, is commonly associated with T cells that have undergone terminal differentiation, and their age-related accumulation is thought to be borne in part from the continued efforts of the immune system to control persistent viral infections such as CMV. T cells from older adults also express receptors more commonly observed on the surface of natural killer (NK) cells. These include the activatory receptors NKG2D and CD16, as well inhibitory members of the killer cell immunoglobulin like receptor (KIR) and C-type lectin family [41;42]. Expression

of these “NK cell associated receptors” has been proposed as a means by which aged T cells not only compensate for their reduced TCR diversity but also their impaired functionality [43].

**B lymphocytes**

B cell generation by the bone marrow (BM) wanes with age. Underlying this reduced productivity are alterations in the BM microenvironment and changes in the number and differentiation potential of B cell precursors. For instance, ageing is associated with impaired release of interleukin (IL)-7 by BM resident stromal cells [44] and a reduced commitment of haematopoietic stem cells to lymphopoiesis [45], which together lead to a decline in the number of BM residing B cell progenitors [46;47].

The effect of age on the size of the peripheral B cell pool is unclear, with some groups suggesting it is reduced with age [48-50], and others reporting it to be unchanged despite the aforementioned reduction in B cell lymphopoiesis [51], a paradox that is explained by an apparent increase in B cell longevity with age [52;53]. Whether the composition of the circulating B cell pool is altered with age is also an area of debate. For example, studies can be found that have shown the percentage and/or numbers of naïve [48;54;55], IgM memory [48;56], switched memory [48;55] and late memory [48] B cells are increased [48;55], decreased [48;54-56] or unchanged [48] with age.

### 1.3.1.2 Age-associated changes in lymphocyte function

**T lymphocytes**

Several studies have demonstrated that when compared to T cells of young controls, those obtained from aged subjects exhibit reduced proliferation [57-60], cytotoxicity [60-62] and cytokine production [57;62;63] following *in vitro* or *in vivo* stimulation. This decrease in

T cell function has been attributed in part to: (i) the aforementioned changes in the composition of the circulating T cell pool, (ii) functional defects in aged dendritic cells [60;64] and (iii) aberrant intracellular signalling. Regarding this latter point, Larbi and co-workers showed the recruitment of lipid rafts, areas of the cell membrane that serve as platforms for the localisation and interaction of signalling molecules, to an engaged TCR was impaired in T cells from aged donors [65]. This defect, which was linked to an age-related decline in membrane fluidity, may explain in part why early signalling events critical for T cell activation, such as calcium mobilisation, phosphatidylinositol metabolism and the activation of proximal kinases are impaired in stimulated T cells from old donors [59;66;67].

It is currently unclear as to whether the function of T regulatory cells (Tregs), a T cell subset that promotes peripheral self-tolerance by suppressing the activity, proliferation and cytokine production of effector T cells, is altered with age. For instance, results from *in vitro* studies that have employed murine [68;69] or human [70-72] Tregs suggest the suppressive nature of this T cell subset is increased [68;70], decreased [69;72] or maintained [69-71] with age. Similar inconsistencies have also been reported in the small number of studies that have examined age-associated changes in Treg function *in vivo* [68;69;73;74]. For example, whilst Sun and colleagues demonstrated a reduced ability of aged Tregs to regulate IL-$17^+$ T cells in a murine model of chronic autoimmune colitis [74], a defect they proposed may contribute to the increased incidence of autoimmunity in the elderly, Lages et al [68] reported an age-related increase in Treg activity in a model of *Leishmania major* infection, which they proposed may contribute some part to the increased reactivation rates of latent viruses with age.

**B lymphocytes**

Compared to that elicited by younger adults, the vaccination response of elderly individuals is characterised by decreased antibody concentrations, delayed peak antibody titres and lower seroprotection [29;30;75], features that suggest the humoral immune response wanes with age. Indeed, studies employing murine and human B lymphocytes have shown the proliferative nature of these cells, as well as their ability to differentiate into antibody secreting plasma cells is reduced with age [56;76;77]. Furthermore, the antibodies produced by aged B cells are weaker and of lower affinity when compared to those of younger subjects [78;79].

Critical events in the generation of a robust humoral immune response include: (i) isotype switching and somatic hypermutation (SHM), which are essential for the production of high affinity class-switched antibodies, and (ii) B cell interaction with T lymphocytes and follicular dendritic cells (FDCs), which is important for antigen recognition as well as B cell proliferation, selection and survival. With age, aberrations in both these pathways have been reported, suggesting the weak humoral immune response of elderly individuals is the result of both B cell intrinsic and extrinsic defects. Indeed, the levels of activation-induced cytidine deaminase and the transcription factor E47, which are both essential for the induction of SHM are markedly lower in stimulated B cells from aged individuals [55;80;81], whilst FDCs from aged mice fail to trap and present immune complexes as efficiently as those from young mice [82;83]. In terms of T cell dysfunction, Eaton and colleagues found that transferring aged $CD4^+$ T cells into young mice led to reduced levels of antigen-specific IgG antibodies post vaccination, whereas young $CD4^+$ T cells transferred into aged mice fully supported B cell expansion and IgG production [83]. An age-related decrease in the expression of CD40 ligand on the surface of activated T cells, as well as the aforementioned

accumulation of $CD57^+$ and $CD28^-$ T cells in old individuals are two possible explanations for this reduced helper activity of aged T cells [83-86].

### 1.3.2 Innate immunesenescence

The innate immune system is the body's first line of defence against infection. Consisting of anatomical barriers and a humoral and cellular arm, the innate immune response provides immediate non-specific protection against invading pathogens.

Only a small number of groups have examined the effect of age on barrier function, with the majority reporting its protective capacity to be reduced with age. Compared to younger adults, old individuals exhibit reduced nasal mucociliary clearance, and struggle to expel inhaled particles trapped within small airways of the lungs, defects that have been attributed to age-associated changes in the structure and beat frequency of the ciliated cells that line the respiratory tract [87-90].

The humoral arm of innate immunity consists of a wide array of fluid-phase proteins that belong to different molecular families, which include pentraxins, collectins, complement and ficolins [91]. Of these, it is the complement system that has received the most attention from immune gerontologists. However, the impact of age on this arm of innate immune defence is unclear. For instance, with the exception of C1q, whose serum levels have consistently been reported to increase with age [92;93], the circulating levels of other key complement proteins such as C3 and C4 have reported to be either increased [92;94] or unaltered [94;95] with age. Similarly, contradictory data exists with regards to the lytic capacity of the complement system, with some groups suggesting this function is decreased with age [96] and others reporting no difference [95].

The cellular arm of innate immunity is represented by a diverse group of cells that includes monocytes, macrophages, dendritic cells, neutrophils, NK cells and NKT cells. In addition to mediating direct cytotoxicity, these cells via their ability to secrete immunoregulatory cytokines and chemokines influence the developmental stages of an adaptive immune response. Thus, a change in the function of innate immune cells with age has the potential to impact not only upon a host's immediate response to infection but also their ability to establish long-lasting immunity.

#### 1.3.2.1 Monocytes and macrophages

Monocytes constitute approximately ten percent of the circulating leukocyte pool and perform two important roles in host defence. In addition to mediating direct cytotoxicity, which they accomplish in part through phagocytosis and the production of toxic radicals, monocytes serve as precursors to macrophages. Following extravasation into lymphoid and non-lymphoid organs, monocytes respond to local environmental signals by differentiating into macrophages, who by generating immunoregulatory cytokines and presenting antigens in the context of major histocompatibility complex (MHC) find themselves at the interface of innate and adaptive immunity.

The majority of studies that have compared the percentage [97;98] or absolute number [99;100] of monocytes in the peripheral blood of young and old individuals have reported no age-related differences. However, when the monocyte pool is split into its two major subsets: the $CD14^+$ $CD16^-$ classical subset and the $CD14^+$ $CD16^+$ non-classical subset, a differential effect for age has been described. Whilst both the frequency and total number of non-classical monocytes increase with age [99;101], the proportions and absolute counts of classical monocytes are significantly lower in old individuals [101], suggesting that the composition of the monocyte pool changes with age.

Due to the difficulty in obtaining macrophages from human tissue, only two studies to our knowledge have investigated the effect of human ageing on macrophage number. In both instances, an age-related reduction in the frequency of alveolar [102] and BM resident [103] macrophages was observed, although only the former reached statistical significance.

Whether the recruitment of monocytes/macrophages to inflammatory sites is altered with age is currently unclear. Whilst Swift et al [104] and Smallwood and colleagues [105] reported an age-related increase in macrophage infiltration at sites of thermal injury and foreign challenge respectively, results from *in vitro* experiments [106;107] and a single *in vivo* human study [108] suggest the chemotactic response of aged monocytes/macrophages is markedly impaired. Interestingly, Ashcroft et al, who performed cutaneous punch biopsies to assess monocyte migration *in vivo* [108], reported an age-related delay in the appearance of adhesion molecules on the endothelial cell surface as well as a reduction in their staining intensity, suggesting the impaired migration they observed was due to age-associated changes in the endothelium rather than an intrinsic cell defect.

Monocytes and macrophages eliminate invading microbes primarily through phagocytosis and the production of toxic radicals. Macrophages obtained from aged mice have been reported to perform phagocytosis at a level that is higher [109;110], lower [104;111] or comparable [112] to that of their younger counterparts, making it difficult to determine whether the phagocytic capacity of macrophages is altered with age. In contrast, the majority of studies that have examined the generation of toxic radicals have shown this function to be markedly impaired with age. When stimulated with phorbol 12-myristate 13-acetate (PMA) [113-116], interferon-gamma (IFN-γ) [113], lipopolysaccharide (LPS) [117] or opsonised pathogens [113], monocytes from aged humans [115;117] and macrophages from long-lived

mice [113;114;116] have been shown to generate significantly lower levels of superoxide [114-116], hydrogen peroxide [113;117] and reactive nitrogen intermediates [113;117].

After pathogen engulfment, macrophages assist in the development of an adaptive immune response by presenting antigen-derived peptides to T lymphocytes. Following IFN-γ stimulation, Herrero et al [118] found macrophages from aged mice expressed significantly lower levels of MHC class II molecules on their surface when compared to macrophages from younger littermates, a reduction borne from an age-associated impairment in transcription of the MHC class II gene. In addition, activated macrophages from aged mice express lower levels of CD80 and CD86 [119], two co-stimulatory molecules whose presence is critical for optimal activation of T cells. Thus, taken together, these data suggest the capacity of macrophages to present antigens and stimulate T cells is impaired with age.

The effect of age on cytokine and chemokine production by monocytes and macrophages is not entirely clear. Whilst some studies have shown the production of tumor necrosis factor-alpha (TNF-α) [120-122], IL-6 [120-123], IL1-β [123], and IL-8 [124] to be markedly reduced with age, others have reported the generation of these pro-inflammatory mediators to be increased [125;126] or unchanged [124;127]. This marked variation in study outcome may be attributable in part to the maturity of the cell type used. For instance, when one focuses upon the results of studies that have used fully differentiated tissue resident macrophages rather than immature monocytes from peripheral blood, it is evident that pro-inflammatory cytokine production is reduced with age [120-123].

The majority of studies that have investigated age-associated changes in cytokine production by macrophages have stimulated these cells with ligands for Toll-like receptors (TLR), a family of pattern recognition receptors that recognise structurally conserved molecules shared by viruses, bacteria and fungi [128]. Although it is currently unclear as to whether expression

of TLR on the surface of macrophages is altered with age [120-122;129], a number of studies have reported impaired signal transduction post TLR ligation, suggesting that the reduction in cytokine production that accompanies physiological ageing is borne from defective intracellular signalling. For example, in two separate studies, Boehmer and colleagues [121;122] investigated mitogen-activated protein kinase (MAPK) activation in LPS-stimulated macrophages and found the levels of phosphorylated p38 and c-Jun N-terminal kinase (JNK) to be markedly lower in macrophages from aged mice. Furthermore, following LPS stimulation, aged macrophages fail to up-regulate key components of the TLR pathway as efficiently as those from young controls [129].

#### 1.3.2.2 Dendritic cells

Dendritic cells (DCs) can be categorised into two distinct subsets, those of myeloid origin, which include Langerhan cells (LCs) in the skin and conventional DCs (cDCs) in the blood, and those of lymphoid descent, which are represented by circulating plasmacytoid DCs (pDCs). Whilst pDCs contribute to a host's anti-viral response by secreting copious amounts of interferon (IFN)-α, LCs and other tissue-resident DCs play an important role in the initiation of an adaptive immune response by influencing T cell priming and differentiation.

Studies that have investigated the impact of human ageing on DC number have focused primarily upon those subsets present in peripheral blood: cDCs and pDCs. Whilst age appears to have no effect upon the frequency of cDCs [130-132], its impact on pDCs is unclear, with some groups demonstrating a marked reduction in the percentage and/or absolute number of this DC subset with age [131-134], and others reporting no significant difference between young and old donors [130;135].

**pDCs**

Several groups have shown that following engagement of TLR7, 8 or 9, pDCs from young individuals generate significantly more IFN-α, TNF-α and IL-6 than pDCs obtained from older adults [98;131;132;134;136]. This age-related reduction in TLR-induced cytokine production has been attributed to a decline in TLR expression and aberrant intracellular signalling [136]. Indeed, although not observed by all groups [137], ageing appears to be associated with a reduction in the expression of TLR7 and TLR9 [131;132], whilst impaired up-regulation of phosphoinositide 3-kinase (PI3-K) and the transcription factor IFN regulatory factor-7 has been described in TLR9-stimulated pDCs from aged donors [138].

Through the production of IFN-α, pDCs augment a host's anti-viral response by enhancing the cytotoxic activity of NK and $CD8^+$ T cells. Thus, reduced secretion of IFN-α may contribute to the increased incidence of viral infection with age. In support of this theory, Panda et al reported TLR-induced production of IFN-α to be positively associated with the generation of a protective antibody response following influenza vaccination [132], whilst Sridharan and colleagues found that through reduced production of IFN-α, pDCs from aged donors were unable to enhance the cytotoxicity of resting $CD8^+$ T cells [137].

**Myeloid DCs (mDCs)**

Found at such sites as the skin, mucosa and airways, tissue-resident DCs continually survey and sample their local environment for invading microbes. Upon pathogen recognition, DCs capture and process the antigen, triggering their maturation, which is characterised by phenotypic changes, cytokine secretion and DC migration to secondary lymphoid organs (SLO), where upon arrival they help drive an adaptive immune response by presenting processed Ag to naïve T cells.

Based on current data, it appears that antigen uptake by DCs is not affected by the ageing process [139-142]. Whether this is also true for DC migration however is unclear as whilst some studies have shown this feature of DCs to be comparable between young and old subjects [143-145], others have reported a significant reduction with age [130;141;142]. For example, whereas Sprecher et al reported the capacity of LCs to deliver antigen to draining lymph nodes (DLNs) was comparable between young and old mice [144], Zhao and colleagues observed a marked and progressive age-related decline in both the frequency and number of DCs migrating to DLNs in response to respiratory virus infection, a defect they attributed to elevated levels of prostaglandin D2 in the lungs of aged mice [142].

Increasing experimental evidence suggests that the stimulatory capacity of mDCs wanes with age. Compared to those isolated from young controls, aged mDCs have been shown to be less effective at inducing T cell proliferation [136;141;146;147], IFN-γ secretion [139;147] and T cell cytotoxicity [139;141]. Together, these defects would be expected to lead to a diminished T cell-mediated immune response. Indeed, in an *in vivo* model of tumour growth, Grolleau-Julius et al [141] demonstrated young tumour bearing mice immunised with aged DCs presented with tumours that were significantly larger in size at 7 day follow up than those mice who had received DCs from young mice, suggesting that the weakened T cell response elicited by aged DCs impacts upon the hosts immune response [141]. However, whether these defects in T cell behaviour are the direct result of impaired DC function is unclear. For example, although some studies have found DCs from old people to be impaired in antigen presentation [140], and to express lower levels of the co-stimulatory markers CD80/86 following stimulation [147;148], the majority that have investigated these features of DC biology have shown them to be comparable between young and old donors

[139;141;149;150]. Thus, the weakened T cell response of aged individuals may result from intrinsic T cell defects rather than impaired DC function [150].

Whether ageing is associated with alterations in pro-inflammatory cytokine production by mDCs is an area of debate within the literature with studies reporting increased [130], decreased [132;135;146;149] or comparable [131;140;150] generation of TNF-α, IL-6 and IL-12 by aged mDCs in response to TLR stimulation when compared to that secreted by young mDCs. Studies that have reported a decline in cytokine production have attributed it to an age-related reduction in TLR expression [132], impaired intracellular signalling [149] and an age-associated increase in basal cytokine levels [132]. In the latter case, Panda et al compared cytokine expression in mDCs from young and old donors in the absence of stimulation and found those from older adults exhibited elevated levels of TNF-α and IL-6 [132]. High expression at baseline would therefore mean a smaller net-fold increase in cytokine production upon TLR stimulation.

#### 1.3.2.3 Invariant NKT cell function with age

Comprising 0.5-1% of the circulating T lymphocyte pool, invariant NKT (iNKT) cells are innate lymphocytes defined by the expression of a semi invariant TCR (Vα24/Vβ11 in humans) that recognises glycolipid antigens presented by the MHC class I-like molecule CD1d [151]. Upon recognition of ligands, which include bacterial glycolipids [152], the self-lipid β-D-glucopyranosylceramide [153] and the pharmacological agent α–galactosylceramide (α-GalCer) [154], iNKT cells secrete IL-4, IL-13 and IFN-γ, and via the expression of perforin, granzyme B and death receptor ligands exhibit direct cytotoxicity towards tumour cells [151].

Physiological ageing in humans is accompanied by a marked decline in the frequency of iNKT cells. Whether defined as $V\alpha24^+$ [155] or $V\alpha24^+/V\beta11^+$ [156-158], the proportion [155;156;158] or absolute number [157] of iNKT cells in the peripheral blood of older adults has been shown to be significantly lower when compared to that of younger adults. Murine studies that have investigated numerical changes in iNKT cells with age have reported a significant increase in the proportion [159;160] and absolute number [159] of these cells in the lymph nodes, liver and spleen of aged mice. Thus, the age-related decrease in human iNKT frequency in peripheral blood may reflect a re-distribution of these cells to other organs [161].

Ageing is also associated with alterations in the composition of the circulating iNKT pool. Based on the presence or absence of CD4 and CD8, iNKT cells can be divided into three distinct subsets ($CD4^+$ $CD8^-$, $CD8^+$ $CD4^-$ and $CD4^-$ $CD8^-$). With age, a significant increase in the percentage of $Va24^+$ $CD8^+$ $CD4^-$ iNKT cells has been reported [155], as has an increase in the percentage of $CD4^+$ $CD8^-$ $V\alpha24^+/V\beta11^+$ cells, the latter being accompanied by a marked reduction in the proportion of $CD4^-$ $CD8^-$ $V\alpha24^+/V\beta11^+$ iNKT cells [156].

The effect of age on the function of iNKT cells is not entirely clear, due in part to the small number of studies that have investigated this issue. For example, the proliferation of iNKT cells in response to α-GalCer stimulation has been reported to be significantly reduced [158] or unaffected [156] with age. More consistent findings have however been reported for cytokine production, where two independent groups have shown a marked age-dependent decrease in IFN-γ secretion by iNKT cells following α-GalCer treatment [156;157].

### 1.3.2.4 Inflammageing

Alongside the aforementioned changes in immune cell number and function, ageing is associated with a low-grade systemic up-regulation in circulating inflammatory mediators. Termed inflammageing, this inflammatory state is characterised by elevated levels of pro-inflammatory cytokines (e.g. IL-6 and TNF) and acute phase proteins (e.g. C reactive protein (CRP)), as well as reduced levels of the anti-inflammatory cytokine IL-10 [162-165].

Results of longitudinal based studies suggest inflammageing is deleterious to human health. Studies in elderly cohorts have shown the low-grade increase in the circulating levels of TNF-α [166;167], IL-6 [167-169] and CRP [169;170] are associated with both all-cause [166-169] and cause-specific [168;170] mortality. In addition, inflammageing is a predictor of frailty [171], has been associated with functional disability [172], and is considered a major factor in the development of several age-related pathologies, such as atherosclerosis [173], Alzheimer's disease [165] and sarcopenia [174].

Life-long infection with the persistent herpes virus CMV is thought by many to be the major driver of inflammageing. Indeed, associations have been reported between CMV seropositivity and high circulating levels of CRP [175], whilst *in vitro,* CMV infection of monocytes has been shown to increase their production of both IL-6 and TNF-α [176]. However, a recent longitudinal study has challenged this long-standing belief by demonstrating that over time, circulating levels of TNF-α and IL-6 increase and IL-10 decrease in elderly subjects who are CMV negative [177]. Thus, based on this data it would appear that factors other than CMV infection drive the gradual increase in systemic inflammation that accompanies physiological ageing. These may include the age-related

re-distribution of and increase in fat tissue [163], an individuals genetic background [178] and the age-associated decline in sex steroid synthesis [179].

### 1.4 Clinical relevance of immunesenescence

As the primary role of the immune system is to recognise and eliminate pathogen-infected cells, immunesenescence is often cited as a major factor underlying the increased incidence and severity of infection in older adults. However, this assumption is based primarily on the findings of cross-sectional studies, which often fail to control for external factors that influence immune function independent of the ageing process (e.g. environment, genetics, previous pathogen exposure and nutritional status). In an attempt to overcome this issue and establish whether immunesenescence is of clinical significance, a small number of groups have performed longitudinal based studies on older adults.

Focusing on a population of free-living seniors aged 85 years and over, Wikby and colleagues identified a set of immune parameters that were associated with future mortality [180-183]. The group found that subjects, who at baseline presented with poor T cell mitogen responses, low numbers of $CD4^+$ T cells and $CD19^+$ B cells, an inverted CD4/CD8 ratio and an increased number of $CD8^+$ $CD28^-$ T cells, a phenotype they termed the immune risk profile (IRP), exhibited reduced survival rates at follow-up [180]. In one particular study, it was reported that 100% of aged individuals who presented with an IRP at baseline were deceased at six-year follow-up, whilst 80% of individuals who developed the IRP during the study were also deceased at follow-up [184]. Other studies have focused on specific features of the IRP and have shown that an inverted CD4/CD8 ratio and increased circulating inflammatory activity (e.g. high IL-6 and CRP levels) are themselves predictors of mortality [182].

Away from the IRP, it has been shown that thymic function failure at baseline can predict mortality at 2-year follow-up [185], whilst an impaired inflammatory response by peripheral blood mononuclear cells (PBMCs) following LPS challenge was reported to be associated with a 2-fold increase in mortality risk [186].

Despite demonstrating that features of immunesenescence can predict mortality, the associations made in the aforementioned studies were between immune function and all-cause mortality. To date, few groups have investigated in a longitudinal setting whether immunesenescence is associated with an increased risk of infection and/or death due to infection in older adults [187-190]. In one particular study, Plonquet and co-workers assessed the prevalence of nosocomial infections in a cohort of 252 aged individuals and found a greater proportion of those who fulfilled the IRP criteria at baseline went on to contract pneumonia [190].

### 1.5 Natural Killer cells

Comprising 10-15% of the circulating lymphocyte pool, as well as residing in such peripheral tissues as the liver, lungs and peritoneal cavity, natural killer (NK) cells are critical effector cells of the innate immune system renowned for their ability to recognise and eliminate virally-infected and tumorigenic cells [191]. The importance of NK cells in host protection is illustrated by studies that have shown the selective absence of these cells or an impairment in their function results in increased episodes of viral infection [192-194] and a reduced ability to control the development of certain tumours [195;196]. However, the role of NK cells in host immunity appears to extend beyond eliminating virally-infected and malignant cells as recent *in vivo* and *in vitro* studies have shown NK cells also elicit direct cytotoxicity towards bacteria [197], fungi [198] and parasites [199].

### 1.5.1 NK cell effector functions

NK cells directly eliminate malignant and pathogen-infected cells through two contact-dependent mechanisms: granule exocytosis and death receptor ligation (Figure 1.1).

#### 1.5.1.1 Granule exocytosis

Granule exocytosis is the predominant mechanism by which NK cells eliminate transformed cells [200;201]. Following target cell recognition and formation of an activating immunological synapse, NK cells release an array of cytolytic proteins that induce target cell death through the induction of apoptosis. Of the many proteins released, it is the membrane-disrupting protein perforin and a family of serine proteases termed granzymes that are the critical effector molecules.

Mature human perforin is a 534 amino acid protein consisting of three domains: an N-terminal membrane attack complex/perforin domain, an epidermal growth factor like domain and a C-terminal calcium binding C2 domain [202]. Following its release from secretory lysosomes, perforin binds in a calcium dependent manner to phospholipid components of the target cell membrane where it facilitates the entry of apoptosis-inducing serine proteases into the target cell cytosol. Mice rendered perforin deficient or humans that carry mutations in their perforin gene fail to clear viral infections and eliminate developing tumours as efficiently as their controls [203-205]. As well as demonstrating the importance of granule exocytosis in host defence, this data illustrates the non-redundant role of perforin in this form of NK cell cytotoxicity (NKCC).

Although the role of perforin in NKCC is firmly established, how it delivers granzymes into the cytosol of target cells is still not fully understood (Figure 1.2). Based on its structural similarity to the complement protein C9, perforin was originally proposed to function solely

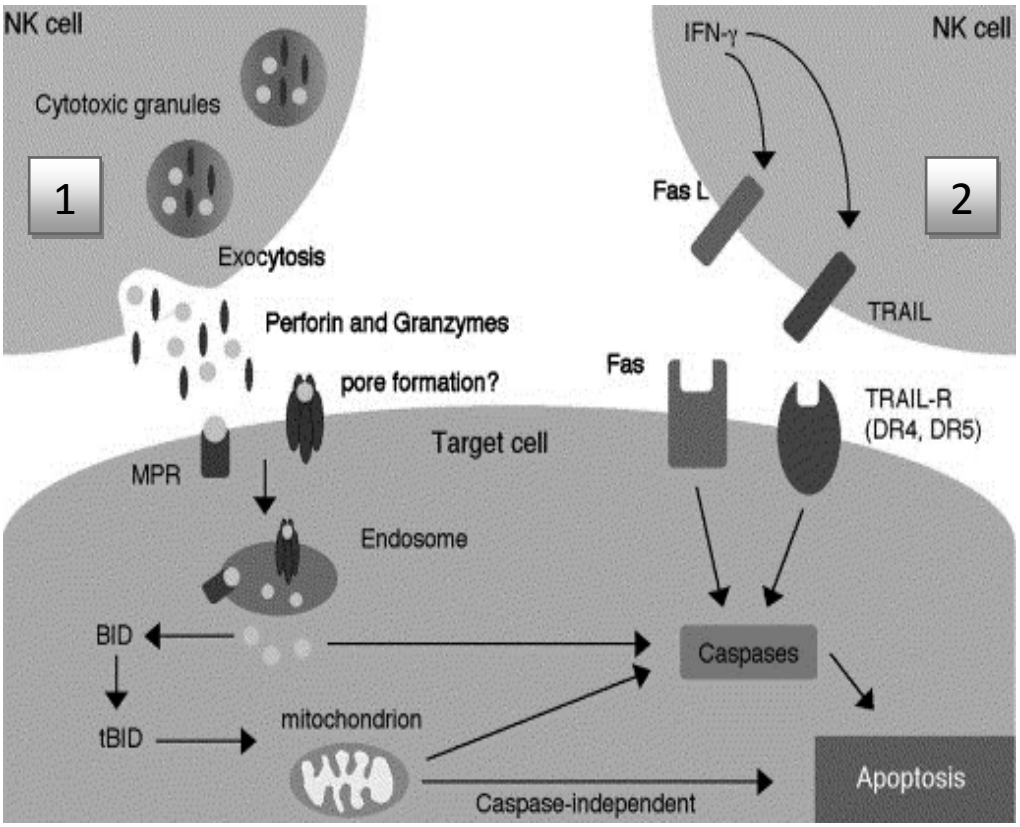



**Figure 1.1 NK cell cytotoxicity (NKCC).** NK cells directly eliminate transformed cells through one of two contact-dependent mechanisms. **(1)** *Granule exocytosis*: Upon interaction with its cellular target, NK cells secrete a range of lytic effector molecules. These include the pore-forming protein, perforin, and a number of serine proteases (granzymes). Following entry into the cytoplasm of target cells, granzymes activate the apoptotic cascade in either a caspase-dependent or independent manner. **(2)** *Death receptor engagement*: In response to direct cell contact and/or cytokine stimulation, NK cells express on their surface Fas ligand (FASL) and TNF-related apoptotic inducing ligand (TRAIL), two members of the TNF family, which upon interaction with their cognate receptors (Fas and TRAIL-R respectively), promote cell death through the direct activation of cellular caspases. *Figure taken directly from review article by Smyth et al [206]*.

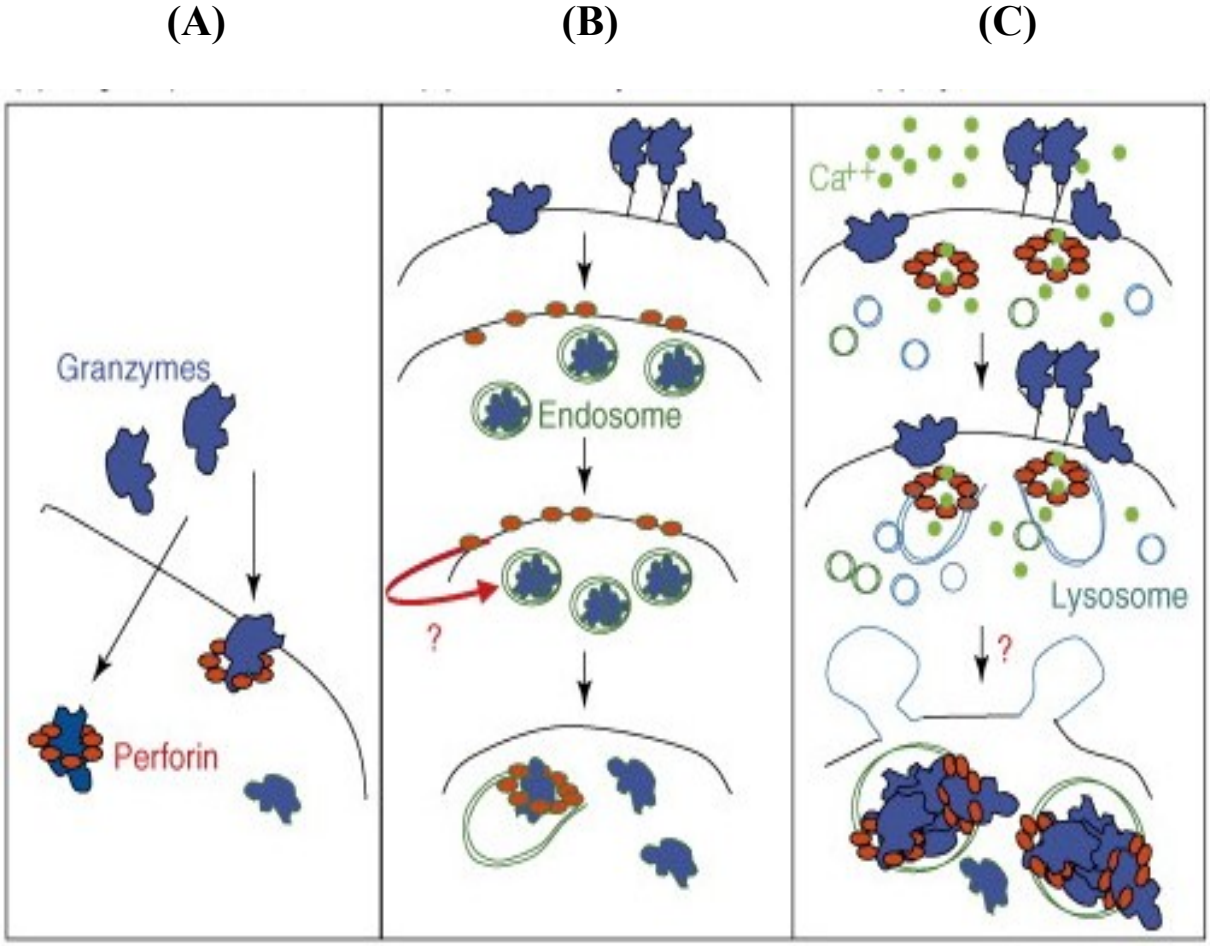



**Figure 1.2. Proposed models for how perforin mediates granzyme entry into the target cell cytosol. (A)** The classic model, which suggests that after binding to the plasma membrane of target cells, perforin undergoes oligomerisation, forming channels through which granzymes passively diffuse. **(B)** The endosomolysin model proposes granzymes enter the target cell independently of perforin (possibly through interaction with a receptor on the target cell surface) and reside in endosomes. Perforin then acts upon the endosomal membrane to release granzymes into the cytoplasm. **(C)** Derived predominantly from the work of Theiry et al [207;208], the hybrid model suggests perforin acts on both plasma and endosomal membranes. By forming pores in the plasma membrane, perforin induces a cellular wound healing response, which results in the co-endocytosis of perforin and granzymes into endosomes. Perforin then acts upon the endosomal membrane, allowing granzymes to enter the target cell cytosol, where they trigger caspase-dependent and independent cell death. In the figure, black lines represent the plasma membrane, endosomal membranes are green and lysosomal membranes are blue. *Figure taken directly from review article by Pipkin et al [209].*

at the plasma membrane of its target cell, where following oligomerisation it acted as a conduit through which granzymes passively diffused [210;211] (Figure 1.2A). Whilst accepted at first, this model was questioned when it was demonstrated that granzymes could enter target cells independently of perforin [212-215], possibly via direct interaction with the cell surface receptor mannose 6-phosphate [215]. However, granzyme entry alone did not induce target cell death, which was observed only when perforin was added to targets that had been pre-exposed to granzymes [212], suggesting that it was the endosomal membrane and not the plasma membrane that was the site of perforin action (Figure 1.2B). Supporting this idea were experiments that substituted perforin with replication-deficient adenovirus or bacterial proteins (e.g. streptolysin O or listeriolysin), agents that disrupt endosomal membrane integrity, and found granzyme B dependent apoptosis still occurred in the absence of cell membrane damage [212;214;216]. However, in spite of this evidence, this model has not gained total acceptance within the field, with one particular group suggesting its basic principles are borne from methodological error. Rather than binding to specific receptors, Shi et al [217], found granzyme B bound to cells mainly by charge and that the washing procedure used in the aforementioned studies did not remove it from the cell surface. Using an approach that completely removed granzyme B from the target cell membrane, Shi and co-workers showed the addition of perforin to target cells did not trigger target cell apoptosis [217], suggesting that rather than releasing endocytosed granzyme B, perforin was in fact delivering surface bound granzyme B into the cytosol. Indeed, only when perforin and granzyme B were added to target cells together did the group observe granzyme B release from endosomes [217]. Based on this and more recent observations [207;208;218], Lieberman and colleagues have proposed the “hybrid model”, which they believe explains more convincingly the mechanism by which perforin delivers granzymes into the target cell cytosol (Figure 1.2C). Taking ideas from both the traditional pore theory and the

endosomolysin model, the group have hypothesised that upon binding to the target cell surface, perforin, via forming transmembrane pores, triggers a membrane-repair response that results in the co-endocytosis of perforin and granzymes. Perforin then mediates granzyme release into the target cell cytosol by undergoing oligomerisation in the endosomal membrane [208].

Granzymes are a family of structurally-related serine proteases, which following perforin-mediated entry trigger caspase-dependent and independent target cell death. Human NK cells express five granzymes, namely A, B, H, K and M, which possess various substrate specificities and exhibit a level of functional redundancy [206;219-221]. Whilst little is known regarding the role of granzymes H, K and M in granule-dependent killing [222;223], great progress has been made in elucidating the mechanisms by which granzymes A and B induce target cell death.

As an aspase, granzyme B cleaves its substrates after aspartic acid residues. Unsurprisingly therefore, several members of the caspase family, including caspases 3, 7, 8 and 10 are direct targets of this protease [224;225]. As caspases 3 and 7 target numerous proteins for degradation, many of which are involved in deoxyribonucleic acid (DNA) preservation, their direct activation by granzyme B underlies the rapid induction of target cell death mediated by the granule exocytosis pathway [226]. Indeed, in the absence of granzyme B, target cell death mediated by cytotoxic lymphocytes is markedly delayed [220]. In addition to direct activation, granzyme B can activate caspases indirectly by driving mitochondrial permeabilisation. This is mediated in part through granzyme B cleaving the BH-3 family protein BH3-interacting domain death agonist, which in its truncated form drives activation of caspase 9 by facilitating the release of pro-apoptotic proteins from the mitochondria into the target cell cytosol [227].

Caspase activation, whether direct or indirect, is not the only mechanism through which granzyme B triggers apoptosis, as pharmacological inhibition of these proteases does not prevent granzyme B from inducing target cell death [228;229]. Other routes through which this serine protease triggers apoptosis include directly cleaving proteins involved in DNA repair (e.g. poly (ADPribose) polymerase (PARP)) [230;231], and preservation [232] as well as proteins important for maintaining the integrity of the nuclear membrane such as lamin B [233].

Granzyme A induces a cell death program that is independent of caspase activation. Following entry into the target cell cytosol, granzyme A travels to both the mitochondrial matrix and nucleus. Within the mitochondria, granzyme A cleaves NDUFS3, a complex I protein, whose degradation results in the generation of reactive oxygen species (ROS) [234;235]. An increase in cellular ROS levels triggers the translocation of the SET complex, an endoplasmic reticulum-associated oxidative stress response complex, to the nucleus, where upon arrival, several of its components, which include: Ape1 (a base excision repair enzyme), HMGB2 (a protein that recognises damaged DNA) and SET (an inhibitor of the exonuclease NM23-H1) are cleaved by nuclear residing granzyme A [236-238]. Degradation of these proteins results in the activation of NM23-H1 and TREX-1, nucleases, which mediate the single-stranded DNA damage that is characteristic of granzyme A-induced target cell death [236;239]. The SET complex however is not the only target of granzyme A, as it also cleaves a range of proteins that are involved in maintaining the integrity of cellular DNA. These include but are not limited to nuclear lamins, core histones and PARP [233;240;241].

#### 1.5.1.2 Death receptor ligation

In addition to granule exocytosis, NK cells directly eliminate transformed cells through death receptor engagement (Figure 1.1). In response to cytokine stimulation [242;243] or following ligation of activatory receptors [244;245], NK cells express on their surface Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Through binding their cognate receptor on the target cell surface, FasL and TRAIL induce the formation of a death induced signalling complex, which by promoting activation of caspase 8 triggers a signalling cascade that culminates in the activation of caspases 3 and 7, and the induction of target cell apoptosis [246].

In resting NK cells, FasL is pre-formed and resides in the same lytic granules as perforin and granzymes [247;248], meaning target cells are exposed to these effector molecules simultaneously following NK cell degranulation. In contrast, NK cells must drive transcription of the TRAIL gene to express this death receptor ligand on its surface [242], a prerequisite that maybe responsible for the slower kinetics reported for death receptor-mediated apoptosis when compared to target cell death induced by granule exocytosis [242;245].

#### 1.5.1.3 NK cell immunological synapse (NKIS)

A critical event that precedes the induction of NKCC is the formation of an activating immunological synapse (IS). Formed at the NK-target cell interface, the NKIS is characterised by "*the orderly rearrangement of NK signalling molecules into micrometer-scale three-dimensional domains*" [249;250], and is the site at which ligand recognition, signal amplification and granule exocytosis occurs. Through the formation of an IS, NK cells deliver cytotoxic effector molecules directly onto the target cell surface. Thus, as well as

ensuring the rapid elimination of transformed cells, the creation of an NKIS protects neighbouring healthy cells from bystander damage.

Formation of an NKIS begins with NK-target cell interaction. Mediated by such adhesion molecules as CD2, lymphocyte function-associated antigen 1 and macrophage receptor 1, close cell-cell contact enables NK activatory receptors to recognise and bind their cognate ligands expressed on the target cell surface [251]. NK cell activation leads to the recruitment of a number of signalling elements to the NK-target cell interface. These include the protein tyrosine kinases Src, Lck, Fyn and Syk, the guanine nucleotide exchange factor Vav1, and PI3-Kinase [250], which together drive the next stage of NKIS formation: actin polymerisation and reorganisation.

The recruitment and activation of Vav1 at the NK-target cell interface leads to the formation of a multi-subunit protein complex consisting of the Rho family GTPase cell-division control protein 42 (Cdc42), Wiskott-Aldrich syndrome protein (WASp) and Arp2/3 [252]. By inducing actin nucleation and branching, this complex mediates re-organisation of the actin cytoskeleton, a step that is characterised by the segregation of signalling elements into two distinct zones: an outer zone, referred to as the peripheral supramolecular activation cluster, where adhesion receptors localise, and an inner ring, known as the central SMAC (cSMAC), where molecules critical for NK cell signalling reside [251]. As well as augmenting NK cell activation by inducing activatory receptor clustering and lipid raft aggregation [249;253], this rearrangement of the actin cytoskeleton acts as a trigger for the polarisation of the microtubule organising centre (MTOC) towards the developing NKIS.

MTOC polarisation is co-ordinated with re-organisation of the actin cytoskeleton through the actions of Cdc42 interacting protein 4 (CIP4) and the multi-domain protein IQGAP1 [254;255]. Functioning downstream of Cdc42, CIP4 interacts with WASP and the

microtubule component α-tubulin [256-258], and is important not only for the directed movement of the MTOC towards the NKIS but also its stabilisation. Indeed, NK cells depleted of CIP4 exhibit impaired MTOC polarisation following target cell interaction [254]. Similarly, silencing of IQGAP1, which interacts with both Filamentous actin (F-actin) and microtubules (MTs), has been shown to markedly reduce MTOC polarisation during NKIS formation [255]. In addition to CIP4 and IQGAP1, MAPK signalling is essential for re-orientation of the MTOC. Studies have shown phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2), JNK and p38 occurs in NK cells following target cell contact [259-262], and that these signalling molecules drive MTOC polarisation, lytic granule mobilisation and ultimately NKCC. Indeed, selective MAPK inhibition greatly reduces MTOC movement to the NK-target cell interface and is associated with impaired NK lytic activity [259;260;263].

Following MTOC polarisation, secretory lysosomes are delivered to the NK-target cell interface. Alongside the aforementioned MAPKs, the actin motor proteins dynein and kinensin, and the regulatory protein Wasp-interacting protein (WIP), which assist in secretory lysosome movement along MTs and lytic granule polarisation respectively [264-266] play an important role in this phase of NKIS formation. Upon arrival at the cSMAC, secretory lysosomes dock to the plasma membrane, a step that requires the action of the small GTPase Rab27a [267]. Once tethered to the membrane, granules are primed for fusion by Munc13-4, a binding partner of Rab27a [268]. Lysosomal priming is thought to allow soluble N-ethylmaleimide-sensitive protein attachment protein receptor (SNARE) proteins, which include syntaxin-11, VAMP4 and VAMP7, to interact with each other, and drive the fusion of lytic granule membranes with the plasma membrane, resulting in the release of perforin and granzyme onto the target cell surface [269;270]. Interestingly, two recent studies reported

the novel finding that F-actin is present in the cSMAC [271;272]. As lytic granules express the actin binding protein myosin IIA on their surface [273], lysosomes may detach from MTs when in the vicinity of the NKIS and associate with F-actin prior to degranulation. In support of this claim, Andzelm et al [274] found that inhibition of myosin IIA activity had no effect upon actin accumulation or lytic granule polarisation to the NKIS but was associated with a marked reduction in lytic granule fusion with the NK cell membrane.

#### 1.5.1.4 Production of immunoregulatory cytokines and chemokines

As well as mediating direct cytotoxicity, NK cells are a rich source of immunoregulatory cytokines and chemokines. Studies have shown that following cytokine or target cell stimulation, NK cells are capable of secreting a range of pro and anti-inflammatory mediators, which include TNF-α, IFN-γ, IL-10, macrophage inflammatory protein-1-alpha (MIP-1α), MIP-1β and IL-8 [275-278].

Primarily through the production of IFN-γ and the aforementioned chemokines, NK cells influence the activity of bystander cells at sites of developing inflammation, and thereby amplify on-going immune responses. For example, in addition to enhancing the antigen presenting capacity of macrophages by up-regulating surface expression of MHC class II molecules, NK cell-derived IFN-γ increases both the microbicidal and tumoricidal activity of these tissue-resident cells [279;280]. Furthermore, by secreting MIP-1α/β and IL-8, NK cells would not only induce the migration of immune cells to the site of antigenic challenge, but would enhance the cytolytic activity of surrounding NK cells, as exposure to MIP-1α and MIP-1β has been shown to trigger both calcium mobilisation and granule exocytosis in resting NK cells [281;282].

Although thought of as separate entities, the innate and adaptive arms of the immune system undergo considerable cross-talk and reciprocal interaction. Through their production of IFN-γ and TNF-α, NK cells place themselves at the interface of this bi-directional relationship by influencing DC maturation and T cell differentiation. In co-culture with resting [283] or IL-2 activated [284] NK cells, immature DC's (iDC's) undergo maturation, as evidenced by their increased expression of CD83, CD86 and HLA-DR, and their ability to induce proliferation of naïve $CD4^+$ T cells [283;284]. This maturation was shown to be attributable in part to NK cell-derived IFN-γ and TNF-α, as neutralising antibodies against both these cytokines markedly reduced the maturation of iDCs [285]. With regards to NK cells and T cell differentiation, Martin-Fontecha et al [286] showed in an elegant study that following subcutaneous injection of LPS-matured DC's, peripheral NK cells travel to the DC draining lymph node, where through the production of IFN-γ they help drive T helper 1 cell polarisation [286].

### 1.5.2 Regulation of NKCC

Given the direct toxic nature of NK cells and their ability to potentiate ongoing immune responses via the production of pro-inflammatory cytokines and chemokines, NK cell activation is tightly regulated. Mechanisms controlling the induction of NK cell activity are present both in the BM, where only NK cells capable of recognising self MHC class I molecules have functional competence bestowed upon them, a phenomenon referred to as NK cell "licensing", and in the periphery where NK cell activation is governed by priming signals and an array of germline-encoded activatory and inhibitory receptors.

### 1.5.2.1 NK cell licensing

In 2005, Yokoyama and colleagues reported that NK cells isolated from MHC class $I^{-/-}$ mice not only failed to generate IFN-γ when stimulated with antibodies against activating receptors, but exhibited reduced cytotoxicity towards tumour targets when compared to NK cells from wild type controls [287]. This aberration in NK cell function was attributed to an intrinsic defect borne from the inability of developing NK cells to interact with self MHC class I molecules, an association that the group suggested was critical for the generation of functionally competent effector cells [287]. Subsequent studies supported this claim by confirming that NK cells isolated from MHC class $I^{-/-}$ mice [288;289] and humans [290;291] were hyporesponsive to stimulatory signals, a trait that is shared by NK cells that do not express the surface receptors that recognise self MHC class I molecules [288;292].

To date, several models have been proposed to explain how developing NK cells have functional competence bestowed upon them by MHC class I molecules, a process referred to as NK cell "licensing". Whilst sharing some common features, these models, whose concepts are outlined below, differ in how they believe licensing signals are delivered to and interpreted by NK cells.

*(i) Arming model* – According to the arming model, recognition of self-MHC class I molecules by cell surface inhibitory receptors delivers positive signals to developing NK cells which promote their functional maturation. In the absence of such signals, NK cells remain hyporesponsive to activatory stimuli.

*(ii) Disarming model* – The disarming model proposes that NK cells develop in a functional responsive state. Without interaction between inhibitory receptors and MHC class I molecules, NK cells are "disarmed" to prevent potentially self-reactive cells entering the

peripheral NK pool. Recognition of MHC class I molecules by inhibitory receptors prevents this process, allowing for the generation of functionally competent NK cells.

*(iii) Cis-interaction model* – The cis-interaction model of NK cell "licensing" proposes that functional competence can be acquired by developing NK cells without the need for inhibitory receptor engagement by MHC class I molecules. Rather, this theory, which is based on the work of Chalifour et al, who found unengaged MHC class I receptors could deliver inhibitory signals to NK cells [293], suggests that by associating with inhibitory receptors in the same cell membrane, MHC class I molecules render NK cells functionally mature by preventing the localisation of unengaged inhibitory receptors to the immunological synapse, who upon arrival would otherwise dampen the response of NK cells to activatory stimuli.

*(iv) Rheostat model* – A feature that distinguishes the rheostat theory from those described above is that rather than viewing NK cell "licensing" as an event with a binary outcome, i.e. developing NK cells are either "licensed" or "unlicensed", it considers it to be a quantitative phenomenon. The model postulates that the functional responsiveness of peripheral NK cells is related to the strength of signal they receive through surface expressed inhibitory receptors during their development in the BM [294;295]. Thus, assuming that the host possesses the cognate MHC class I ligands, the more inhibitory receptors an NK cell expresses, the greater its functional response upon stimulation.

For many years, NK cell "licensing" was considered a fixed event restricted to the BM. However, the findings of two recent studies have challenged both these concepts by showing that changes in peripheral MHC class I expression can influence the functional competence of mature circulating NK cells. In an elegant study, Elliott and co-workers demonstrated that when hyporesponsive unlicensed NK cells from MHC class $I^{-/-}$ mice were transferred into

wild type hosts, these cells acquired functional competence, producing cytokines and exhibiting cytotoxicity at a level that was comparable to that of NK cells from their wild type littermates [296]. Conversely, when functionally responsive NK cells from wild type mice were transferred into MHC class $I^{-/-}$ hosts, these cells became hyporesponsive to activatory receptor stimulation [296;297]. Thus, in addition to suggesting that continual exposure to self-MHC class I molecules is needed for mature circulating NK cells to retain their functional competence, it is apparent from both these studies that NK cell "licensing" is not solely a developmentally associated process.

According to the "licensing" hypothesis, NK cells are functionally defective if they do not receive signals from self-MHC class I molecules. However, the recent work of Orr and colleagues suggests that under certain circumstances, "unlicensed" NK cells are more efficient than their "licensed" counterparts at eliminating transformed cells [298]. The group showed using a model of murine CMV (MCMV) infection that depleting mice of "unlicensed" NK cells resulted in viral titres that were comparable to those in mice that had been depleted of all NK cells, and significantly greater than those in wild type controls. In contrast, no difference in viral titre was observed between control mice and mice lacking "licensed" NK cells, suggesting that it was "unlicensed" NK cells that conferred host protection [298]. Indeed, in an adoptive transfer model, only recipients of "unlicensed" NK cells were able to survive infection with MCMV [298]. As exposure to inflammatory cytokines enhances the responsiveness of "unlicensed" NK cells [287], current thinking is that the activation of these effector cells is driven by the pro-inflammatory environment created in times of infection [299].

#### 1.5.2.2 NK cell priming

For many years, NK cells were thought to eliminate transformed cells in a manner that was independent of prior sensitisation. However, it is now apparent from the findings of recent murine [300] and human [301-303] studies that NK cells must be "primed" before they can initiate an effective cytotoxic response.

Tumour cells and cytokines have both been shown to provide NK cells with priming signals. In two separate studies, Lowdell and colleagues [302;303] found that by binding to the NK cell adhesion molecule CD2, a protein associated with CD15 on the surface of NK-resistant tumour cells could deliver to resting NK cells priming signals, which enabled these lymphocytes to lyse in subsequent cytotoxicity assays target cells that normally display resistance to NK-mediated killing. Detailed examination of the priming process revealed it to be associated with a marked up-regulation of CD69 on the NK surface and to occur in the presence and absence of signals that negatively regulate the induction of NKCC [302;303]. With respect to cytokine-mediated priming, Bryceson et al [301] demonstrated in a series of re-directed lytic assays that, with the exception of ligation of the Fc receptor CD16, pre-treatment with the activating cytokine IL-2 was required for resting NK cells to eliminate targets cells that expressed ligands for only a single NK cell activating receptor [301]. In a similar manner, Hart and colleagues reported that exposure to IL-12 was needed for resting NK cells to mediate cytotoxicity or produce IFN-γ upon engagement of TLR7 and TLR8 [304]. Data showing that cytokine-induced priming enhances intracellular perforin levels and NK cell degranulation upon target cell contact [305] provides a possible mechanism for the more robust cytotoxicity mediated by cytokine-primed NK cells.

In the absence of priming signals, NK cells exhibit minimal cytotoxic activity. This was demonstrated elegantly in an *in vivo* model of infection by Lucas et al [300], who found that

by trans-presenting IL-15 to NK cells in times of pathogenic challenge, lymph node resident $CD11c^{high}$ dendritic cells bestowed upon resting NK cells the ability to perform cytotoxicity at a level significantly greater than that of NK cells from mice deficient in $CD11c^{high}$ dendritic cells [300]. In line with the aforementioned *in vitro* data, the enhanced cytotoxicity of "primed" NK cells was associated with an increase in intracellular granzyme B levels and surface CD69 expression [300]. Thus, it appears that priming enhances the cytotoxicity of resting NK cells by increasing their cytotoxic armoury and lowering their activatory threshold.

#### 1.5.2.3 NK cell receptors

Although derived from the same progenitor cell as B and T lymphocytes, NK cell activity is not regulated by signals transmitted through one dominant receptor. Rather, NK cell activation is governed by the balance of signals received through a multitude of surface expressed germline-encoded activatory and inhibitory receptors [306].

##### 1.5.2.3.1 Inhibitory receptors

Based on an *in vivo* experiment that found NK cells could reject tumour cells that had lost MHC class I expression but could not prevent tumour development by MHC class I positive cells, Karre and colleagues formulated the "missing-self" hypothesis, which put forward the idea that the loss of self MHC class I molecules rendered cells susceptible to NK cell-mediated lysis [307;308]. To explain this concept, it was proposed that NK cells possessed on their surface a receptor specific for self-MHC class I molecules, which upon ligand recognition prevented NK cell activation by transmitting inhibitory signals [309]. Whilst at first this theory was difficult to believe, it is now established that NK cells express on their surface an array of inhibitory receptors, that after binding to either MHC class I or non MHC

class I molecules negatively regulate NK cell activity (Table 1.1) [306]. Common to all inhibitory receptors is the presence in their cytoplasmic domain of an immunoreceptor tyrosine-based inhibitory motif (ITIM), which serves as the focal point through which inhibition of NK cell activity is mediated. Upon ligand recognition, tyrosine residues present within ITIMs are phosphorylated by Src family kinases [310]. Once phosphorylated, these residues are bound by the Src homology 2 (SH2) domains of the protein tyrosine phosphatases: Src homology region 2-containing protein tyrosine phosphatase-1 (SHP-1), SHP-2 or SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP)-1 [311-313], who by targeting an array of intracellular signalling molecules promote NK cell inhibition by interfering with numerous steps involved in the induction of NKCC (Table1.2).

SHP-1 has been proposed to negatively regulate NK cell activity by dephosphorylating an array of intracellular signalling molecules. These include the adaptor proteins SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells (LAT) [314;315], the guanine nucleotide exchange factor Vav [316], the Src family kinase Lck [317] and the Syk family kinases Zeta-chain-associated protein kinase 70 (ZAP70) and Syk [310;318]. Whether the dephosphorylation of these proteins is mediated directly or indirectly is currently unclear [314;316]. Nevertheless, by targeting LAT, SHP-1 has been shown to disrupt its association with phospholipase C-γ (PLC-γ) [315], which may underlie the decrease in inositol 1,4,5-triphosphate ($IP_3$) release and calcium mobilisation that occurs in NK cells following recognition of MHC class I molecules [319]. Moreover, through interfering with the rearrangement of the actin cytoskeleton by dephosphorylating Vav [316], SHP-1 could be involved in inhibitory receptor-mediated blocking of activatory receptor clustering and lipid raft aggregation [320].

By hydrolysing the PI3-kinase product phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-$P_3$)

**Table 1.1 Inhibitory receptors expressed on the surface of NK cells**

| | Name | Ligand | Signalling Motif |
|---|---|---|---|
| **MHC Class I specific receptors** | KIR2DL | HLA-C | ITIM |
| | KIR3DL | HLA-A,B | ITIM |
| | LILR1/2 | HLA Class I molecules | ITIM |
| | CD94/NKG2A/B | HLA-E | ITIM |
| **Receptors for non MHC Class I molecules** | LAIR | Collagen | ITIM |
| | Siglec 7,9 | Sialic Acid | ITIM |
| | KLRG1 | Cadherin | ITIM |
| | NKR-P1 | Lectin-like transcript 1 | ITIM |

**Abbreviations;** HLA, Human leukocyte antigen; ITIM, Immunoreceptor tyrosine based inhibitory motif; KIR, Killer cell immunoglobulin-like receptor; KLRG1, Killer cell lectin-like receptor G1; LAIR, Leukocyte associated immunoglobulin-like receptor; LILR, Leukocyte immunoglobulin-like receptor; NKR-P1, Inhibitory NK cell receptor protein 1; Siglec, Sialic acid binding immunoglobulin-like lectins.

**Table 2.2. Steps in NKCC that are targeted by NK cell inhibitory receptor signalling**

| Site of action | Description | Reference(s) |
|---|---|---|
| **Conjugate formation** | Ligation of KIR2DL1 disrupts NK cell adhesion to its target by recruiting SHP-1 to the NK–target cell interface. | [321] |
| **Activation receptor clustering** | KIR2DL1 ligation hampers NKG2D mediated NK cell activation by reducing NKG2D translocation to lipid rafts.<br><br>KIR2DL1 and KIR2DL2 signalling suppresses activation receptor clustering at the immunological synapse by disrupting the recruitment of actin binding proteins. | [320]<br><br>[252;322] |
| **Proximal signalling events** | **Activatory receptors** – KIR2DL1 or CD94/NKG2 ligation prevents phosphorylation of tyrosine motifs within the co-stimulatory receptor CD244.<br><br>**Adapter molecules** – The transmembrane adapter molecules SLP-76 and LAT are thought to be direct substrates of SHP-1.<br><br>**Phosphorylated proteins** – Inhibitory receptor ligation interferes with the phosphorylation status of molecules that propagate activatory receptor signalling. These include Syk family kinases, PLC and Vav. Inhibiting PLC activity prevents inositol phosphate turnover and calcium mobilisation | [323]<br><br>[314;315]<br><br>[316;319;324] |
| **Polarisation** | Overexpression of the phosphatase SHP-2 in NK cell lines suppressed polarisation of the microtubule organising centre.<br><br>KIR or CD94/NKG2 ligation inhibits LFA-1 or NKG2D induced granule polarisation. | [325]<br><br>[326] |
| **Degranulation** | Degranulation induced by CD16 was reduced upon co-ligation of the inhibitory receptor CD94/NKG2A | [327] |

**Abbreviations:** KIR, Killer-cell immunoglobulin-like receptor; PLCγ, Phospholipase Cγ; SHP, Src homology phosphatase-1; SLP-76, Src homology 2 domain-containing leukocyte protein of 76 kDa; Syk, Spleen tyrosine kinase.

into phosphatidylinositol-3,4-bisphosphate (PI-3,4-$P_2$) [328], SHIP-1 is a negative regulator of PI3-kinase dependent signalling, and therefore influences many aspects of NK cell biology. For example, SHIP-1 mediated breakdown of PI-3,4,5-$P_3$ has been shown to reduce IFN-γ production by monokine-stimulated NK cells [329] and to negatively regulate antibody dependent cell cytotoxicity (ADCC) induced by the engagement of the activating receptor CD16 [330]. Furthermore, by reducing PI-3,4,5-$P_3$ levels, SHIP-1 would also affect the recruitment into the developing IS of such pleckstrin homology-domain containing activatory proteins as protein kinase B, pyruvate dehydrogenase kinase 1 and PLC- γ.

#### 1.5.2.3.2 Activatory receptors

If, according to the missing-self hypothesis, NK cell activity can be induced simply by the "*absence or reduced expression of self molecules"* [308], then why don't NK cells elicit cytotoxicity towards self cells with low or no MHC class I expression? One reason is that, with the odd exception [331;332], the induction of NKCC requires not only the absence of inhibitory signals but also the ligation of activatory receptors [333].

NK cell activatory receptors belong in the main to one of two families: (i) the immunoglobulin superfamily, members of which include the low affinity IgG Fc receptor CD16 (FcγRIII), the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, and the activatory KIRs, KIR2DS and KIR3DS or (ii) the C-type lectin family, to which the receptors CD94/NKG2C, CD94/NKG2E and NKG2D belong [333]. Through the expression of these receptors, NK cells recognise a range of targets, which include IgG coated cells that are detected by CD16 and transformed cells, whose expression of the stress-inducible glycoproteins MHC class I-chain-related protein A (MICA) and MICB is recognised by NKG2D [331]. Moreover, by possessing the NCRs NKp44 and NKp46, whose ligands

include viral haemagglutinin [334;335] and bacterial surface proteins [336], NK cells can recognise directly the presence of invading pathogens.

All of the NK cell activatory receptors mentioned above possess short cytoplasmic tails, and therefore possess no intrinsic signalling activity. Thus, in order to transmit information, these receptors, with the exception of NKG2D, associate with signalling polypeptides that contain immunoreceptor tyrosine based activatory motifs (ITAMs). These adaptor molecules include CD3ζ and FCRγ, which associate with CD16, NKp30 and NKp46, and DNAX-activation protein-12 (DAP12), which couples to NKp44, the activatory KIRs as well as CD94/NKG2C and CD94/NKG2E [337]. Following ligand recognition, tyrosine residues within ITAMs are phosphorylated by Src-family kinases (e.g. Lck and Fyn), which leads to the recruitment and subsequent activation of the Syk family kinases Syk and ZAP70. Once activated, these kinases phosphorylate various transmembrane adaptor molecules, including LAT and SLP-76, which propagate activatory receptor signalling by serving as docking sites for downstream SH2 domain containing signalling molecules (e.g. PI3-kinase, PLC-γ1) and adaptor proteins (e.g. Son of Sevenless and Vav) (Figure 1.3). Interestingly, whilst activatory receptors differ in proximal signalling modules, activatory signals converge on common distal signalling molecules to induce NKCC and cytokine production (Figure 1.3). For example, Chen and colleagues recently demonstrated that despite utilising different proximal signalling molecules, ligation of NKp30, NKp46 or CD94/NKG2C resulted in the phosphorylation of the MAPK family members ERK2 and JNK1, whose activation was crucial for lytic granule polarisation and NKCC [338].

As mentioned above, unlike the majority of activatory receptors, NKG2D does not interact with an ITAM containing adaptor. Instead, it associates with DAP10, a transmembrane adaptor that possesses a YINM motif in its cytoplasmic tail [339]. Phosphorylation of

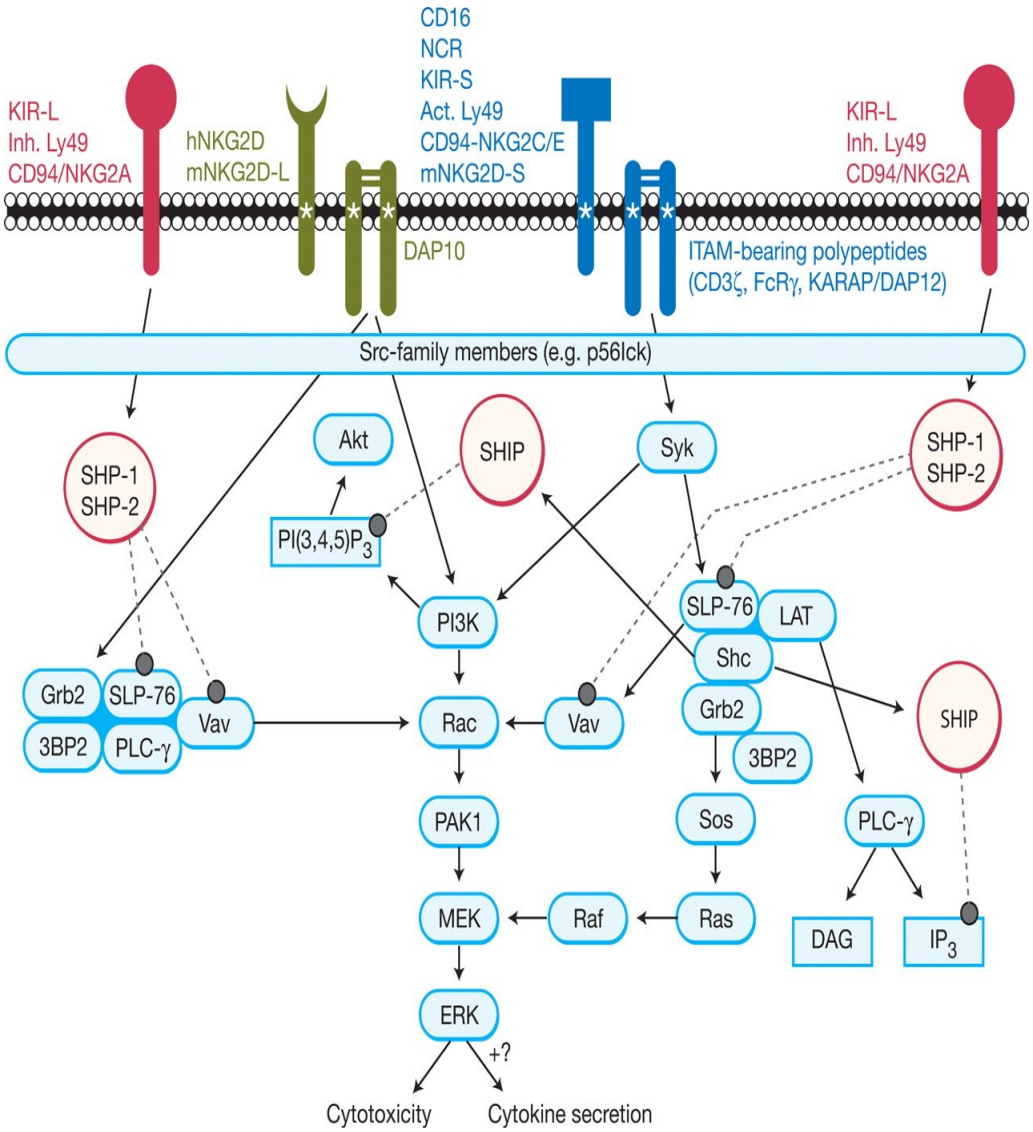



**Figure 1.3 Signalling pathways emanating from ITAM-associated NK activatory receptors.** Figure taken directly from review article by Vivier et al [337].

tyrosine residues within this domain leads to the recruitment of PI3-kinase and growth factor receptor-bound protein 2 [340;341], who initiate signalling pathways that ultimately feed into those utilised by ITAM-associated receptors (Figure 1.3). For instance, NK cells activated through NKG2D exhibit strong MAPK signalling and PLC-γ1 activation [260]. In contrast to other activatory receptors, signals transmitted to NK cells through NKG2D can override those delivered by inhibitory receptors, allowing NK cells to eliminate MHC class I positive targets [331;332]. This phenomenon, which appears to be unique to NKG2D, may reflect the fact that this receptor resides permanently in lipid rafts, membrane domains renowned for eliciting and sustaining signalling activity [320].

### 1.5.3 NK cell subsets

Human NK cells are phenotypically characterised by the surface expression of CD56, an isoform of the human Neural Cell Adhesion Molecule, and the absence of CD3. However, they are not a homogenous population, as based on the differential surface expression of CD56 and the low affinity IgG Fc receptor CD16, NK cells can be categorised into one of five distinct subsets: (1) $CD56^{DIM}$ $CD16^{BRIGHT}$, (2) $CD56^{DIM}$ $CD16^{-}$, (3) $CD56^{BRIGHT}$ $CD16^{DIM}$, (4) $CD56^{BRIGHT}$ $CD16^{-}$ and (5) $CD56^{-}$ $CD16^{BRIGHT}$. Numerically, the dominating subsets within peripheral blood are $CD56^{DIM}$ $CD16^{BRIGHT}$ and $CD56^{BRIGHT}$ $CD16^{DIM/-}$, which differ markedly in both phenotype and function (Figure 1.4).

#### 1.5.3.1 $CD56^{BRIGHT}$ NK cells

Via the expression of the chemokine receptors CCR7 and CXCR3, as well as the adhesion molecule CD62L, peripheral $CD56^{BRIGHT}$ NK cells possess a surface phenotype that allows them access to SLO [342]. Indeed, whilst amounting to only around 5-10% of the circulating NK cell pool, $CD56^{BRIGHT}$ NK cells constitute 15% of the total NK cell population present

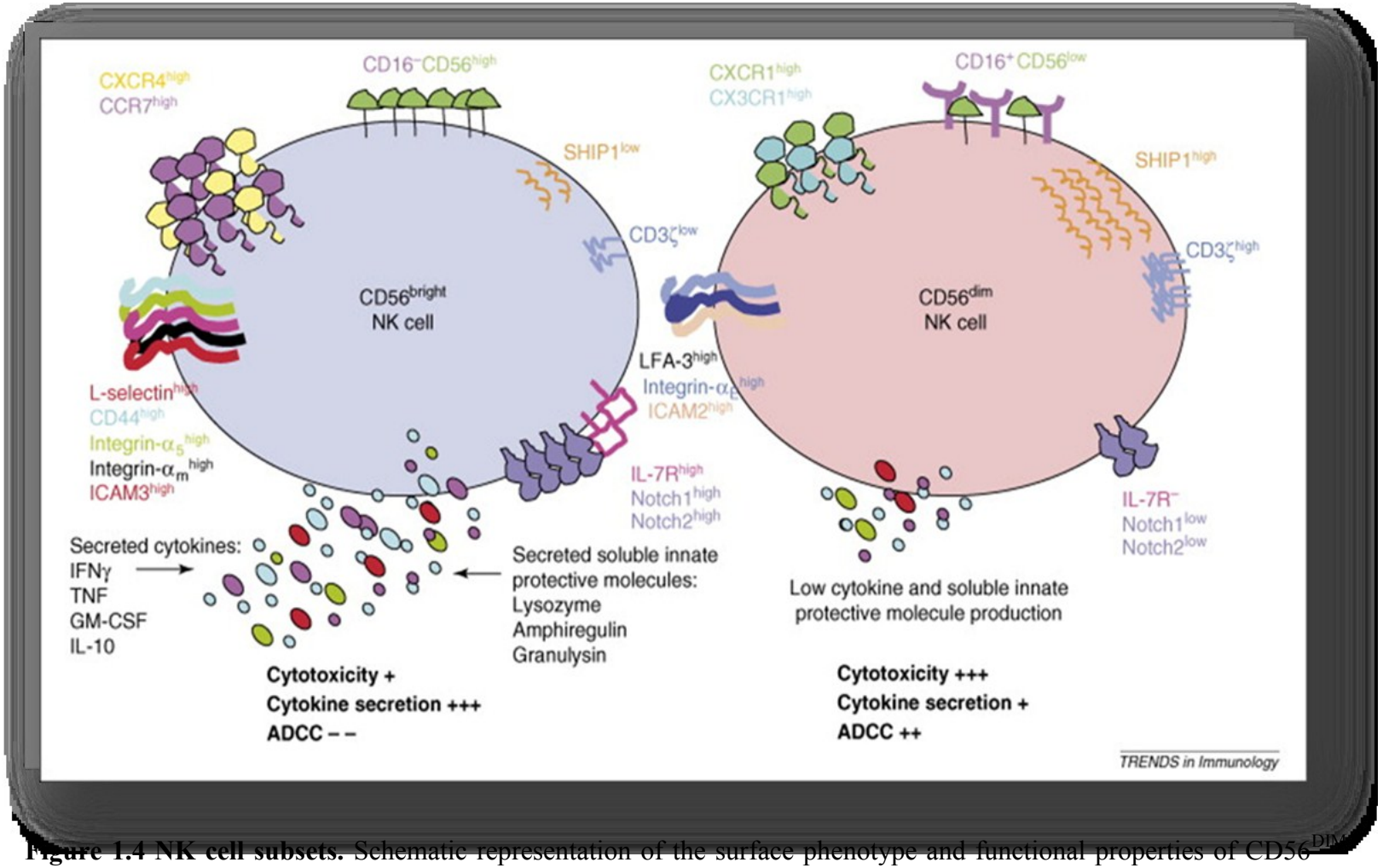



**Figure 1.4 NK cell subsets.** Schematic representation of the surface phenotype and functional properties of $CD56^{DIM}$ $CD16^{+}$ and $CD56^{BRIGHT}$ $CD16^{-}$ NK cells, the two predominant subsets of NK cells within human peripheral blood. *Figure taken directly from review article by Hanna et al [343].*

within the spleen and 75-95% of the NK cells resident in the lymph nodes and tonsils [276;344]. Interestingly, SLO resident $CD56^{BRIGHT}$ NK cells do not express CCR7 or CD62L [276], suggesting that the surface phenotype of this NK subset differs depending upon its location. Indeed, compared to their peripheral counterparts, $CD56^{BRIGHT}$ NK cells in SLO are $NKp30^{-}$ and show reduced expression of NKp46 and CD94/NKG2A [276]. In addition to location, the activation status of $CD56^{BRIGHT}$ NK cells also influences their surface phenotype, as exposure to IL-2 has been shown to not only increase surface NKp46 levels but induce expression of NKp30, NKp44, CD16 and KIR [14734722].

Irrespective of anatomical location and activation status, $CD56^{BRIGHT}$ NK cells express receptors for a range of cytokines including IL-1, IL-2, IL-10, IL-12, IL-15 and IL-18 [345]. The IL-2 receptor is expressed constitutively in both its intermediate (IL-2Rβγ) and high affinity (IL-2Rαβγ) conformations, enabling $CD56^{BRIGHT}$ NK cells to respond to picomolar concentrations of this cytokine [344;346].

Classically, $CD56^{BRIGHT}$ NK cells are viewed as the predominant source of the NK cell-derived immunoregulatory cytokines and chemokines described in section 1.5.1.2. However, recent evidence suggests that this is dependent upon the type of stimulation. For example, $CD56^{BRIGHT}$ NK cells secrete significantly more IFN-γ, GM-CSF, IL-10 and IL-13 than $CD56^{DIM}$ NK cells following stimulation with IL-2, IL-12 or IL-15 [275;276;292;344], but their production of both cytokines (TNF-α and IFN-γ) and chemokines (e.g. MIP-1α and MIP-1β) is negligible following target cell stimulation [277;292], suggesting that $CD56^{BRIGHT}$ NK cells are a "cytokine responsive" subset [292;347].

In keeping with their preference to respond to cytokines, resting $CD56^{BRIGHT}$ NK cells show little cytotoxic activity against tumour targets, which is attributable in part to their low perforin content and weak capacity to form conjugates [348;349]. However, following

stimulation with IL-2, $CD56^{BRIGHT}$ NK cells exhibit a level of NKCC that is comparable to that of the cytotoxic $CD56^{DIM}$ NK cell subset, an enhancement borne in part from an IL-2 induced increase in NCR and NKG2D expression [276;350].

#### 1.5.3.2 $CD56^{DIM}$ NK cells

$CD56^{DIM}$ NK cells comprise approximately 90% of the circulating NK cell pool, and are therefore the most abundant NK cell subset in human peripheral blood [276]. Through the expression of the chemokine receptors $CX_3CR1$ and CXCR1, $CD56^{DIM}$ NK cells are attracted to sources of IL-8 and fractalkine [342]. By responding strongly to such signals, $CD56^{DIM}$ NK cells home to sites of inflammation, where due to their high expression of both perforin and granzyme A, and a strong capacity to form conjugates, they mediate potent cytotoxicity [348;349]. The induction of NKCC is tightly controlled through the expression of several inhibitory and activatory receptors, which include KIR family members, NKG2D, NKp30, NKp46, CD94 and CD16 [351;352]. Thus, $CD56^{DIM}$ NK cells can respond directly to pathogens through NKp46, recognise transformed cells via NKG2D and mediate ADCC through CD16.

For many years, the role of $CD56^{DIM}$ NK cells in host protection was thought to be restricted to the removal of transformed cells through direct cytotoxicity. However, a series of recent studies have shown that following target cell recognition or crosslinking of activating receptors, $CD56^{DIM}$ NK cells secrete substantial levels of IFN-γ, TNF-α, MIP-1α and MIP-1β [277;278;353], suggesting that in addition to mediating NKCC, $CD56^{DIM}$ NK cells are an important early source of immunoregulatory cytokines and chemokines. Interestingly, the generation of these soluble mediators is marginal when this subset is subjected to cytokine stimulation, suggesting that $CD56^{DIM}$ NK cells are a "target responsive" subset [347].

### 1.5.4 Age associated changes in NK cell function and phenotype

#### 1.5.4.1 NK cell proportions and absolute number

A significant increase in the percentage and/or absolute number of $CD3^-$ $CD56^+$ NK cells within the peripheral lymphocyte pool is the general finding reported by studies that have investigated the effect of age on NK cell frequency [354-357]. When the composition of the circulating NK cell pool has been studied, age has been shown to have a differential effect on the two main circulating subsets. Whilst the proportions and/or number of $CD56^{DIM}$ NK cells increase with age [351;354], old subjects possess significantly fewer $CD56^{BRIGHT}$ NK cells [351;354;355], resulting in a marked age-related increase in the circulating $CD56^{DIM}$:$CD56^{BRIGHT}$ ratio [352;358]. As physiological ageing is accompanied by a reduction in both NK cell production and proliferation, the increase in NK cells that occurs with age has been proposed to result from an accumulation of long-lived NK cells in older adults [359].

#### 1.5.4.2 NK cell phenotype

Flow cytometric analysis of peripheral blood NK cells has shown physiological ageing to be accompanied by changes in surface phenotype. It has been reported by a number of groups that $CD3^-$ $56^+$ $57^+$ cells constitute a greater proportion of the circulating NK pool in old individuals when compared to younger adults [355;356;360], whilst $CD56^{DIM}$ NK cells isolated from old donors express higher levels of the antigenic determinant HLA-DR and the death receptor Fas, but lower levels of the activatory marker CD69 when compared to $CD56^{DIM}$ NK cells from their young counterparts [361]. As CD57 expression is considered to be a sign of NK cell maturity, the increased percentage of CD57 positive NK cells in the periphery of old subjects suggests ageing is accompanied by a shift in the maturity status of

the circulating NK pool.

The effect of age on the expression levels of certain NK cell activatory receptors is controversial. For example, whilst some studies have reported a decline with age in the percentage of NK cells expressing NKp30 or NKp46 [351;362], which in the case of NKp30 is accompanied by a reduction in its surface density [362], other studies have demonstrated similar proportions of NK cells bearing these receptors in young and old donors [355]. More consistent findings have however been reported for NKG2D, CD16 and the co-activatory receptor CD2 whose levels are all maintained with age [352;355;356].

With regards to human ageing and NK cell inhibitory receptors, expression of KIR on the surface of NK cells from old subjects has been shown to be either increased or comparable to the levels present on NK cells isolated from young donors [351;354;356], whilst marked age-associated reductions in KLRG-1 and NKG2A have been documented [352;354;356], as has a decline in CD94, the binding partner of NKG2A [351;352;356].

Inter-study differences in experimental protocol is the reason most frequently cited to explain the contrasting effects that human ageing has been proposed to have on the expression of activatory and inhibitory receptors. However, it is now being recognised that in a manner similar to that reported for T lymphocytes, infection with CMV can influence NK cell receptor expression. Studies have shown that the percentage of NK cells expressing KLRG-1, NKp30 or NKp46 is markedly reduced in CMV seropositive individuals when compared to their seronegative counterparts [363;364]. Conversely, $CD57^{+}$ NK cells have been reported to form a greater proportion of the circulating NK cell pool in subjects positive for CMV [364]. Interestingly, this ability of CMV to modify NK cell receptor expression has been described by studies performed solely on young adults, suggesting CMV can induce a surface phenotype that is reminiscent of NK cells isolated from aged subjects. Thus, rather than being

a consequence of the ageing process, the alterations reported in NK surface phenotype between young and old subjects may simply reflect cohort differences in the number of CMV positive subjects [364].

#### 1.5.4.3 NK cell function

##### 1.5.4.3.1 NKCC and ADCC

Groups that have investigated the effect of age on the natural cytotoxicity of resting NK cells have focused exclusively upon target cell death induced by the granule exocytosis pathway. Whilst some studies have demonstrated a marked age-related decline in NKCC [42;357;365], others have reported the lytic activity of NK cells to be increased [366-368] or unchanged [351;365;369] with age. Underlying these contrasting observations are marked inter-study differences in experimental design. For example, whilst some groups performed NKCC assays with PBMCs [351;365;367;369], others used peripheral blood lymphocytes (PBL) [357;368] or purified NK cells [42;365] as their effector population. Furthermore, studies differed in the length of time they cultured NK cells with their targets before assessing NKCC, the significance of which was illustrated in the work of Rukavina and colleagues, who found NKCC exhibited an age-related impairment in short-term (2 hours) assays, but after prolonged co-culture (18 hours) NK lytic activity was comparable between young and old donors [370].

In spite of these conflicting findings, the general belief within the NK cell field is that at the single cell level, NKCC is reduced with age. Indeed, an age-related increase in the proportions and/or absolute numbers of circulating NK cells was reported by many of the studies that found NKCC to be comparable between young and old donors when PBMCs were used as effector cells [351;365;371]. Moreover, an age-associated decrease in lytic

activity has been reported by the majority of groups that have used purified NK cells at fixed concentrations in their cytolytic assays [42;365;371].

In an attempt to unearth the mechanism(s) responsible for the age-related decline in NKCC at the single cell level, a number of groups have examined the effect of age on the expression of NK cell receptors and cytotoxic proteins, as well as key events in the process of NKCC. An observation that has been reported by several groups is that NK cells from older adults recognise and bind to tumour targets as effectively as those from younger subjects, suggesting the age-related impairment in NKCC is the result of a post-binding defect [365;372-374]. However, based on current data it is not entirely clear where this defect lies. For example, whilst some groups have reported perforin expression is markedly reduced with age [370], others have shown the levels of this pore-forming protein, as well as its mobilisation and utilisation in response to target cell contact to be comparable between NK cells from young and old donors [375]. Likewise, whilst some studies have reported reduced expression of the activatory receptor NKp30 on the surface of NK cells from aged subjects, and proposed that this would have an adverse effect upon NK cell killing [351;362], other groups that have studied this receptor have reported no age-related difference in its expression [355]. Thus, despite the general acceptance that NKCC is impaired at the single cell level with age, the mechanism(s) responsible are currently unknown.

In contrast to natural cytotoxicity, ADCC is unaffected by the ageing process. Studies have shown no difference in the surface expression of the Fc receptor CD16 with age [352] or in the ability of NK cells from old donors to induce lysis of antibody-coated target cells when compared to their younger counterparts [356;376;377].

#### 1.5.4.3.2 Response to and production of immunoregulatory cytokines and chemokines

Whilst resting NK cells efficiently lyse MHC class I deficient K562 tumour cells, they exhibit little cytotoxicity against Daudi tumour cells due to the absence of NK cell priming signals on the surface of the latter cell line [303]. The resistance of Daudi cells to NKCC is overcome by pre-treating NK cells with pro-inflammatory cytokines, a procedure that generates lymphokine activated killer (LAK) cells. Ageing appears to have no effect upon the ability to make LAK cells, as a number of studies have shown NK cells from old donors treated with IFN-α [358] IFN-γ [358], IL-2 [378;379] or IL-12 [379] exhibit greater cytotoxicity against Daudi targets than their untreated counterparts. However, whether ageing impacts upon the cytotoxic capacity of LAK cells is currently unclear, with some groups demonstrating comparable LAK activity between young and old subjects [358;378;380], and others reporting a marked age-associated reduction in LAK-mediated cytotoxicity following IFN-α, IFN-γ or IL-2 treatment of resting NK cells [367;379].

Although NK cells isolated from old individuals are capable of up-regulating their production of cytokines (e.g. IFN-γ) and chemokines (e.g. MIP-1α, IL-8 and regulated on activation normal T cells expressed and secreted (RANTES)) in response to cytokine stimulation [358;378;380;381], the levels generated are often significantly lower than those produced by NK cells from younger subjects [378;380-382]. The aforementioned age-related decline in the frequency and absolute number of cytokine-responsive $CD56^{BRIGHT}$ NK cells [351;354;355] is likely to be one mechanism contributing to this impaired functional response.

### 1.5.5 Clinical significance of age-associated changes in NK cell number and function

The recurrent viral episodes experienced by individuals with selective NK cell deficiency [192-194] highlights the *in vivo* importance of NK cells in host protection. However, what impact do the aforementioned age-related changes in NK cell number and function have on human health, and are they clinically relevant?

Having observed in a small-scale retrospective study that low NK cell activity in elderly people was associated with a past history of severe infection [188], Ogata and colleagues undertook a prospective study to confirm a relationship between low NK activity and an increased susceptibility to infection. Supporting their previous work, they noted low NKCC at baseline was associated not only with an increased risk of developing infection but with a reduced probability of surviving infectious episodes [189], a finding in line with an earlier longitudinal study that had shown persistently low NK cell activity to be a predictor of infectious morbidity in old individuals [187]. In a five-month follow-up study investigating the immunomodulatory effect of influenza vaccination in aged subjects, Mysliwska et al [383] found NKCC post vaccination to be significantly higher in those individuals who attained protective antibody titres, whilst those with low vaccination-induced NK cell activity exhibited an increased frequency of respiratory tract infection [383]. Failure to counteract the age-associated reduction in NKCC by increasing the circulating number of NK cells also appears to be detrimental to health. A three-fold increase in mortality risk was reported in the first 2 years of follow-up for elderly individuals with low NK cell numbers when compared to those with high NK cell numbers [384].

Further evidence, albeit indirect, highlighting the importance of NK cells in healthy ageing is the results of studies that have been performed on centenarians. Given their extended longevity and avoidance of major age-associated diseases, immune gerontologists consider

centenarians to be a model of successful human ageing. NK cells isolated from these individuals have been shown to exhibit a level of cytotoxicity that is comparable to that of young adults, and significantly greater than that of middle-aged subjects [385;386], suggesting that preservation of NKCC is important for healthy ageing.

### 1.6 Neutrophils

As the first immune cell recruited to a site of infection, neutrophils are a hosts front-line defence against invading pathogens. Unsurprisingly therefore, these cells are equipped with an array of anti-microbicidal mechanisms, which enable them to contain and directly eliminate foreign antigens. Pathogens are contained via phagocytic uptake and entrapment within recently described neutrophil extracellular traps (see section 1.6.2), and eliminated by exposure to free radicals and proteolytic enzymes and peptides [387]. However, it has become increasingly apparent over recent years that the role of neutrophils in host defence extends beyond simply containing and eliminating foreign pathogens. Although terminally differentiated, neutrophils secrete a plethora of immunoregulatory chemokines such as MIP-1α, MIP-1β, IL-8 and growth-related oncogene-α [388-390], and although contested by some groups [388;391] also appear to be an early source of pro-inflammatory cytokines [392-394]. Through the secretion of these soluble mediators, neutrophils are able to orchestrate immune responses by regulating the recruitment and activity of bystander immune cells. Furthermore, it is now evident that neutrophils influence the initial stages of an adaptive immune response, with studies demonstrating neutrophils are able to drive DC maturation [395], transport antigens to secondary lymphoid tissue [396;397], serve as antigen presenting cells [398;399] and modulate both T helper cell differentiation and $CD8^+$ immune responses [399;400].

### 1.6.1 Effect of age on neutrophil biology

#### 1.6.1.1 Neutrophil production

Produced by the bone marrow at a rate of 1-2x$10^{11}$ per day, neutrophils are the most abundant leukocyte in human circulation. With age the proliferative response of neutrophil precursor cells to granulocyte colony stimulating factor (G-CSF) deteriorates [401], whilst the response to granulocyte macrophage colony stimulating factor (GM-CSF) and IL-3 stimulation is maintained [401]. Despite a reduced sensitivity to G-CSF, studies have shown that in both the steady-state [402] and in times of acute infection [403], circulating numbers of neutrophils are comparable between young and old individuals, suggesting that the process of granulopoiesis is unaltered with age.

#### 1.6.1.2 Neutrophil adherence and chemotaxis

To arrive at sites of antigenic challenge, neutrophils must first adhere to activated endothelium, a step that requires interactions between an array of endothelial ligands and their cognate receptors on the neutrophil surface. Studies that have examined the effect of age on neutrophil receptors have reported no differences in the levels of CD15 [404] or in the expression of the integrins CD11a and CD11b [404-406], which mediate rolling adhesion and firm adhesion respectively, suggesting aged neutrophils would be capable of binding the activated endothelium. Indeed, several *in vitro* studies have shown the binding of stimulated neutrophils obtained from aged donors to gelatin [407], fibronectin [408], nylon fibres [409] and endothelial monolayers [407] is comparable or even increased when compared to that of neutrophils isolated from younger adults.

Once across the vascular endothelium, neutrophils migrate to the site of antigenic challenge along chemotactic gradients comprised of complement factors (e.g. complement component

5a (C5a)), chemokines (IL-8, MIP-1α) and bacterial products (e.g. formyl-methionine-leucine-phenyalanine (fMLP)). Based on the findings of *in vitro* studies it would appear that neutrophil migration is impaired with age, as numerous studies have reported defects in the chemotactic response of aged neutrophils to fMLP [409-412] and GM-CSF [412]. However, these findings are not supported by *in vivo* data. With the exception of a single study, which reported an age-associated reduction in the arrival of neutrophils to sites of damaged skin [413], both murine [104;414] and human [415] studies that have investigated *in vivo* neutrophil migration have shown this function to be comparable between young and old subjects.

#### 1.6.1.3 Anti-microbial defence

Neutrophils express on their surface a multitude of receptors that enable them to detect and attach to infecting microbes. These include complement receptors (e.g.CD35, CD18/CD11b), TLRs (e.g.TLR4) and the Fc receptor CD16. Neutrophil activation induced by ligation of these receptors can trigger pathogen uptake, a crucial first step in neutrophil-mediated defence.

Whilst neutrophils from aged individuals are as efficient as those from young donors at ingesting non-opsonised particles [416], their phagocytic activity towards opsonised pathogens is markedly reduced [404;405;409;417;418]. Studies have shown that physiological ageing is associated with a decline in both the percentage of neutrophils capable of phagocytosing opsonised microbes [404;405;409;418], and in the number of microbes ingested per cell [405;409;418], two defects that together result in a reduced phagocytic efficiency. From current data, it would appear that the age-related decline in the uptake of complement coated microbes is the result of an intrinsic signalling defect since expression of complement receptors are unaltered with age [419], and serum from aged

individuals opsonises pathogens as effectively as that obtained from young subjects [405]. In contrast, the reduced efficiency by which aged neutrophils ingest antibody-coated microbes has been attributed to an age-associated loss of CD16 from the neutrophil surface, although this view is not shared by all groups [405;419].

Pathogens engulfed by neutrophils are exposed to a harsh microbicidal environment. Playing an important role in the creation of this environment is nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a membrane bound multi-subunit enzyme whose reduction of NADPH generates superoxide ($0^{2-}$). When challenged with *Candida albicans* (*C.albicans*), zymosan or *Escherichia coli* (*E.coli*), aged neutrophils generate $0^{2-}$ levels that are comparable to those produced by neutrophils from young subjects [418;420;421]. However, their production of $0^{2-}$ following stimulation with fMLP, GM-CSF or opsonised *Staphylococcus aureus* (*S. aureus*) is markedly lower [408;412;415;418;420;421]. Since the surface expression of fMLP and GM-CSF receptors is unaltered with age [422-424], the impaired response elicited by aged neutrophils towards these stimuli may be borne from abnormalities in the neutrophil cell membrane and/or defects in proximal intracellular signalling. In support of this idea, no study to date has reported an age-related difference in $0^{2-}$ production following stimulation with PMA [408;425;426], a pharmacological agent that bypasses the cell membrane to activate NADPH oxidase. Furthermore, when analysing the lipid composition of peritoneal neutrophil membranes from young and old rats, Alvarez and colleagues discovered a significant age-related decrease in cholesterol content as well as a marked increase in phospholipids, changes that were associated with increased membrane fluidity and reduced $0^{2-}$ production upon fMLP stimulation [425].

Through the process of degranulation, neutrophils are able to eliminate pathogens extracellularly. Based on the work of two independent studies, which found that when

compared to fMLP-stimulated neutrophils from young controls, those isolated from old donors released significantly fewer proteases into the extracellular environment [410;427], it seems that this form of neutrophil defence also wanes with age.

#### 1.6.1.4 Priming and apoptosis

Circulating neutrophils are short-lived cells, possessing an estimated half-life of 7-10 hours. At sites of infection and inflammation, neutrophil lifespan is increased, primarily as a result of exposure to pro-inflammatory cytokines (e.g. GM-CSF, G-CSF) and microbial products (e.g. LPS). Indeed, when treated *in vitro* with these pro-inflammatory mediators, neutrophils are rescued from apoptosis. However, GM-CSF and LPS are not as efficient at prolonging the lifespan of neutrophils from elderly donors when compared to those of young individuals [423;428-431], suggesting that the sensitivity of neutrophils to these survival signals declines with age.

GM-CSF promotes neutrophil survival by increasing the expression of the anti-apoptotic protein induced myeloid leukaemia cell differentiation protein (Mcl-1), and reducing the expression of the pro-apoptotic protein Bax. However, in aged neutrophils, the levels of both proteins are unchanged following GM-CSF treatment [428]. This inability to respond to GM-CSF has been attributed to defective intracellular signalling. For instance, whilst stimulation of neutrophils from young donors with GM-CSF is associated with a significant decline in the activity of the tyrosine phosphatase SHP-1, a negative regulator of GM-CSF signalling, no reduction in SHP-1 activity is observed in aged neutrophils [429]. Consequently, the activity of signalling pathways that promote neutrophil survival in response to GM-CSF, such as the Janus kinase/signal transducer and activator of transcription pathway as well as ERK and AKT signalling is significantly lower in neutrophils from aged donors when compared to those from their younger counterparts [422;423;432].

As well as a survival factor, GM-CSF is a priming agent needed for the optimal activation of neutrophils. Thus, the age-related decline in neutrophil sensitivity to GM-CSF impacts not only upon their lifespan but also their microbicidal capacity. Indeed, two independent studies have shown GM-CSF primed neutrophils from young donors generate significantly more $0^{2-}$ following fMLP stimulation than those from older individuals [412;433]. When these findings are considered alongside those regarding neutrophil apoptosis, it is reasonable to suggest that *in vivo*, neutrophils from elderly donors would respond less effectively to stimulation and die more rapidly, which would adversely affect their ability to eradicate foreign pathogens.

#### 1.6.1.5 Clinical relevance of age-associated changes in neutrophil function

Neutrophils isolated from elderly subjects exhibit reduced phagocytic killing of bacteria and fungi when compared to neutrophils from younger individuals [418;419;434], illustrating that the age-related changes described above do impact upon the protective nature of these cells. However, are these *in vitro* observations of clinical significance? To our knowledge, only one study to date has addressed this issue. In this study, in which a cohort of elderly individuals were followed over seven years, Niwa and colleagues found that when compared to those who survived to the end of the study, "non-survivors" presented with significantly impaired neutrophil chemotaxis at baseline [411]. Importantly, defective neutrophil migration was observed in all subjects in whom infection was the cause of death, suggesting that aberrant neutrophil function may contribute some part to the increased incidence and severity of infection amongst the elderly population.

### 1.6.2 Neutrophil extracellular traps (NETs)

In a seminal paper published in 2004, Brinkmann and colleagues described that upon stimulation with pro-inflammatory cytokines and whole pathogens, neutrophils released into the extracellular environment their nuclear DNA coated with granule-derived peptides and enzymes [435]. Termed neutrophil extracellular traps (NETs), these structures are ejected from dying neutrophils, and as such are seen as the "last resort" in neutrophil-mediated protection, where they play an important role in pathogen containment. As an unusual form of cell defence, NETs have received considerable attention from a number of groups, meaning that over the last eight years our understanding of how these structures are formed, their function, and their relevance *in vivo* has grown considerably.

#### 1.6.2.1 Structure and function of NETs

Consisting of a DNA backbone decorated with a multitude of anti-microbial peptides and enzymes, NETs are mesh-like structures approximately 100 nm in diameter. With the exception of histones, the anti-microbial proteins present on NETs are derived almost exclusively from neutrophil granules and include: myeloperoxidase (MPO), lactoferrin, bacterial permeability increasing protein, neutrophil elastase (NE), and cathepsin G [435;436]. By binding these proteins, NETs prevent their dissemination, and thus protect surrounding tissue from collateral damage.

As would be predicted from their name, NETs act as physical barriers, trapping a range of microbes, which include both gram positive and gram negative bacteria [435;437;438], the fungal pathogen *C. albicans* [439], and the parasites *Leishmania amazonensis* and *Plasmodium falciparum* [440;441]. Microbial attachment to NETs is mediated predominantly by ionic forces between the negatively charged pathogen surface and the positively charged

anti-microbial proteins that coat NETs. However, other factors can influence pathogen entrapment, and include surfactant protein D, a member of the collectin family that has recently been shown to augment pathogen trapping by binding simultaneously to NET fibres and carbohydrate ligands present on the surface of bacteria [438].

Numerous studies have reported reduced survival rates for pathogens cultured with neutrophils that have been pre-treated with NET-inducing stimuli [439;440;442;443]. As anti-microbial peptides present on NETs retain their functionality [444-446], this reduction in pathogen viability has been assigned to direct microbial killing by NETs. Indeed, treating neutrophils with deoxyribonucleases (DNase) to digest NETs prior to or in conjunction with the addition of micro-organisms markedly increases pathogen survival [435;439;440;447]. However, as pathogen viability is often determined by enumerating colony forming units, could it be that pathogens are simply trapped in NETs but still alive? Recent data from two independent groups [445;447], who dismantled NETs with DNase prior to measuring pathogen viability, suggest that this is the case. In both instances, NET degradation resulted in a greater recovery of viable bacteria, suggesting that pathogens trapped in NETs remain alive. On this evidence it has been proposed NETs lack microbicidal activity, and that these structures are important only for pathogen containment [445;447;448].

Whilst NETs may not eliminate pathogens directly, they may contribute to the clearance of infectious organisms by modulating innate and adaptive immune responses. For instance, activation of the contact system, a branch of humoral innate immunity, occurs on NET fibres and would result in the generation of pro-inflammatory and anti-microbial peptides [449]. In addition, pentraxin 3, a soluble pathogen recognition receptor that binds the complement component C1q, is present in NETs, where it could activate this innate form of host defence [450]. With regards to adaptive immunity, Tillack and colleagues found that by priming T

cells, NETs lower the activatory threshold of these cells allowing them to elicit functional responses to suboptimal stimuli [451].

#### 1.6.2.2 Molecular mechanisms underlying the formation of NETs

Three distinct pathways have been described for how neutrophils generate and release extracellular traps. The first is an oxidant dependent process defined by (i) degradation of the nuclear envelope, (ii) disintegration of granule membranes and (iii) cell lysis [443]. These steps are triggered by a range of stimuli (e.g. PMA and bacteria), and are complete within 3-4 hours of neutrophil activation. The second pathway, which is oxidant independent, generates NETs within 30-60 minutes, and is characterised by the fusion of nuclear membrane derived vesicles with the plasma cell membrane [452]. The third pathway of NET generation stands alone in as much as it drives neutrophils to expel DNA that is of mitochondrial rather than nuclear origin [453]. Throughout the remainder of this introduction, only NET formation that occurs through the first pathway outlined above will be discussed.

Pro-inflammatory cytokines (e.g. IL-8, TNF-α), pharmacological agents (e.g. PMA), bacteria, fungi and protozoa are stimuli that trigger NET release [454], a multi-step process that culminates in neutrophil death. Termed "NETosis", this form of cell death is distinct from both apoptosis and necrosis [443], and is defined by unique changes in neutrophil morphology. For example, NET release is preceded by homogenisation of eu- and heterochromatin, chromatin decondensation and disintegration of both the nuclear envelope and granule membranes [443]. These changes allow for the mixing of NET components before NETs are released concurrent to rupture of the cell membrane, an event for which a functional actin cytoskeleton is crucial [443;455].

Two non-redundant processes that underpin these morphological changes and thus NET formation are: histone hypercitrullination and the generation of ROS (Figure 1.5).

#### 1.6.2.2.1 Histone citrullination

Neutrophils that lack peptidylarginine deiminase 4 (PAD4), a calcium dependent enzyme that catalyses the citrullination of histones H2A, H3, and H4 [456;457], fail to generate NETs, suggesting that citrullination of histones is important for NET formation [458]. Indeed, all NET-inducing stimuli examined to date, such as IL-8, TNF-α, LPS and the bacteria *Shigella flexneri* increase histone citrullination by activating PAD4 [455;458;459].

Histone citrullination is critical for chromatin decondensation, an early event in NET formation and a defining feature of NETosis. Studies have shown regions of highly decondensed chromatin, which form the backbone of NETs, stain positive for citrullinated histones [459], whilst pharmacological inhibition of PAD4 prevents the chromatin decondensation normally observed in neutrophils following treatment with NET-inducing stimuli [459]. Unsurprisingly therefore, PAD4 inhibition prevents NET formation [459].

#### 1.6.2.2.2 Generation of reactive oxygen species

Pre-treating neutrophils with the NADPH oxidase inhibitor diphenylene iodonium or the anti-oxidant enzyme catalase prevents NET formation to a wide array of stimuli [443;460], suggesting that NET production is dependent upon the generation of ROS. Indeed, when challenged with PMA or pathogens, neutrophils isolated from patients with chronic granulomatous disease (CGD), an inherited disorder in which subunits of NADPH oxidase are either absent or dysfunctional [461], fail to generate NETs and show no signs of the aforementioned morphological changes that accompany NET formation [443;462-464].

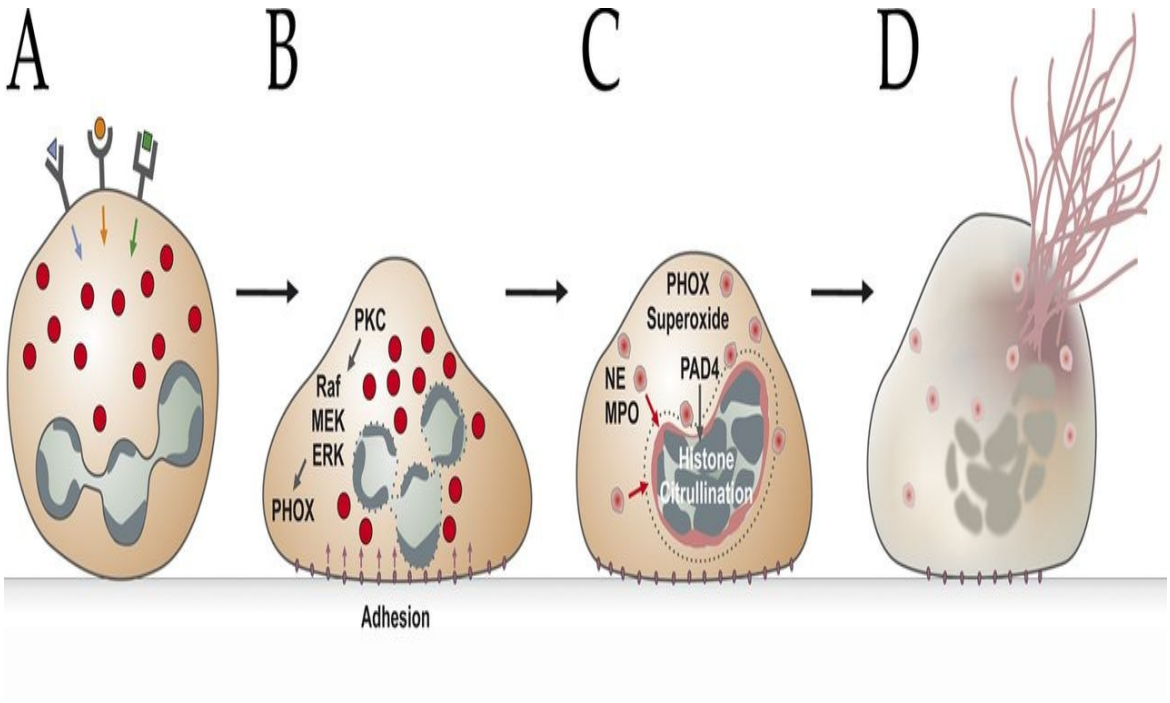



**Figure 1.5. Schematic representation of the molecular processes involved in the formation of neutrophil extracellular traps (NETs).** Ligation of cell surface receptors **(A)** triggers via the serine/threonine protein kinase, protein kinase C (PKC), activation of the ROS generating enzyme phagocytic oxidase (PHOX) **(B)**. Production of ROS has been proposed to trigger the translocation of neutrophil elastase (NE) and myeloperoxidase (MPO) from azurophilic granules (depicted as red circles) to the nucleus, where upon arrival they work together to drive degradation of histones **(C)**. Furthermore, ROS have been suggested to prolong the activity of peptidyl arginine deiminase 4 (PAD4), an enzyme that drives chromatin decondensation by promoting histone hypercitrullination **(C)**. These molecular processes lead to the disintegration of both nuclear and granule membranes, allowing for the intracellular mixing of NET components prior to rupture of the cell membrane and NET release **(D)**. Figure taken directly from review article by Brinkmann et al [465].

However, the cells do produce NETs when treated with exogenous hydrogen peroxide ($H_2O_2$) [443]. $H_2O_2$ however is not the active oxygen species that triggers NET generation. Instead, this function has been attributed to its downstream products hypochlorous acid and singlet oxygen [443;460;462;464]. Indeed, neutrophils devoid of MPO, a lysosomal protein whose enzymatic activity is critical for the generation of these two oxygen species, fail to release NETs in response to PMA and fungal challenge [443;466].

If the generation of ROS is critical for NET formation then what role do they play in this process? Currently the answer to this question is not known, although two roles have been proposed (Figure 1.5). Based on data showing elevated calcium levels induce NET formation [467], Remijsen and co-workers have suggested ROS may increase intracellular calcium levels by damaging mitochondria and/or the endoplasmic reticulum, and that this would promote NET production by sustaining the activity of PAD4 [468]. The second hypothesis that has been put forward proposes that ROS generation serves as a trigger for the translocation of NE from azurophilic granules to the nucleus, where upon arrival it would drive chromatin decondensation by degrading histone H4 [469].

Although clearly essential for NET production, ROS alone are not sufficient to trigger it, suggesting other cellular processes are required. This thinking is based on several lines of evidence, which include the inability of fMLP, a potent activator of NADPH oxidase, to induce NET formation [470], and the fact that despite generating ROS levels comparable to those of young adults, neutrophils isolated from neonates generate significantly fewer NETs following LPS stimulation [471].

### 1.6.2.3 Relevance of NETs *in vivo*

Despite a significant number of studies demonstrating that NETs are generated *in vivo* in times of infection [435;440;441;472-476], few groups have examined the contribution of NET-mediated immunity to a hosts overall immune response [472;473;477-479]. Given that no specific inhibitor of NET formation currently exists, this issue has been tackled either by administering DNase to pathogen-infected mice in order to digest pre-formed NETs [478;479], or infecting mice with bacterial strains that through expression of DNases can escape from NETs [472;473;477].

In a murine model of polymicrobial sepsis, Meng and colleagues administered DNase to evaluate the effect of NETs on bacterial spreading and host survival [478]. They found mice treated with DNase exhibited enhanced bacterial dissemination and increased mortality rates 24 hours post infection when compared to their untreated counterparts, although no difference in overall survival rates was observed between the two groups [478]. In other murine models of infection, it has been shown that bacterial isolates that express DNases and escape from NETs *in vitro*, are more virulent *in vivo* [472;473;477]. For instance, nuclease expressing bacteria are not cleared as effectively from host tissue as isolates devoid of DNases, and induce significantly higher rates of mortality [477].

Based on their findings, these groups have proposed that NETs make a significant contribution to the innate immune response. In light of the aforementioned studies by Parker et al [445] and Menegazzi and colleagues [447] that showed NETs do not directly kill pathogens (see section 1.6.2.1), it is likely that NETs confer protection by preventing microbial dissemination and degrading pathogen associated virulence factors [435].

### 1.6.2.4 Pathological consequences of NET formation

Via their ability to capture, contain and possibly eliminate invading micro-organisms, NETs may seem at first glance a useful addition to the defensive armoury of neutrophils. However, recent findings suggest their production may lead to some unwanted side effects. Firstly, *in vitro* studies have shown NET-resident histones and MPO directly induce epithelial and endothelial cell death [446;474;480]. Secondly, NET production in the absence of microbial stimulation, or an impaired ability to remove NETs from inflammatory sites has been linked to the development and/or pathology of a number of autoimmune diseases and pathological conditions such as small vessel vasculitis [481;482], cystic fibrosis [483;484], atherosclerosis [485], pre-eclampsia [486] and systemic lupus erythematosus (SLE) [480;487-490]. Of these, it is the potential association between NETs and the development of SLE that has been the subject of considerable attention.

Chronically activated pDCs secreting copious amounts of IFN-α are heavily implicated in the pathogenesis of SLE. As self DNA activates pDCs from SLE patients [491;492], NETs have been proposed to play a role in the propagation of this autoimmune disease, a claim based in part on data showing: (i) increased NET production by resting neutrophils from SLE patients [480], (ii) impaired degradation of NETs by lupus serum [489;490] and (iii) the presence of immune complexes consisting of self DNA and the NET-associated anti-microbial peptide LL37 in the sera of SLE patients but not healthy controls, which *in vitro* trigger pDC activation and IFN-α secretion [487;488]. When these data are viewed alongside experiments showing IFN-α induces LL37 expression on the surface of circulating neutrophils, whose recognition by autoantibodies triggers NET production [487], one can envisage the existence of a viscous cycle in SLE patients, in which NETs are the main protagonist.

Taken together, this data suggests that NET production is a double-edged sword. On the one hand, these structures appear useful for the immediate capture and containment of invading pathogens, but if produced in excess or their clearance impaired, NETs are a source of self-antigen that may drive the development of autoimmune disease as well as exacerbate ongoing pro-inflammatory responses.

## 1.7 Summary and thesis aims

When compared to younger subjects, older adults report an increased incidence of infection. As well as being more frequent, infectious episodes in the elderly are longer lasting, more severe and are associated with greater morbidity and mortality rates. Whilst numerous factors contribute to the increased susceptibility of older adults to infection, many consider age-associated changes in immune function to be primarily responsible. Despite the importance of innate immunity in preventing infections, only in the last decade have we begun to understand the impact ageing has on its function, with numerous studies reporting considerable age-related modification. However, these studies have mainly been observational, with very few identifying the mechanisms responsible for the functional changes they describe. Furthermore, the effect of age on many novel aspects of the innate immune response such as NET formation has not yet been examined.

Thus, the aims of this thesis were to:

1. Identify the mechanism(s) responsible for the age-related decline in NKCC at the single cell level.

2. Determine the effect of age on the generation of neutrophil extracellular traps.

# CHAPTER 2

# Materials and Methods

## 2.1 Materials and Methods

### 2.1.1 Subjects and blood sample collection

In total, 95 young (mean age 27.2±0.5 years; range 20-35 years; 41 males, 54 females) and 167 old (mean age 71.7±0.5 years; range 60-91 years; 74 males, 93 females) volunteers were enrolled into this study, which was approved by the local research ethics committee. Blood samples were collected after obtaining written informed consent from donors, who at the time of participation were in good health, free of immunological illness and significant co-morbidity, and were not taking any medication known to interfere with immunity.

### 2.1.2 Tissue culture

For cell culture and cell suspensions, the standard media (unless otherwise stated) was RPMI-1640 (Sigma-Aldrich, Dorset, UK) supplemented with 10% (volume/volume (v/v)) heat-inactivated (HI) fetal calf serum (FCS; Sera Laboratories International, Sussex, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (GPS; all purchased from Sigma-Aldrich), hereafter referred to as complete media (CM). To prepare HI-FCS, FCS was incubated for 30 minutes in a waterbath at $56^0$C. Phosphate buffered saline (PBS) was prepared by dissolving one PBS tablet (Sigma-Aldrich) in 200 ml distilled water and was autoclaved prior to use.

### 2.1.3 Flow Cytometry

Flow cytometry was performed using a $CyAn_{ADP}^{TM}$ bench top cytometer (Dako, Cambridgeshire, UK). Excitation of the flurochromes Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE) and PE-Cy-7 (PE-CY7) was achieved using an argon laser at 488 nm, and emission recorded through specific band pass filters: 530 nm for FITC (FL1), 575 nm for PE (FL2) and 750 nm for PE-CY7 (FL5). Pacific Blue (PcB)-conjugated antibodies were excited

using a blue-violet laser at 405 nm with emission recorded using a band pass filter at 450 nm (FL6). When multi-colour flow cytometry was performed, spectral overlap of flurochromes was compensated for by running individual cell populations stained with each dye.

Flow cytometry data was analysed using Summit v4.3 software (Dako, Colorado, USA). Data relating to antigen expression was recorded as both staining intensity and percentage of antigen positive cells. Staining intensity was measured as mean fluorescent intensity (MFI) or median fluorescent intensity (MedFI) and is presented after subtracting the values of samples stained with irrelevant isotype controls.

### 2.1.4 Immune cell purification

#### 2.1.4.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Venous blood was collected into heparinised tubes and diluted 1:1 with RPMI-1640 media supplemented with GPS. This mixture was layered gently over 6 ml of Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) and centrifuged at 1200 revolutions per minute (rpm) for 30 minutes at $20^0$C in a bench top centrifuge with no brake (JOUAN CR3*i*, DJB Labcare Limited, Buckinghamshire, UK). Post centrifugation, mononuclear cells were transferred into a universal tube (Sarstedt, Leicester, UK), and washed twice (1200 rpm for 10 minutes at $20^0$C) in magnetic assisted cell sorting buffer (MACS®; PBS, 2 mM Ethylenediamine-Tetraacetic Acid (EDTA), 0.5% bovine serum albumin (BSA) and 0.09% azide; Miltenyi Biotec, Gladbach, Germany). PBMC numbers were then determined and cells resuspended in CM at a concentration of 1-2x$10^6$ /ml for functional and phenotypic analysis or in MACS® buffer for NK cell isolation.

### 2.1.4.2 NK cell isolation

NK cells were obtained from PBMC samples by negative selection using MACS® technology (Human NK cell isolation kit; Miltenyi Biotec). Briefly, freshly isolated PBMCs that had been resuspended in MACS® buffer (see section 2.1.4.1) were spun at 300 x g for 10 minutes at $4^0$C (JOUAN MR22*i*), and the subsequent pellet re-constituted in 40 µl of MACS® buffer per $10^7$ cells. To this suspension, 10 µl of NK cell biotin-antibody cocktail per $10^7$ cells was added and the sample incubated for 15 minutes at $4^0$C with occasional mixing. Post incubation, 30 µl of MACS® buffer and 20 µl of NK cell microbead cocktail per $10^7$ cells was added and the sample incubated for a further 15 minutes at $4^0$C, after which cells were washed in MACS® buffer (2 ml per $10^7$ cells; 300 x g for 10 minutes at $4^0$C) and the pellet prepared for magnetic separation by resuspension in 500 µl of MACS® buffer.

For magnetic separation, an LS column (Miltenyi Biotec) was prepared by placing the column in the magnetic field of a QuadroMACS (midi) separator (Miltenyi Biotec) and washing with 3 ml of MACS® buffer. The PBMC suspension was applied to the column, followed by 9 ml of MACS® buffer, and the elute, which represented the purified NK cell population collected. Cells were pelleted by centrifugation (300 x g for 10 minutes at $4^0$C) and resuspended to a final concentration of 1, 2 or $5x10^6$ /ml in CM.

To determine the percentage of NK cells within the isolated cell population, 100 µl aliquots of the cell suspension were dispensed into a 96-well flexi plate (BD Falcon™, New Jersey, USA) and the plate spun at 250 x g for 5 minutes at $4^0$C (JOUAN MR22*i*). Post centrifugation, supernatants were decanted and pellets resuspended in 100 µl of PBS/1%BSA that contained either 1 µg/ml of a FITC-conjugated mouse anti-human CD3 (Dako, Cambridgeshire, UK; Clone UCHT1) and 0.3 µg/ml of a PE-conjugated mouse anti-human CD56 (Dako; Clone C5.9) or their concentration-matched isotype controls (mouse IgG1-

FITC (Dako; Code X0927) and mouse IgG2b-PE (Dako; Code X0951)). After a 20-minute incubation on ice, cells were washed once in PBS (250 x g for 5 minutes at $4^0$C), transferred to polypropylene tubes (Becton Dickinson Labware Europe, Meylan Cedex, France) and analysed by flow cytometry, where 15,000 cells were collected and the number that fell within a $CD56^+$ $CD3^-$ gate recorded. On a routine basis, this population comprised ≥97% of the isolated sample.

#### 2.1.4.3 Isolation of peripheral blood neutrophils

Peripheral blood was collected by venepuncture and transferred to sterile 50 ml Falcon™ tubes (Becton Dickinson) containing the anti-coagulant EDTA (3 mg/ml blood; Sigma-Aldrich). To this, 2% (w/v) dextran (reconstituted in 0.9% saline; Amersham Biosciences, Uppsala, Sweden) was added (1 ml per 6 ml of blood) and samples incubated at room temperature (RT) to sediment red blood cells. After 30 minutes, leukocyte-rich plasma was carefully layered onto a Percoll® density gradient consisting of 80% Percoll® (2.5 ml) and 56% Percoll® (5 ml) in a sterile 15 ml Falcon™ tube. To prepare gradients, a working stock of Percoll® was made by mixing 45 ml of Percoll® (Sigma-Aldrich) with 5 ml of 9% (w/v) sodium chloride (NaCl; Sigma-Aldrich). From here, 80% Percoll® was prepared by diluting 40 ml of the Percoll® stock with 10 ml 0.9% (w/v) NaCl, whilst 56% Percoll® comprised of 28 ml of the working Percoll® stock and 22 ml of 0.9% (w/v) NaCl. Gradients were then prepared by carefully layering 5 ml of 56% Percoll® on top of 2.5 ml 80% Percoll®.

Once loaded with leukocyte-rich plasma, gradients were spun for 20 minutes at 1100 rpm in a freestanding centrifuge with no brake at 20°C (JOUAN, MR211*i*). Post centrifugation, neutrophils, which resided at the 80-56% interface were removed, placed into a sterile 50 ml Falcon™ tube and washed once at 1600 rpm for 10 minutes in RPMI-1640 media supplemented with GPS. Post centrifugation, the supernatant was discarded and cells

resuspended in RPMI-1640 media supplemented with GPS or Hank's balanced salt solution (HBSS) supplemented with calcium and magnesium (Gibco®, Invitrogen, Paisley, UK) to a final concentration of $1x10^6$ /ml.

Purity of neutrophil preparations was determined by cytospin and differential staining using a commercially available Giemsa stain (Diff-Qik; Gentaur Europe, Brussels, Belgium). Routinely, neutrophils comprised ≥98% of the cell population.

### 2.1.5 Phenotypic analysis

#### 2.1.5.1 Determination of lymphocyte proportions, absolute numbers and surface phenotype

200 µl aliquots of isolated PBMCs ($1x10^6$ /ml in CM) were dispensed into wells of a 96-well flexi-plate and pelleted by centrifugation at 250 x g for 5 minutes at $4^0$C. Post centrifugation, supernatants were decanted and cells resuspended in 100 µl PBS/1%BSA containing surface specific antibodies (Table 2.1) or their concentration-matched isotype controls (Table 2.2). Following a 20-minute incubation on ice, cells were washed once in PBS (250 x g, 5 minutes, $4^0$C) and transferred to polypropylene tubes for flow cytometric testing.

To determine lymphocyte proportions, 15,000 lymphocytes were gated on a forward scatter (FS)/ sideward scatter (SS) flow cytometry plot and the percentage displaying a $CD3^+$, $CD3^-$ $CD56^+$, $CD3^-$ $CD56^+$ $CD16^{+/-}$ or $CD3^-$ $CD56^-$ $CD16^+$ surface phenotype recorded (Figure 2.1A-B). Using this data and readings of total lymphocytes, which were obtained from a full blood count on EDTA-treated whole blood using a fully automated Coulter $ACT^{diff}$ haematology analyser (Beckman-Coulter, High Wycombe, UK), the absolute number of circulating T and NK cells were calculated. NK cell receptor expression was studied on 5000 $CD3^-$ $CD56^{DIM}$ NK cells, where both the percentage of positive cells and the MedFI values

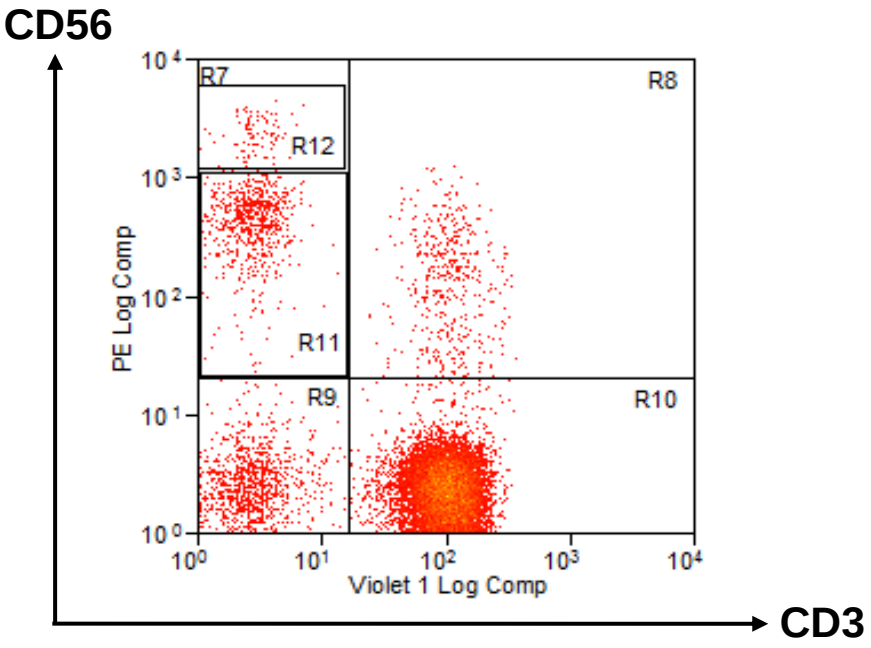
**Table 2.1 Anti-human primary antibodies**

| Antigen | Label | Isotype | Stock concentration | Dilution | Clone | Source |
|---|---|---|---|---|---|---|
| CD3 | PcB | IgG1κ | 0.2 mg/ml | 1:100 | UCHT1 | BD Biosciences |
| CD3 | FITC | IgG1κ | 100 mg/L | 1:100 | UCHT1 | Dako |
| CD16 | FITC | IgG1κ | 100 mg/L | 1:25 | DJ130c | Dako |
| CD56 | PE | IgG2bκ | 30 mg/L | 1:100 | C5.9 | Dako |
| CD57 | FITC | IgMκ | 25 µg/ml | 1:25 | HCD57 | BioLegend UK |
| CD94 | FITC | IgG1κ | 25 µg/ml | 1:5 | DX22 | eBioscience |
| NKp46 | PcB | IgG1κ | 0.5 mg/ml | 1:100 | 9E2 | BioLegend UK |
| NKG2D | PECy7 | IgG1κ | 50 µg/ml | 1:5 | 1D11 | BioLegend UK |

**Table 2.2 Isotype controls**

| | Label | Stock concentration | Clone | Source |
|---|---|---|---|---|
| IgG1κ | PcB | 0.2 mg/L | MOPC-21 | BD Biosciences |
| IgG1κ | PcB | 0.5 mg/L | MOPC-21 | BioLegend UK |
| IgG1κ | FITC | 100 mg/L | DAK-GO1 | Dako |
| IgG1κ | PECy7 | Lot specific | 15H6 | Southern Biotech |
| IgG2bκ | PE | 200 mg/L | DAK-GO9 | Dako |
| IgMκ | FITC | 0.5 mg/ml | MM-30 | BioLegend UK |

**(A)**




**(B)**

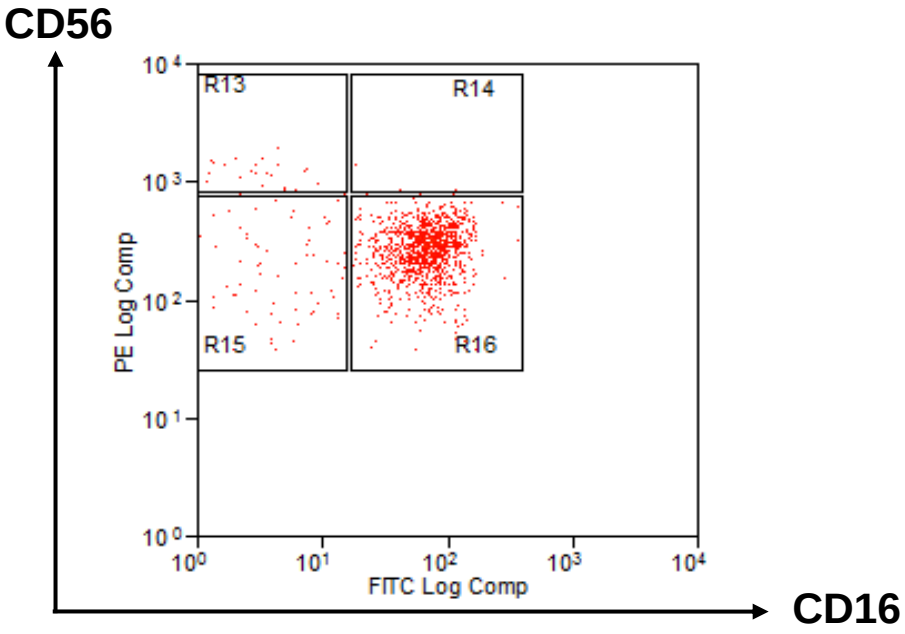



**Figure 2.1. Gating strategy used to identify NK cell subsets within the peripheral lymphocyte pool. (A)** NK cells within peripheral lymphocytes were defined by the surface phenotype $CD3^{-}$ $CD56^{+}$ (Region 7), and based on the differential surface expression of CD56, were categorised as either $CD56^{DIM}$ (Region 11) or $CD56^{BRIGHT}$ (Region 12). **(B)** Gating on NK cells and examining their expression of the low affinity Fc receptor CD16 allowed for the identification of 4 distinct NK cells subsets: $CD56^{BRIGHT}$ $CD16^{-}$ (Region 13), $CD56^{BRIGHT}$ $CD16^{+}$ (Region 14), $CD56^{DIM}$ $CD16^{-}$ (Region 15) and $CD56^{DIM}$ $CD16^{+}$ (Region 16).

were recorded.

#### 2.1.5.2 NKp30 staining on isolated NK cells

100 µl aliquots of NK cells ($1x10^6$ /ml in CM) were dispensed into a 96-well flexi plate and pelleted by centrifugation (250 x g for 5 minutes at $4^0$C). After decanting the supernatant, cells were resuspended in PBS/1%BSA containing 5 µg/ml of purified mouse anti-human NKp30 antibody (BioLegend UK, Cambridge, UK; Clone P30-15) or its concentration-matched isotype control (BioLegend UK; Clone MOPC-21) and incubated on ice for 20 minutes. After a single wash in PBS (250 x g, 5 minutes, $4^0$C), cells were resuspended in 50 µl of 20% (v/v) goat serum (Sigma-Aldrich) in PBS/1%BSA and incubated for 20 minutes on ice, after which, samples were washed in PBS (250 x g, 5 minutes, $4^0$C) and pellets resuspended in PBS/1%BSA containing 10 µg/ml of FITC goat anti-mouse IgG (BioLegend UK; Clone Poly4053). Following a 20-minute incubation on ice, cells were washed in PBS (250 x g, 5 minutes, $4^0$C), transferred to polypropylene tubes and analysed by flow cytometry, where 10,000 NK cells were counted and the percentage of receptor positive cells and their MedFI value recorded.

#### 2.1.5.3 Measurement of perforin and granzyme B expression in isolated NK cells

100 µl aliquots of NK cells ($1x10^6$ /ml in CM) were transferred into wells of a 96-well flexi plate and washed once in PBS at 250 x g for 5 minutes at $4^0$C. Post centrifugation, cells were subjected to a 30-minute incubation at RT in the presence of 50 µl fixation medium (Life Technologies, Paisley, UK), after which samples were washed in PBS (250 x g, 5 minutes, $4^0$C). After decanting the supernatant, cell pellets were resuspended in 50 µl of permeabilisation medium (Life Technologies), to which either 10 µg/ml of a PE-conjugated monoclonal antibody against human perforin (Ancell, Minnesota, USA; Clone δG9), 5 µg/ml

of a FITC-labelled mouse monoclonal antibody against human perforin (BioLegend; Clone δG9), 20 µg/ml of a FITC-conjugated mouse anti-human granzyme B (Biolegend UK; Clone GB11) antibody or their concentration-matched isotype controls (mouse IgG2b-PE, IgG1-FITC (both from Dako) or mouse IgG1-FITC (Biolegend UK; Clone MOPC-21)) were added. Following a 30-minute incubation at RT in the dark, cells were washed once in PBS (250 x g, 5 mins, $4^0$C) and analysed by flow cytometry, where the percentage of positive cells and MFI values of 5000-10,000 NK cells were recorded. The absolute number of perforin positive NK cells was calculated from cell percentages and total NK cell numbers, which were obtained by haematological analysis of whole blood samples (see section 2.1.5.1).

### 2.1.6 Analysis of NK cell function

#### 2.1.6.1 Assessment of NKCC and conjugate formation

Cytotoxicity of resting NK cells against the human erythroleukaemic cell line K562 was assessed by two-colour flow cytometry using an adapted version of the protocol described by Godoy-Ramirez and colleagues [493]. The MHC class I deficient NK-sensitive cell line K562 was used as the target population. Obtained from the American Type Culture Collection (ATCC, Middlesex, UK), K562 cells (ATCC number CCL-243) were maintained in CM in 75 $cm^2$ cell culture flasks (Sarstedt) at $37^0$C in a humidified 5% $C0_2$ atmosphere. Cells were split 1 in 3 on the day preceding experimentation and washed once (1600 rpm, 8 minutes, $20^0$C) in PBS prior to use. Cultures of K562 cells were replaced on a monthly basis.

##### 2.1.6.1.1 Cytotoxicity assay

To determine resting NKCC, 200 µl of NK cells ($1x10^6$ /ml in CM) were dispensed into wells of a 96-well round bottomed plate (Sarstedt) and serially diluted in CM. To each well, 100 µl of K562 cells ($1x10^5$ /ml in CM) were added to give effector (E) to target (T) cell ratios of

10:1, 5:1 and 2.5:1. E:T cell contact was increased by centrifugation at 50 x g for 1 minute at $20^0$C, and samples incubated at $37^0$C in a humidified 5% $C0_2$ atmosphere for 4 or 16 hours. To account for the spontaneous lysis of target cells during this period, 100 µl of K562 cells were incubated in the absence of effector cells.

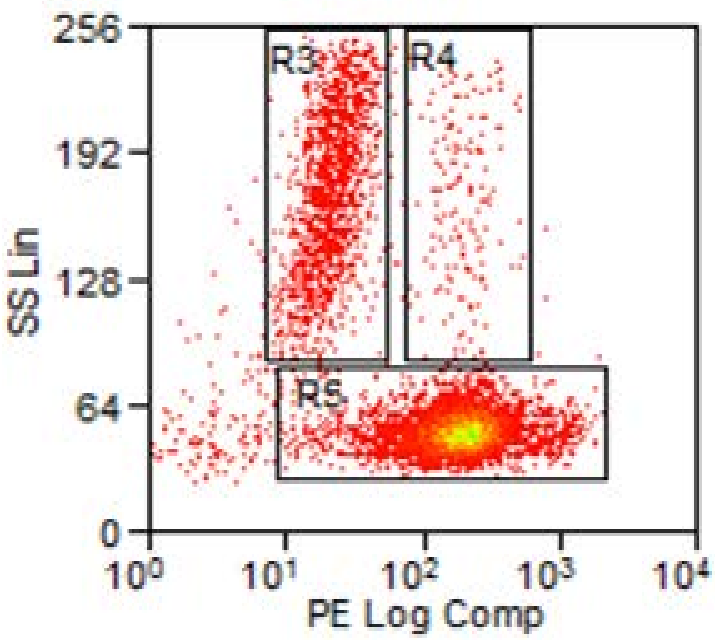
Post incubation, cells were pelleted (250 x g, 5 minutes, $4^0$C), supernatants removed, and samples resuspended in 100 µl PBS/1%BSA containing 0.3 µg/ml PE-conjugated mouse anti-human CD56. After 20 minutes of labelling on ice, cells were washed once in PBS (250 x g, 5 minutes, $4^0$C) and the subsequent pellet resuspended in 90 µl PBS. To detect cell death, 10 µl of SYTOX® Blue dead cell stain (pre-diluted 1:800 in PBS; Life Technologies) was added to each well and the plate incubated in the dark for 5 minutes at $4^0$C. Post incubation, cells were transferred to polypropylene tubes and analysed by flow cytometry.

**2.1.6.1.2 Data acquisition**

To assess the percentage of lysed target cells within samples, NK and K562 cells were distinguished from one another by gating on a PE log comp Vs. side scatter flow cytometry plot (Figure 2.2A). From here, the number of K562 cells (within a total cell population of 2000) with perturbed membranes was examined on a Violet 1 log comp flow cytometry plot (Figure 2.2B) and percentage specific lysis calculated using the following equation:

$$\% \text{ Specific lysis} = \left( \frac{(N^o \text{ of SYTOX}^{®} \text{ +ve cells in test sample} - N^o \text{ of SYTOX}^{®} \text{ +ve cells in spontaneous sample})}{2000} \right) \text{X } 100$$

**(A)**




**(B)**

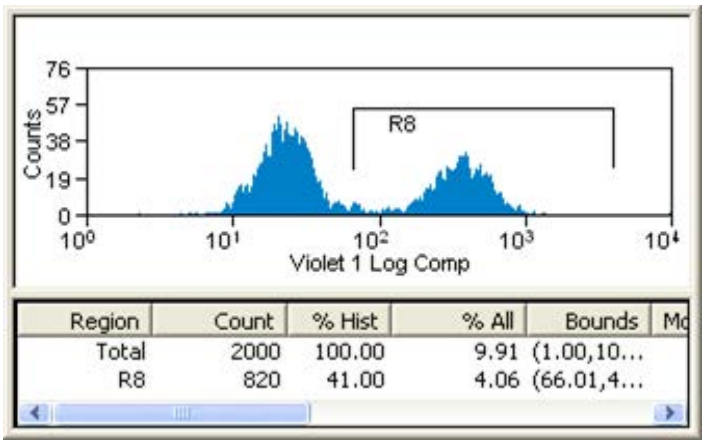



**Figure 2.2. Two-colour flow cytometric analysis of NK cell cytotoxicity and conjugate formation. (A)** Immunostaining of effector and target cell co-cultures with PE-conjugated anti-CD56 allowed for the identification of three distinct cell populations: (1) free K562 cells (Region 3), (2) conjugates formed between NK and K562 cells (Region 4) and (3) free NK cells (Region 5). **(B)** By the addition of the cell impermeable DNA binding dye SYTOX® Blue to NK and K562 co-cultures, the percentage of dead K562 cells was determined by examining the number of SYTOX® positive cells (Region 8).

For the assessment of conjugate formation, conjugates were defined on a PE log comp Vs. side scatter flow cytometry plot as K562 cells positive for PE staining (Figure 2.2A) and the percentage within 2000 K562 cells recorded.

### 2.1.6.2 Assessment of the cytotoxicity of MIP-1α-treated NK cells

4-hour NKCC assays were performed as described in section 2.1.6.1 with the only difference being that at the start of the incubation period, MIP-1α (R and D Systems Europe Limited, Oxford, UK) at a final concentration of 1, 2, 5 and 10 ng/ml or its vehicle control (PBS) was added to the NK-K562 co-cultures.

### 2.1.6.3. Assessment of the role of perforin and death receptor ligands in NKCC

To determine the sensitivity of K562 cells to perforin-induced death, 4-hour NKCC assays at an E:T ratio of 10:1 were carried out as described in section 2.1.6.1 with the sole modification that prior to K562 exposure, NK cells were treated for 2 hours ($37^0$C, 5% $C0_2$) with 10-500 nM of the vacuolar type $H^+$-ATPase inhibitor concanamycin A (CMA; Sigma-Aldrich) [494] or vehicle control. By raising the pH of lytic granules, CMA reduces perforin activity by promoting its degradation and/or interfering with the cleavage of pro-perforin into its mature active form [495].

To assess whether death of K562 cells could be induced through death receptor signalling, 200 µl aliquots of K562 cells ($1x10^6$ /ml in CM) were dispensed into a 96-well flat bottomed plate (Sarstedt) and incubated for 4 hours in the presence or absence of sFasL (10-100 ng/ml), sTRAIL (1-10 ng/ml) (both reagents were a gift from Dr. E. Humphreys, University of Birmingham, UK) or TNF-α (1-100 ng/ml; Sigma-Aldrich). Post incubation, cells were spun at 250 x g for 5 minutes at $4^0$C and the pellet resuspended in 90 µl of PBS. To this, 10 µl of SYTOX® Blue dead cell stain (pre-diluted 1:800 in PBS) was added, and

samples transferred to polypropylene tubes. For flow cytometric analysis, the number of SYTOX® positive cells within a population of 2000 was studied on a Violet 1 log flow cytometry plot and percentage lysis calculated using the equation described in section 2.1.6.1.2.

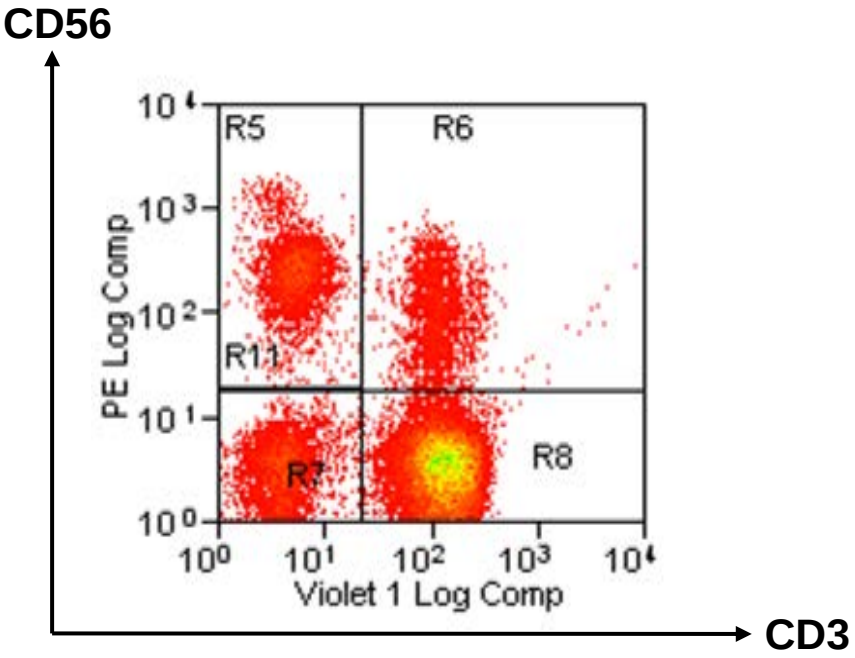
**2.1.6.4 CD107a assay**

PBMC's were isolated as described in section 2.1.4.1 and resuspended in CM to a final concentration of $2x10^6$ /ml. 100 µl aliquots were then dispensed into polypropylene tubes, to which 100 µl of CM or K562 cells ($2x10^5$ /ml or $2x10^6$ /ml in CM) was added. Alongside these stimuli, 1.25 µg/ml of a FITC-conjugated mouse anti-human CD107a monoclonal antibody (eBiosciences, Hatfield, Ireland, UK; Clone eBioH4A3) or its concentration-matched isotype control (mouse IgG1-FITC (Dako; Code X0927) was added, and samples incubated for 1 hour at 37°C in a humidified 5% $C0_2$ atmosphere. Post incubation, 6 µg/ml of monensin (diluted in 100% ethanol; Sigma-Aldrich) was added to all samples, which were then incubated for a further 2 or 5 hours at 37°C to give total incubation times of 3 and 6 hours respectively.

Post incubation, samples were subjected to centrifugation (1600 rpm, 10 minutes, 4°C) and cells stained with 100 µl PBS/1%BSA that contained 1 µg/ml of PcB-conjugated mouse anti-human CD3 (BD Biosciences, Oxford, UK) and 0.3 µg/ml PE-conjugated mouse anti-human CD56. After a 20-minute incubation on ice, samples were washed once in PBS (1600 rpm, 10 minutes, 4°C) and CD107a levels measured by flow cytometry.

For cytometric analysis, the percentage of CD107a positive NK cells was recorded along with the MFI value. CD107a expression was measured by gating on $CD3^-$ $CD56^+$ cells within a total lymphocyte population of 50,000 (Figure 2.3A-B). CD107a expression on NK cells

**(A)**




**(B)**

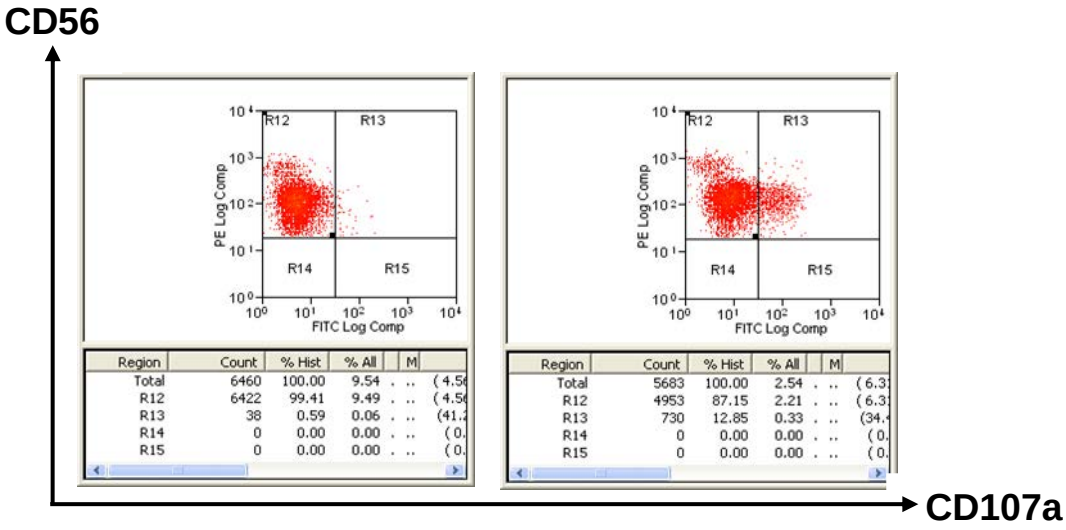



**Figure 2.3. Gating strategy for CD107a expression assay. (A)** PBMC samples were stained with anti-CD56 PE and anti-CD3 PcB to identify NK cells, which are depicted in region 5. **(B)** CD107a expression on unstimulated NK cells (left panel) and on NK cells following a 6-hour co-culture with K562 cells at an E:T ratio of 1:1 (right panel).

cultured with RPMI alone was considered background and the value subtracted from each test sample.

#### 2.1.6.4.1 CD107a assay using isolated NK cells

100 µl aliquots of NK cells ($1x10^6$ /ml in CM) were added to wells of a 96-well round bottomed plate that contained 100 µl of CM or K562 cells ($1x10^5$ /ml or $1x10^6$ /ml), and either 1.25 µg/ml of a FITC-conjugated mouse anti-human CD107a monoclonal antibody or its concentration-matched isotype control. After gentle resuspension, E:T cell contact was increased by centrifugation (50 x g, 1 minute, $20^0$C), and samples incubated at $37^0$C in a humidified 5% $C0_2$ atmosphere for 1 hour. Post incubation, samples were treated with 6 µg/ml of monensin (diluted in 100% ethanol) and incubated for an additional 3 hours at $37^o$C.

Post incubation, samples were subjected to centrifugation (250 x g, 5 minutes, $4^0$C), and pellets resuspended in 100 µl PBS/1%BSA containing 0.3 µg/ml of a PE-conjugated mouse anti-human CD56 monoclonal antibody. After a 20-minute incubation on ice, cells were washed once in PBS (250 x g, 5 minutes, $4^0$C) and analysed by flow cytometry, where 10,000 NK cells were gated and the percentage exhibiting a $CD56^+$ $CD107a^+$ phenotype recorded along with the accompanying MFI value. CD107a expression on NK cells cultured with RPMI alone was considered background and therefore the value was subtracted from all test samples.

#### 2.1.6.5 CD69 staining

100 µl of freshly isolated NK cells ($1x10^6$ /ml in CM) were dispensed into wells of a 96-well round bottomed plate, to which 100 µl of K562 cells ($1x10^5$ /ml in CM) or CM was added. Following centrifugation at 50 x g for 1 minute at $20^0$C to increase E:T cell contact, samples

were incubated for 4 hours at 37$^{o}$C in a humidified 5% $C0_2$ atmosphere. Post incubation, cells were pelleted (250 x g, 5 minutes, 4$^{o}$C), supernatants discarded and samples resuspended in PBS/1% BSA containing 0.3 µg/ml PE-conjugated mouse anti-human CD56 and either 10 µg/ml FITC-conjugated mouse anti-human CD69 (BD Biosciences; Clone L78) or its concentration-matched isotype control (mouse IgG1-FITC). After a 20-minute incubation on ice, cells were washed once in PBS (250 x g, 5 minutes, 4$^{o}$C) and analysed by flow cytometry, where 15,000 NK cells were counted and the percentage expressing CD69 recorded along with the corresponding MFI value.

#### 2.1.6.6 Detection of perforin on the surface of K562 cells

Binding of perforin to the surface of K562 cells was assessed through the use of a FITC-conjugated anti-perforin antibody using a protocol adapted from the work of Lehmann and colleagues [496;497]. Briefly, following a 4-hour co-culture of NK and K562 cells at an E:T ratio of 10:1, samples were subjected to centrifugation (250 x g, 5 minutes, 4$^{o}$C) and pellets resuspended in PBS/1% BSA that contained 0.3 µg/ml of a PE-conjugated mouse anti-human CD56 monoclonal antibody along with either 5 µg/ml of a FITC-labelled mouse monoclonal antibody against human perforin or its concentration-matched isotype control (mouse IgG1-FITC). Following a 20-minute incubation on ice, cells were washed once in PBS (250 x g, 5 minutes, 4$^{o}$C), and analysed by flow cytometry.

To determine the percentage of K562 cells to which perforin had bound, NK and K562 cells were distinguished from one another by gating on unlabelled K562 cells on a PE log comp Vs. side scatter FACS plot, and recording the number within a total population of 2000 that displayed positive FITC fluorescence.

### 2.1.6.7 Analysis of perforin concentration in NK-K562 cell co-culture supernatants

Following a 4-hour co-culture of NK and K562 cells at an E:T ratio of 10:1, samples were transferred to 0.5 ml eppendorf tubes and pelleted by centrifugation (3000 rpm for 5 minutes at RT) in a bench top microcentrifuge (MSE, London, UK). Cell-free supernatants were then harvested, snap-frozen in liquid nitrogen and stored at -80$^{o}$C until use.

Perforin concentrations were determined using a commercially available solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) according to manufacturer's instructions (Abcam, Cambridge, UK). Briefly, after thawing samples on ice, 100 µl aliquots were dispensed into wells of a microtiter plate pre-coated with a monoclonal antibody specific for human perforin. After a 1-hour incubation at RT, liquid was aspirated and wells washed 3 times with 300 µl of wash solution. 50 µl of biotinylated anti-perforin antibody was then added to each well and the plate incubated for 1 hour at RT. After liquid aspiration and 3 washes with 300 µl wash buffer, 100 µl of streptavidin horseradish peroxidise (HRP) solution was added to all wells and the plate incubated at RT for 20 minutes. After emptying the wells and washing 3 times with 300 µl wash buffer to remove any unbound enzyme, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was dispensed into all wells. Following a 10-minute incubation in the dark at RT, colour development was quenched by the addition of 100µl sulphuric acid ($H_2SO_4$). Absorbance of all wells was measured within 5 minutes of adding $H_2SO_4$ using a spectrophotometer with a primary wavelength of 420 nm and a reference wavelength of 620 nm (BioTek® Synergy HT, Northstar Scientific Limited, Leeds, UK). Perforin concentrations within test samples were extrapolated from values obtained from a standard curve that was created from standards of known perforin concentration using GraphPad Prism® software (GraphPad Software Limited, California, USA).

### 2.1.6.8 Measurement of perforin release from NK cells

Freshly isolated resting NK cells ($1x10^6$ /ml in CM) were treated for 4 hours at $37^oC$ with CM, K562 target cells (E:T 10-1) or PMA (2.5 µg/ml; Sigma-Aldrich) and ionomycin (0.5 µg/ml; Sigma-Aldrich). Post incubation, samples were pelleted (250 x g, 5 minutes, $4^0C$) and resuspended in PBS/1% BSA containing 0.3 µg/ml PE-conjugated mouse anti-human CD56. After a 20-minute incubation on ice, cells were washed in PBS (250 x g, 5 minutes, $4^0C$) and resuspended in 50 µl fixation medium. Following a 30-minute incubation at RT, samples were washed in PBS (250 x g, 5 minutes, $4^0C$) and resuspended in 50 µl of permeabilisation medium, to which either 5 µg/ml of a FITC-labelled mouse monoclonal antibody against human perforin or its concentration-matched isotype control (mouse IgG1-FITC) was added. After a 30-minute incubation in the dark at RT, samples were washed in PBS (250 x g, 5 minutes, $4^0C$), and analysed by flow cytometry, where 5000 NK cells were counted and the percentage of perforin positive NK cells recorded along with the corresponding MFI value.

### 2.1.6.9 Immunofluorescence microscopy

#### 2.1.6.9.1 Staining to determine perforin localisation

100 µl of freshly isolated NK cells ($1x10^6$ /ml in CM) were cultured for 30 minutes ($37^oC/5\%$ $C0_2$) in wells of a 96-well round bottomed plate with 100 µl of either K562 cells ($1.5x10^5$ /ml in CM) or CM. Post incubation, samples were cytospun onto microscope slides (300 rpm for 5 minutes at RT; Shandon Cytospin 2), and cells fixed in cold acetone (Fisher Scientific, Leicester, UK) for 20 minutes at $4^0C$. After air-drying the slides for 5 minutes, cells were re-hydrated in PBS and permeabilised by exposure to 0.1% Triton x-100 (Sigma-Aldrich) in PBS for 3 minutes at RT. After 3 x 5 minute washes in PBS, cells

were treated in a humidified chamber for 10 minutes at RT with 10% (v/v) goat serum in PBS/2% BSA, after which slides were subjected to a single 5 minute wash in PBS. Cells were then stained in a humidified chamber for 1-hour at RT with 100 µl of PBS/1%BSA containing 2.5 µg/ml of a FITC-labelled mouse monoclonal antibody against human perforin or its concentration-matched isotype control (mouse IgG1-FITC). Post incubation, slides were washed three times in PBS (5 minutes per wash) before being treated for 30 minutes at RT in a humidified chamber with 100 µl of an anti-FITC goat IgG fraction Alexa Fluor® 488 conjugate (Life Technologies) that had been pre-diluted 1:100 from stock in PBS. Following 3 x 5 minute washes in PBS, samples were stained with 104 ng/ml of DAPI nucleic acid stain (Life technologies) for 2 minutes at RT. After a single wash in PBS, 22x22 mm coverslips (VWR International, Leicestershire, UK) were adhered to slides with fluoromount medium (Sigma-Aldrich), and cells visualised using a LEICA DMI 6000 B microscope (Leica microsystems, Milton Keynes, UK) at X40 and X63 objective.

To determine the percentage of NK cells in which perforin had localised to the NK-target cell interface, three individuals blinded to donor age counted fifty NK-K562 cell conjugates on slides prepared from three young and three old subjects.

**2.1.6.9.2 F-actin staining**

NK-K562 cell co-cultures were set up as described in section 2.1.6.9.1. Post incubation, samples were cytospun onto microscope slides (300 rpm for 5 minutes at RT), and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes at RT. After 3 x 5 minute washes in PBS, cells were treated at RT for 3 minutes with 0.2% Triton x-100, which was followed by 3 x 5 minute washes in PBS, and a 30-minute incubation at RT with PBS/1%BSA. After a single wash in PBS, slides were stained in a humidified chamber for 1-hour at RT with 5 µg/ml FITC-conjugated phalloidin (Sigma-Aldrich). Post incubation slides were washed

3 times in PBS and stained with 104 ng/ml of DAPI nucleic acid stain for 2 minutes at RT. Following a single wash in PBS, 22x22 mm coverslips were mounted onto slides using fluoromount medium and cells visualised using a LEICA DMI 6000 B microscope at X40 and X63 objective.

**2.1.6.10 Assessment of target cell-induced NK cell apoptosis by Annexin V staining**

100 µl aliquots of NK cells ($1x10^6$ /ml in CM) were dispensed into wells of a 96-well round bottomed plate, to which 100 µl of K562 cells ($1x10^5$ /ml) or CM was added. Following centrifugation (50 x g, 1 minute, $20^0$C), samples were incubated for 4 hours at $37^o$C in a humidified 5% $C0_2$ atmosphere. Post incubation, cells were subjected to centrifugation (250 x g, 5 minutes, $4^0$C), and resuspended in PBS/1% BSA that contained 0.3 µg/ml PE-conjugated mouse anti-human CD56. After a 20-minute incubation on ice, cells were washed once in PBS (250 x g, 5 minutes, $4^0$C) and pellets resuspended in 98 µl of 1X Annexin V binding buffer (0.1 M Hepes (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$), to which 2 µl of Annexin V FITC (BD Biosciences) was added. Following a 10-minute incubation on ice, samples were transferred to polypropylene tubes for flow cytometric analysis.

To determine the percentage of Annexin V positive NK cells, 10,000 unconjugated NK cells were gated on a FS/SS flow cytometry plot and the number exhibiting positive FITC fluorescence recorded. The number of Annexin V positive NK cells in samples cultured in CM was considered to represent spontaneous apoptosis and therefore this value was subtracted from all test samples.

### 2.1.6.11 Analysis of cell signalling by Western blotting

#### 2.1.6.11.1 Preparation of K562 cell lysates

K562 cells were washed in PBS (1600 rpm for 8 minutes at $20^0$C) and resuspended at a concentration of $1x10^6$ /ml in CM. 100 µl aliquots were then dispensed into 1.5 ml eppendorf tubes and cells pelleted by centrifugation (200 x g, 5 minutes, $4^0$C). After discarding supernatants, cell lysates were prepared by the addition of 20 µl of sodium dodecyl sulphate (SDS) sample buffer (4% SDS (v/v); Geneflow Limited, Staffordshire, UK), 0.1M dithiothreitol (Sigma-Aldrich), 20% glycerol (v/v; BDH Limited, Poole, UK), 0.0625 M Tris-HCL (pH 6.8), 0.004% bromophenol blue (w/v; Sigma-Aldrich)) and boiled for 10 minutes at 100°C.

#### 2.1.6.11.2 MAPK signalling in antibody-stimulated NK cells

To study MAPK signalling in activated NK cells, the stimulation protocol outlined in the recent article by Al-Hubeshy et al [498] was used. Briefly, 96-well flat-bottomed ELISA NUNC plates (Fisher Scientific) were coated with 5 µg/ml of mouse monoclonal antibodies directed against the activatory receptors NKG2D (R and D Systems Europe Limited; Clone 149810) and NKp30 (R and D Systems Europe Limited; Clone 210847). Following overnight incubation at $4^0$C, plates were washed 3 times with 200 µl PBS, after which 100 µl aliquots of PBS/10%HIFCS (v/v) were dispensed into wells and the plate incubated at RT for 30 minutes. Post incubation, blocking solution was decanted and the plates washed 3 times with 200 µl PBS.

Once plates were prepared for use, 20 µl aliquots of freshly isolated NK cells ($5x10^6$ /ml in CM) were added to wells, and samples incubated for 2, 5, 10, 30, 60 and 90 minutes at $37^o$C in a humidified 5% $C0_2$ atmosphere. Post stimulation, 20 µl of 2X SDS sample buffer was

added to wells, after which lysates were transferred into 0.5 ml eppendorf tubes and boiled for 10 minutes at 100°C. Data corresponding to minute 0 reflects NK cells that were lysed prior to their addition to antibody-coated wells.

**2.1.6.11.3 PMA and ionomycin stimulated NK cells**

20 µl of freshly isolated NK cells ($5x10^6$ /ml in CM) were treated in 96-well flat-bottomed plates (BD Falcon) for 2, 5, 10, 30, 60 and 90 minutes with 2.5 µg/ml PMA and 0.5 µg/ml ionomycin at $37^oC$ in a humidified 5% $C0_2$ atmosphere. Post stimulation, cell lysates were prepared by the addition of 20 µl of 2X SDS sample buffer, after which samples were transferred into 0.5 ml eppendorf tubes and boiled for 10 minutes at 100°C. Data corresponding to minute 0 relates to untreated NK cells.

**2.1.6.11.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting**

After centrifugation at 13,000 rpm for 2 minutes to pellet cell debris, lysates were loaded onto a 10% SDS-polyacrylamide gel (constituents listed in table 2.3) and run for 3 hours at 200 volts with appropriate pre-stained markers (Bio-Rad, Hertfordshire, UK). Proteins were then transferred to methanol-activated 0.45 micron polyvinylidene difluoride membranes (Scientific Laboratory Supplies, Yorkshire, UK) using Bio-Rad turbo transfer technology (Bio-Rad). Once blotted, membranes were incubated on an orbital shaker (Medline Scientific Ltd, Oxford, UK) in 5% BSA/Tris buffered saline (TBS) containing 0.1% tween-20 (Sigma-Aldrich) for 1-hour at RT, after which blots were probed overnight at 4°C with either a rabbit anti-human Phospho-p44/42 or Phospho-p38 antibody (both purchased from Cell Signalling Technology, Hertfordshire, UK; both antibodies were pre-diluted 1:1000 from stock in TBS-0.1% tween-20 containing 2.5% BSA). Post incubation, blots were subjected to 3 x 15 minute washes in TBS-0.1% tween-20 before being probed for 1-hour at RT with a HRP-linked anti-

**Table 2.3 Constituents of a 10% SDS-polyacrylamide gel**

| | Protogel | $H_20$ | 1.5M Tris pH 8.8 | 0.5M Tris pH 6.5 | 10% SDS | 10% APS | Temed |
|---|---|---|---|---|---|---|---|
| **Resolving gel** | 10.0 ml | 12.5 ml | 7.5 ml | n/a | 300 µl | 150 µl | 30 µl |
| **Stacking gel** | 2.6 ml | 12.2 ml | n/a | 5 ml | 200 µl | 100 µl | 20 µl |

**Abbreviations**: APS, Ammonium persulfate; $H_20$, Water; SDS, Sodium dodecyl sulphate.

rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK; antibody was pre-diluted 1:4000 from stock in TBS-0.1% tween-20). Following 3 washes in TBS-0.1% tween-20 (15 minutes each wash), blots were incubated for 5 minutes at RT in enhanced chemiluminescence solution (Geneflow, Staffordshire, UK), after which proteins were visualised using either autoradiography against X-ray film (GE Healthcare) or Bio-Rad chemi-doc technology (Bio-Rad).

To confirm equal loading of proteins, primary and secondary antibodies were first removed from blots with mild stripping buffer (15 g glycine (Sigma-Aldrich), 1 g SDS (Sigma-Aldrich), 10 ml Tween 20 (Sigma-Aldrich) in 1 L ultrapure water; pH 2.2). For this, blots were subjected to 2X 10-minute incubations in stripping buffer at RT, followed by 2X 10-minute washes in PBS. After a further 2X 5-minute incubations at RT in TBS-0.1% tween-20, blots were blocked in 5% BSA/TBS-0.1% tween-20 for 1-hour at RT, before being probed overnight at 4$^{o}$C with an Erk 1/2 or p38 antibody (diluted 1:2000 from stock in TBS-0.1% tween-20 containing 1% BSA; Cell Signalling Technology). Post incubation, blots were washed 3 times (15 minutes each wash) in TBS-0.1% tween-20 and probed with a HRP-linked anti-rabbit IgG antibody (pre-diluted 1:4000 from stock in TBS-0.1% tween-20) for 1-hour at RT. Blots were then washed 3 times in TBS-0.1% tween-20 (15 minutes each wash) and proteins visualised as described above. Densitometry was performed using the National Institute of Health ImageJ software package (http://rsbweb.nih.gov/ij/).

**2.1.6.12 Determination of cytokine and chemokine secretion**

100 µl aliquots of freshly isolated NK cells (1x10$^{6}$ /ml in CM) were dispensed into wells of a 96-well round bottomed plate containing 100 µl of either K562 cells (1x10$^{6}$ /ml in CM; E:T 1:1) or CM. Following centrifugation at 50 x g for 1 minute at 20$^{0}$C, samples were incubated for 6 hours at 37$^{o}$C in a humidified 5% $C0_2$ atmosphere. Post incubation, samples were

transferred to 0.5 ml eppendorf tubes and cells pelleted by centrifugation (3000 rpm for 5 minutes at RT) in a bench top microcentrifuge. Supernatants were then harvested, snap-frozen in liquid nitrogen and stored at -80$^{o}$C prior to analysis.

To determine cytokine and chemokine concentrations, multiplex technology was performed according to manufacturer's instructions (Life Technologies). Briefly, after pre-wetting wells of a 96-well filter plate with wash solution, 25 µl of a 1X antibody bead stock for the cytokines IFN-γ and TNF-α, and the chemokines IL-8 and MIP-1α was added to each well. After a 30-second incubation at RT, the plate was washed once with wash solution and 50 µl of incubation buffer added to all wells. 100 µl of standard solution or tissue culture supernatant was then added to relevant wells and the plate incubated in the dark for 2 hours at RT on an orbital shaker (speed 500-600 rpm; Grant Instruments Limited, Cambridge, UK). Post incubation, the plate was washed twice with wash buffer and 100 µl of a 1X biotinylated antibody stock added to all wells. Following a 1-hour incubation in the dark at RT on an orbital shaker (speed 500-600 rpm), the plate was washed twice and 100 µl of 1X streptavidin-RPE added to all wells. After a further 30-minute incubation in the dark on an orbital shaker (speed 500-600 rpm), the plate was washed 3 times and 100 µl of wash solution added to all wells. Cytokine and chemokine concentrations were then analysed using a Luminex® 100™ instrument (Luminex® Corporation, Austin, Texas, USA**).**

### 2.1.7 Analysis of neutrophil function

#### 2.1.7.1 Quantification of NET formation

For the analysis of NET formation by unprimed neutrophils, 100 µl aliquots of neutrophils ($1x10^6$ /ml in RPMI-1640 media supplemented with GPS, hereafter referred to as assay media) were dispensed into wells of a 96-well flat bottomed plate (Becton Dickinson)

containing 75 µl of assay media. Cells were then stimulated by the addition of 25 µl of either PMA (0.25-25 nM; Sigma-Aldrich), IL-8 (10 ng/ml; R&D Systems, Abingdon, UK) or LPS (100 ng/ml; Sigma-Aldrich) and incubated for 3 hours at 37$^{o}$C in a humidified 5% $C0_2$ atmosphere. All stimuli were prepared from stock solutions in assay media, which alone served as a negative control.

Post incubation, samples were treated with 1 U/ml of micrococcal nuclease (MNase; Sigma-Aldrich) and 1 µM of the cell-impermeable DNA binding dye SYTOX® Green (Life Technologies) in order to digest and stain extracellular DNA respectively. After incubation at RT for 10 minutes, samples were transferred to 0.5 ml eppendorf tubes (Sarstedt) and pelleted at 5000 rpm for 10 minutes. The DNA content of supernatants was then assessed by transferring 170 µl to a flat-bottomed 96-well black plate (Corning, New York, USA) and measuring fluorescence in a Fluoroskan Ascent fluorometric plate reader (Labsystems Affinity sensors, Cambridge, UK) that was equipped with a filter setting of 485 nm (excitation) and 530 nm (emission). Experiments were performed in quadruplicate and background fluorescence values of unstimulated controls subtracted from test samples.

For the assessment of NET formation by primed neutrophils, isolated neutrophils ($1x10^6$ /ml in assay media) were treated with 10 ng/ml TNF-α (Sigma-Aldrich) or vehicle control for 15 minutes at 37$^{o}$C in a humidified 5% $C0_2$ atmosphere. Post incubation, samples were washed once (1600 rpm for 10 minutes) and cells re-suspended to $1x10^6$ /ml in assay media. NET formation in response to LPS (100 ng/ml) or Il-8 (10 ng/ml) stimulation was then examined as described above, with the exception that for the comparison of NET formation between young and old donors, fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, Leeds, UK) equipped with a filter setting of 485 nm (excitation) and 528 nm (emission).

### 2.1.7.2 Visualisation of NETs by fluorescent microscopy

$2x10^5$ neutrophils, resuspended in assay media, were seeded onto 13 mm circular glass coverslips (VWR International) and incubated for 30 minutes at 37°C in a 5% $CO_2$ atmosphere to allow for cell adherence. Post incubation, neutrophils were stimulated with either PMA (25 nM), IL-8 (10 ng/ml), LPS (100 ng/ml) or assay media for 3 hours (37°C, 5% $CO_2$), after which cells were fixed by an additional 30-minute incubation at 37°C in the presence of 4% paraformaldehyde. Once fixed, neutrophils were subjected to the following staining protocol;

- 3X 5-minute washes in PBS at RT
- 1X 1-minute incubation at RT in 0.1% triton x-100
- 1X 5-minute wash in PBS at RT
- 1X 5-minute incubation at RT with 1 µM SYTOX® Green
- 1X 5-minute wash in PBS at RT

Once stained, specimens were mounted in fluoromount medium and visualised using a LEICA DMI 6000 B microscope at X10, X20 or X63 objective.

### 2.1.7.3 Measurement of ROS generation

ROS generation was assessed by luminol-amplified chemiluminescence, a technique that detects both extracellular and intracellular free radicals [499].

To assess ROS generation in unprimed neutrophils, 100 µl aliquots of neutrophils ($1x10^6$ /ml in HBSS supplemented with calcium and magnesium) were dispensed into wells of a 96-well white-bottomed flat plate (Corning) containing 25 µl of luminol (working concentration 1µM; pH 7.3; Sigma-Aldrich) and 50 µl of calcium and magnesium containing HBSS. Cells were then treated with either LPS (100 ng/ml), PMA (25 nM), IL-8 (10 ng/ml) or vehicle

control, and ROS generation assessed at 1-minute intervals for 60 minutes using a Berthold Centro LB 960 luminometer (Berthold Technologies, Hertfordshire, UK). Experiments were performed in quadruplicate, with ROS production calculated as area under the curve (AUC).

To measure ROS production by primed neutrophils, isolated neutrophils ($1x10^6$ /ml in HBSS supplemented with calcium and magnesium) were treated with 10 ng/ml TNF-α or vehicle control for 15 minutes at 37°C in a humidified 5% $C0_2$ atmosphere. ROS production in response to LPS (100 ng/ml) and IL-8 (10 ng/ml) stimulation was then measured as described above.

#### 2.1.7.4 Simvastatin treatment of primed neutrophils

Freshly isolated neutrophils resuspended at $1x10^6$ /ml in either assay media or HBSS supplemented with calcium and magnesium were treated for 40 minutes at 37°C with 1 nM simvastatin (Sigma-Aldrich) or vehicle control. During the last 15 minutes of treatment, TNF-α (10 ng/ml) was added to prime cells.

### 2.1.8 Detection of serum immunoglobulin-G (IgG) antibodies to CMV

The presence of serum IgG antibodies to CMV was determined using an in-house pre-validated ELISA (a gift from the laboratory of P. A.H. Moss, School of Cancer Sciences, University of Birmingham, Birmingham, UK). Briefly, wells of a 96-well flat-bottomed ELISA NUNC plate were coated overnight at $4^0$C with 50 µl of viral or mock lysate that had been pre-diluted 1:4000 from stock in carbonate-bicarbonate coating buffer (Sigma-Aldrich). Stocks of viral lysate were generated from the supernatants of MRC-5 fibroblasts that had been infected for 4 hours at 37°C with the AD169 or Townel125 human CMV strains at a multiplicity of infection of 4:1. Mock infections were performed simultaneously to produce lysates that acted as controls for non-specific IgG binding. Once coated, ELISA NUNC plates

were washed 3 times in wash buffer (PBS/0.05% Tween; 200 µl per wash), after which 25 µl of donor serum samples (pre-diluted 1:150 in dilution buffer (PBS/0.05% Tween/1% BSA)) were added in duplicate to both viral and mock lysate wells alongside 75 µl of dilution buffer. Following a 1-hour incubation at RT, plates were washed 3 times in wash buffer, after which samples were treated for an additional 1-hour at RT with 100 µl of goat anti-human-IgG-HRP conjugated secondary antibody (pre-diluted 1:8000 in dilution buffer; Southern Biotech, Alabama, USA). After 3 washes with wash buffer, 100 µl of TMB substrate solution (Sigma-Aldrich) was dispensed into all wells and the plate incubated for 10 minutes at RT in the dark. Reactions were stopped by the addition of 100µl 2N $H_2SO_4$ (Sigma-Aldrich), and absorbance recorded using a spectrophotometer with a primary wavelength of 420 nm and a reference wavelength of 620 nm.

For analysis, average readings of mock lysate wells were subtracted from the values obtained in viral lysate wells. Using these figures, CMV titres were calculated from a standard curve of known CMV titres in GraphPad Prism® software. Values greater than 10 were considered to be CMV positive.

### 2.1.9 Data handling and statistical analysis

Statistical analyses were performed using GraphPad Prism® software or PASW statistics software version 18.0 (Chicago, Illinois, USA). To test if data came from a Gaussian distribution, the Kolmogorov-Smirnov test was used. For data that followed a normal distribution, unpaired and paired student T-tests were performed to assess differences between two independent groups or matched paired samples respectively. A one-way analysis of variance (ANOVA) with either a Dunnett's or Tukey post hoc test was used to study differences between more than two groups, whilst a repeated measures ANOVA with either a Dunnett's or Tukey post hoc test was used to compare more than two matched observations.

A linear regression was performed to assess the relationship between two variables. For non-normally distributed data, the non-parametric Mann-Whitney U test, a Wilcoxon matched-pairs signed rank test or Kruskal-Wallis test with Dunn's multiple comparison post hoc test were used. The effect of age and CMV status on immune parameters was assessed by a two-way ANOVA, where age and CMV were inserted as dependent variables. When a significant effect for CMV was observed, Bonferroni post hoc analysis was performed to determine its location.

Where data is presented in box and whisker plot format, the middle line depicts the median value and the whiskers represent the minimum and maximum data points. Data presented in histogram format is summarised as mean ± standard error of the mean (SEM). In all instances, the minimum level of confidence at which results were considered significant was $p \leq 0.05$.

# CHAPTER 3

## Studies to identify the mechanism(s) underlying the age-associated reduction in NK cell cytotoxicity

## 3.1 Introduction

A number of studies have shown that physiological ageing is accompanied by a marked reduction in NKCC at the single cell level [42;365;371]. As a key form of frontline defence against virally-infected cells, this age-related decline in NK cell lytic activity is often cited as a factor that contributes to the increased incidence and severity of viral infection reported amongst older adults [9]. In support of this claim, Ogata and co-workers demonstrated in two separate studies that low NKCC was associated with both the development of infection and death due to infection in a cohort of immunologically normal older adults [188;189], findings that were in agreement with an earlier longitudinal study that had shown persistently low NK cell activity to be a predictor of infectious morbidity in old individuals [187].

Despite the significance that the age-related decline in NK cell lytic activity has on human health, no study to date has identified the mechanism(s) responsible. With the exception of a small number of studies that have reported no age-related impairment in NK-target cell interaction [365;372;374], the NK cell literature is riddled with contradictory data concerning the effect of age on key features of the NKCC process. For instance, whereas some groups have suggested perforin levels decline with age [370], others have shown the expression of this pore-forming protein, along with its mobilisation and utilisation in response to target cell contact is comparable between NK cells from young and old donors [375]. Similarly, whilst some studies have reported reduced expression of activatory receptors on the surface of NK cells from aged subjects, and proposed that these changes would have a negative impact upon NK cell killing [351;362], other groups that have studied the same receptors have reported no effect for age on their expression [355]. However, it may be that signalling downstream of activatory receptors is impaired with age since when compared to NK cells from younger

adults, those from older subjects have been shown to produce significantly lower levels of the calcium-mobilising second messenger $IP_3$ in response to target cell contact [373].

Given that despite numerous attempts, no study to date had fully established why the lytic activity of NK cells declines with age, the aim of this chapter was to elucidate the underlying cause(s) of this phenomenon. To do this, NK cells were isolated from healthy young (≤35 years) and old (≥60 years) donors, and the effect of age on each of the events fundamental to the process of NKCC was examined.

## 3.2 Results

### 3.2.1. Effect of age on the proportions and absolute numbers of circulating NK cells

Immunostaining and multi-colour flow cytometric analysis of PBMC samples isolated from young and old donors was performed to assess the effect of age on the composition of the circulating lymphocyte pool (Table 3.1). A greater proportion of both $CD3^{-}$ $56^{+}$ and $CD3^{-}$ $56^{+}57^{+}$ NK cells was found amongst the lymphocyte pool of old individuals when compared to young adult controls, changes which were accompanied by an age-related reduction in the percentage of $CD3^{+}$ lymphocytes (Table 3.1).

Based on the differential surface expression of CD56, NK cells can be divided into two major subsets, $CD56^{DIM}$ and $CD56^{BRIGHT}$ [345]. When analysed according to this criteria, a differential effect of age was observed, with older adults possessing a significantly higher percentage of $CD56^{DIM}$ cells amongst their lymphocyte pool but a significantly lower proportion of $CD56^{BRIGHT}$ cells, which culminated in an increased $CD56^{DIM}$ to $CD56^{BRIGHT}$ ratio (Table 3.1). Further analysis based on the co-expression of CD56 and the low affinity Fc receptor CD16 revealed the accumulation of $CD56^{DIM}$ cells to be the result of an increased proportion of $CD3^{-}16^{+}56^{DIM}$ NK cells, whilst a significant decline in the percentage of $CD3^{-}$ $16^{-}56^{BRIGHT}$ cells accounted for the reduction in the $CD56^{BRIGHT}$ subset (Table 3.1) [500]. In addition to $CD56^{DIM}$ and $CD56^{BRIGHT}$ subsets, the frequency of $CD3^{-}56^{-}16^{+}$ NK cells was assessed and an age-related reduction observed (Table 3.1).

In a smaller cohort of individuals, full blood counts were performed in addition to flow cytometry in order to examine the effect of age on the absolute number of circulating lymphocytes (Table 3.2). Compared to their younger counterparts, old individuals presented

**Table 3.1. Age-associated changes in the circulating lymphocyte pool**

| | Young (n = 21) | Old (n = 21) | p Value |
|---|---|---|---|
| $CD3^{+}56^{-}$ | 64.44±1.8 | 58.40±1.8 | **0.02** |
| $CD3^{-}56^{+}$ | 10.60±0.9 | 17.21±1.2 | **0.0001** |
| $CD3^{-}56^{+}57^{+}$ ¥ | 55.62±2.9 | 66.10±2.1 | **0.03** |
| $CD56^{DIM}$ | 10.40±0.9 | 16.75±1.2 | **<0.0001** |
| $CD56^{BRIGHT}$ | 0.56±0.1 | 0.40±0.04 | **0.03** |
| $CD3^{-}16^{+}56^{DIM}$ ¥ | 85.7±1.5 | 91.8±1.0 | **0.001** |
| $CD3^{-}16^{-}56^{DIM}$ ¥ | 3.8±0.5 | 3.8±0.8 | 0.537 |
| $CD3^{-}16^{+}56^{BRIGHT}$ ¥ | 1.6±0.2 | 1.5±0.3 | 0.392 |
| $CD3^{-}16^{-}56^{BRIGHT}$ ¥ | 8.3±1.1 | 2.6±0.4 | **<0.0001** |
| DIM:BRIGHT | 23:1±3.5 | 51:1±5.7 | **0.0001** |
| $CD3^{-}56^{-}16^{+}$ ∞ | 3.5±0.6 | 7.8±1.0 | **0.001** |

Values are presented as mean ± SEM. ¥Data for CD16 and CD57 expression reflects the proportion of antigen positive cells within the NK cell pool. ∞The frequency of $CD3^{-}56^{-}16^{+}$ NK cells is presented as a percentage of $CD3^{-}56^{-}$ lymphocytes. All other data relates to the percentage of positive cells within the lymphocyte pool. Differences between groups were assessed by an unpaired student T test or Mann-Whitney U test. Significant differences are indicated in bold font.

**Table 3.2. Effect of age on the absolute number (x$10^6$/L) of circulating lymphocytes**

| | Young (n = 11) | Old (n = 15) | p Value |
|---|---|---|---|
| Lymphocytes | 2036±119.30 | 1520±120.00 | **0.004** |
| $CD3^+56^-$ | 1429±78.74 | 1077±101.80 | **0.005** |
| $CD3^-56^+$ | 231±23.82 | 176±21.07 | 0.058 |
| $CD56^{DIM}$ | 217±23.20 | 170±21.01 | 0.161 |
| $CD56^{BRIGHT}$ | 14±2.10 | 6±0.65 | **0.0003** |
| $CD3^-16^+56^{DIM}$ ¥ | 206±22.68 | 165±20.83 | 0.206 |
| $CD3^-16^-56^{DIM}$ ¥ | 7±1.45 | 4±0.47 | 0.083 |
| $CD3^-16^+56^{BRIGHT}$ ¥ | 4±0.69 | 2±0.40 | **0.001** |
| $CD3^-16^-56^{BRIGHT}$ ¥ | 14±2.70 | 5±0.62 | **<0.0001** |

Values are presented as mean ± SEM. ¥Data for CD16 expression relates to the number of antigen positive cells within the $CD3^-56^+$ NK cell pool. Differences between groups were assessed by an unpaired student T test or Mann-Whitney U test. Significant differences are indicated in bold font.

with significantly lower numbers of total lymphocytes and $CD3^{+}56^{-}$ T cells, whilst a trend was observed for an age-related reduction in the circulating numbers of $CD3^{-}$ $56^{+}$ NK cells (Table 3.2). When NK cells were analysed by subset, no differences were found between young and old subjects in the absolute number of circulating $CD56^{DIM}$, $CD56^{DIM}$ $16^{+}$ or $CD56^{DIM}$ $16^{-}$ subsets. In contrast, a marked age-related decline was observed in the total number of $CD56^{BRIGHT}$ NK cells, which was attributable to a significant reduction in both the $CD56^{BRIGHT}$ $16^{+}$ and $CD56^{BRIGHT}$ $16^{-}$ subsets (Table 3.2).

### 3.2.2. Effect of age on NKCC

In line with the work of previous groups, who had reported a marked age-related decline in NKCC at the single cell level [42;365;371], preliminary studies revealed that at a range of effector:target (E:T) ratios, NK cells isolated from old donors exhibited reduced cytotoxicity towards the MHC class I deficient K562 cell line when compared to those obtained from younger controls (Figure 3.1). As maximal killing and the greatest age-related difference in lytic activity was observed at 10:1, additional killing assays were performed at this E:T ratio in a larger cohort. Confirming our preliminary data, the cytotoxicity of resting NK cells from old donors against K562 target cells was markedly lower than that of NK cells from their younger counterparts following a 4-hour co-culture (percentage target cell lysis, Young = 41±3.1% Vs. Old = 21±2.4%, P = <0.0001; Figure 3.2 A-B), an impairment that was still observed when NK-K562 cell co-cultures were extended to 16 hours (Figure 3.3A-B).

**(A)**

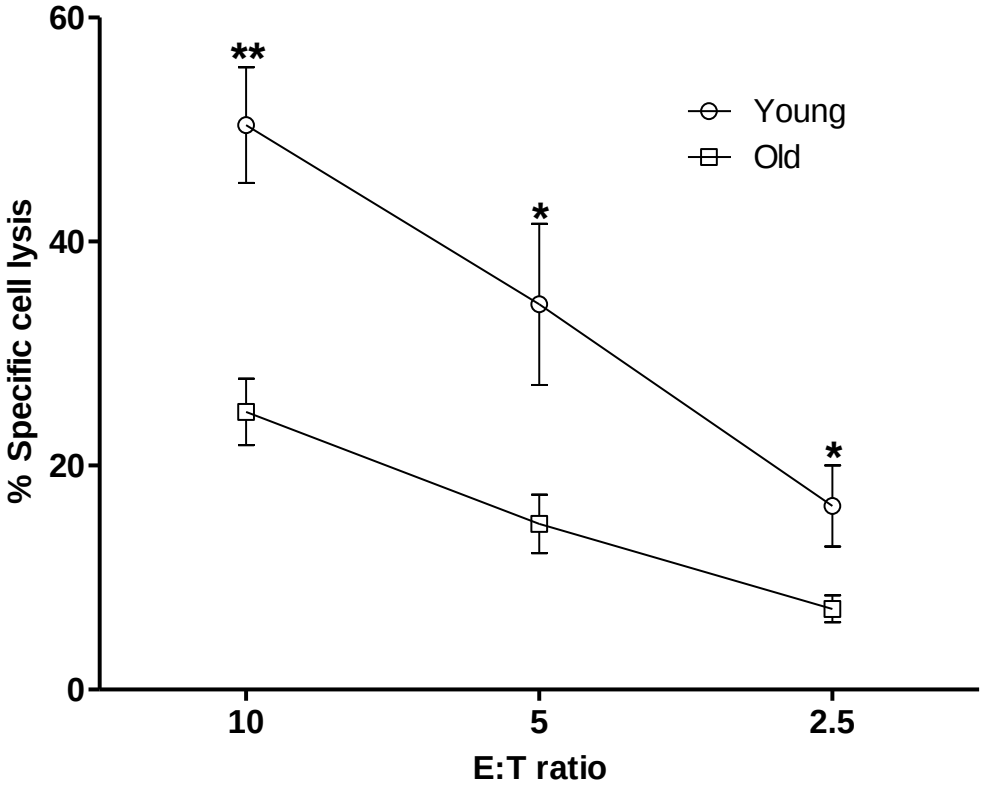
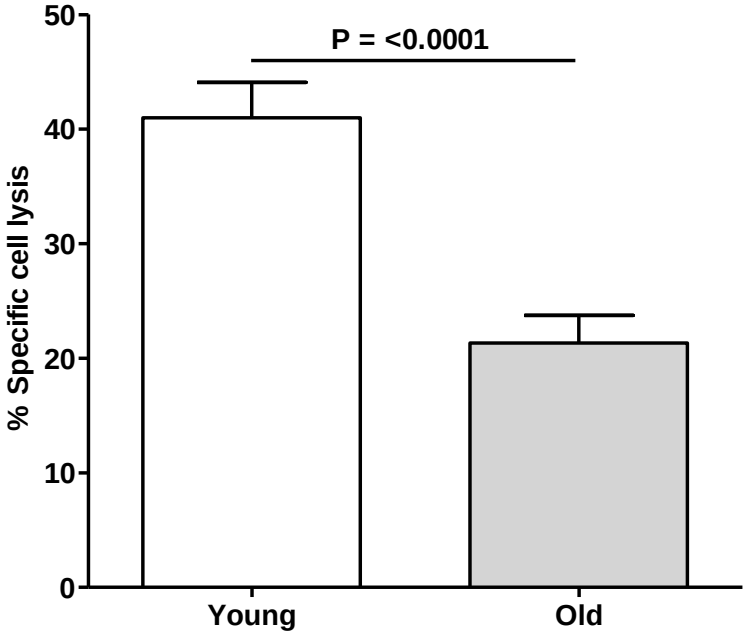



**Figure 3.1. NKCC at the single cell level is reduced with age across a range of effector:target cell ratios.** Cytotoxicity of resting NK cells purified from 5 young and 5 old donors towards the MHC class I deficient K562 tumour cell line was measured at a range of effector:target (E:T) cell ratios by two-colour flow cytometry following a 4-hour co-culture. Data is expressed as mean ± SEM with differences between the two groups at each E:T ratio assessed by an unpaired student T test. ** indicates $p \leq 0.005$, * indicates $p \leq 0.05$.

**(A)**




**(B)**

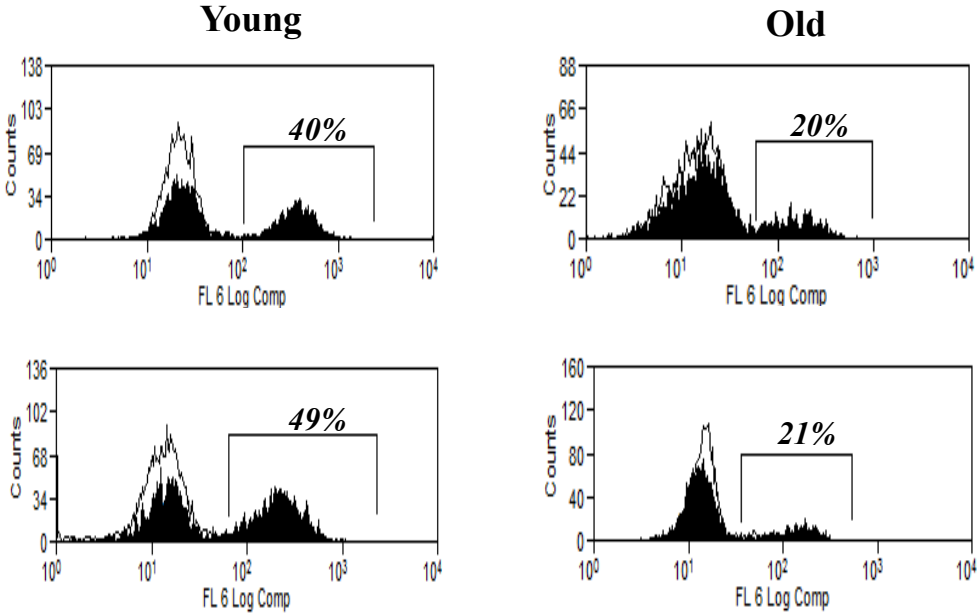



**Figure 3.2. Age-related decline in NK cell lytic activity in short-term cytotoxicity assays.** **(A)** Cytotoxicity of resting NK cells isolated from 12 young and 12 old donors against the MHC class I deficient K562 tumour cell line at an E:T of 10:1 was assessed by two-colour flow cytometry following a 4-hour co-culture. Data is expressed as mean ± SEM, with differences between the two groups assessed by an unpaired student T test. **(B)** Representative flow cytometry histograms for 2 young and 2 old donors highlighting the age-related decline in NKCC. Open histograms depict K562 cells cultured in the absence of NK cells, filled histograms represent SYTOX® positive target cells.

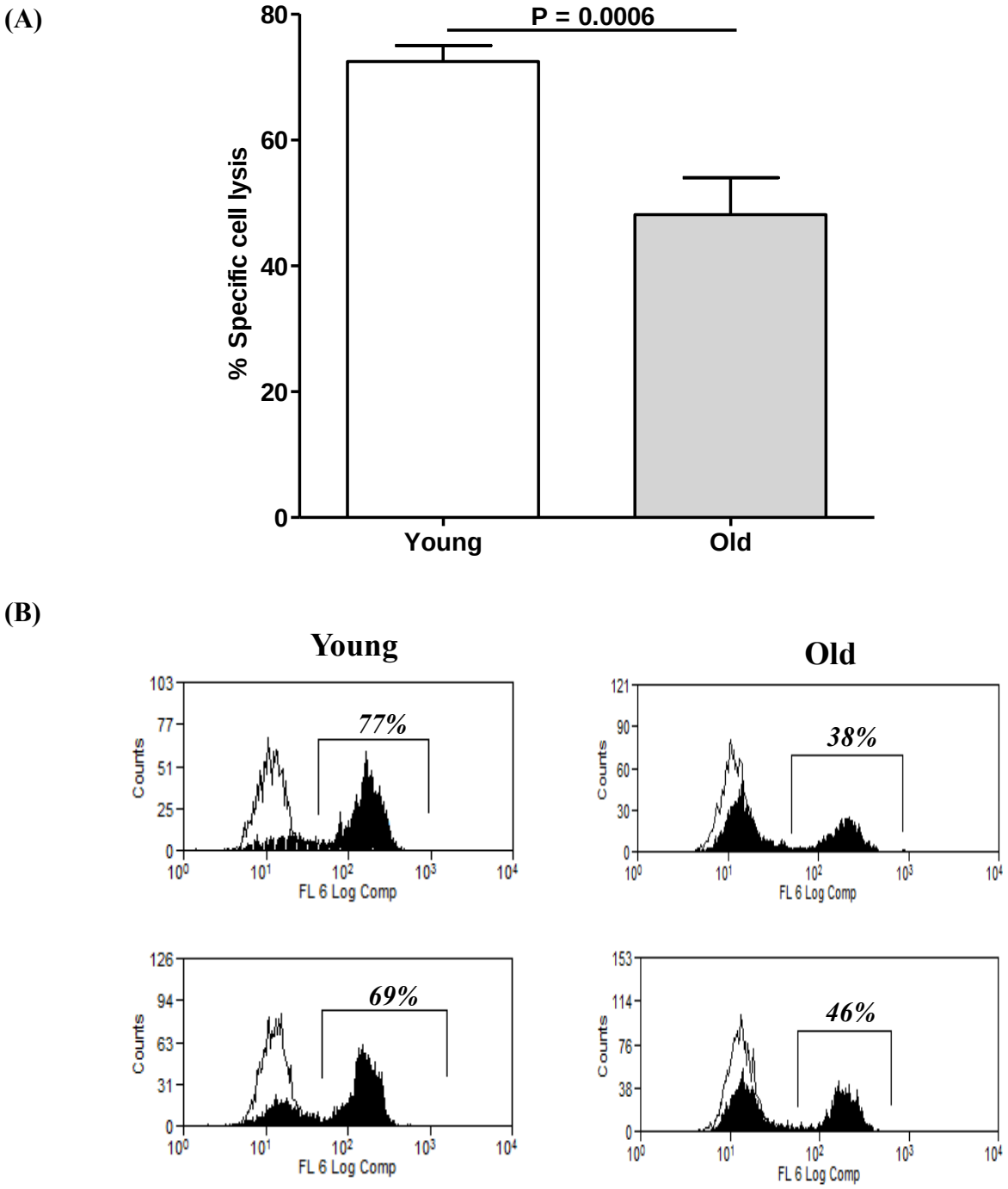



**Figure 3.3. Age-related decline in NK cell lytic activity in long-term cytotoxicity assays.** **(A)** Cytotoxicity of resting NK cells towards the MHC class I deficient K562 cell line (E:T, 10:1) was measured after a 16-hour co-culture. Values represent mean ± SEM of 11 young and 8 old donors with differences between the two groups assessed by an unpaired student T test. **(B)** Representative flow cytometry histograms for 2 young and 2 old donors demonstrating the age-associated reduction in NKCC. Open histograms depict K562 cells cultured in the absence of NK cells, filled histograms represent SYTOX® positive target cells.

To aid in our understanding of the mechanism(s) underlying this age-related impairment in NKCC, it was important to determine which of the two contact-dependent pathways utilised by NK cells to eliminate their targets (granule exocytosis or death receptor ligation) was responsible for inducing K562 cell lysis. Treating NK cells with concanamycin A (CMA), a potent inhibitor of perforin activity [495], significantly reduced K562 cell lysis (Figure 3.4). In contrast, no target cell lysis was observed when K562 cells were treated with soluble ligands for the death receptors Fas (Figure 3.5A), TRAIL (Figure 3.5B) or TNF (Figure 3.5C).

Together, these data demonstrate that NK cell-mediated lysis of K562 cells is dependent upon granule exocytosis, and that a defect in this pathway is responsible for the age-related impairment in NKCC.

### 3.2.3. Effect of age on the formation of NK-K562 cell conjugates

To study NKCC, a previously described flow cytometric protocol that involved labelling NK cells post K562 co-culture with a PE-conjugated antibody specific for CD56 was used [493]. In addition to providing information on NK cell lytic activity, this approach enabled assessment of the formation of NK-K562 cell conjugates, which were classified as K562 target cells exhibiting positive red fluorescence (Figure 3.6A).

It was found that whilst NKCC exhibited a marked age-related decline, NK cells from old donors were as efficient in binding their target cells as those isolated from young adult controls (Figure 3.6B), suggesting the impairment in NKCC that accompanies human ageing is the result of a post-binding defect.

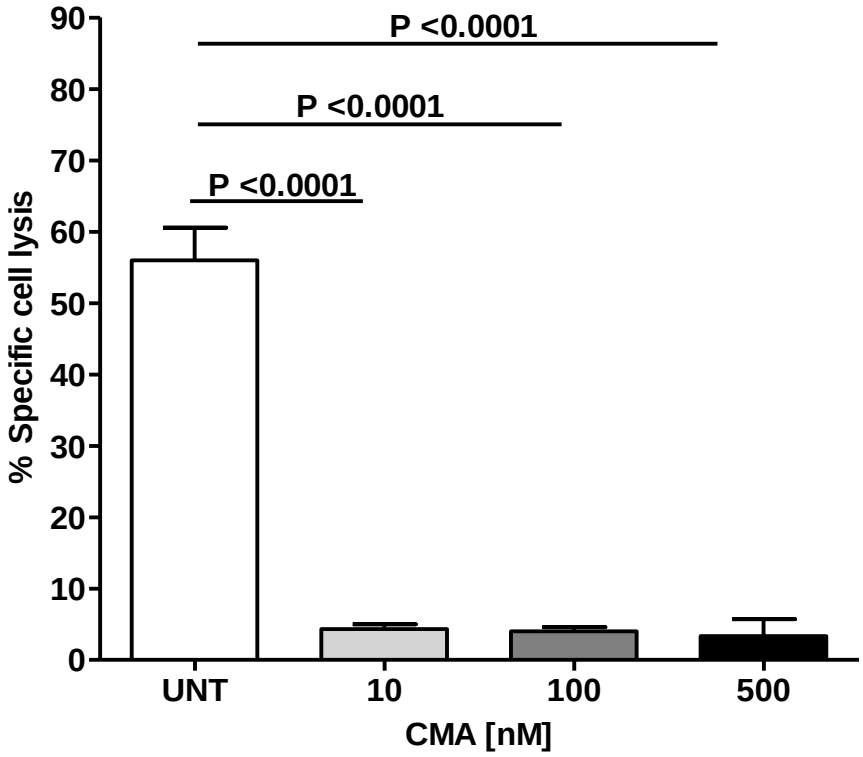
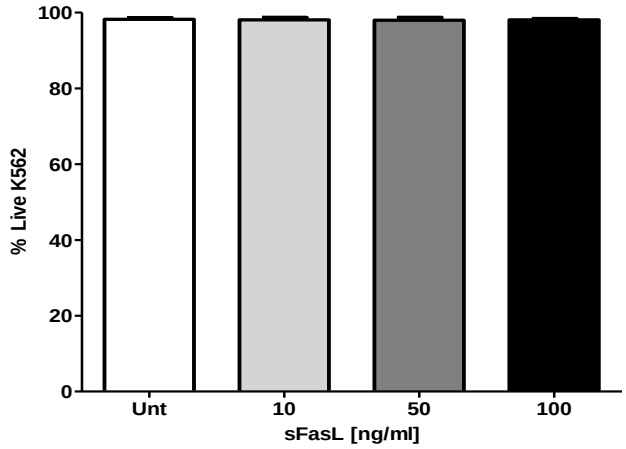
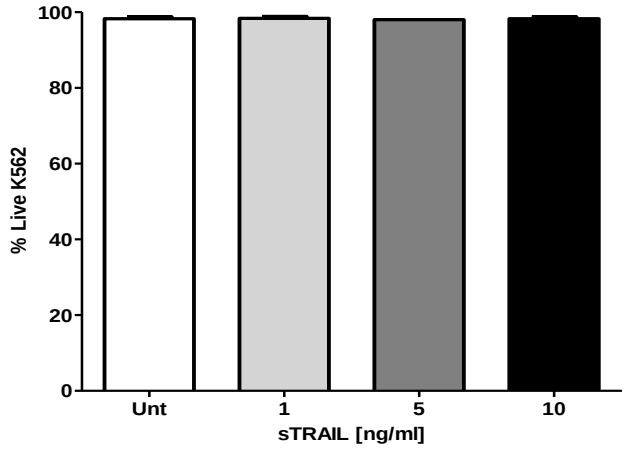



**Figure 3.4. NK cells induce lysis of K562 target cells through the granule exocytosis pathway.** Resting NK cells isolated from 3 young donors were treated for 2 hours with 10-500 nM concanamycin A (CMA), a potent inhibitor of perforin based cytotoxicity, after which their lytic activity towards K562 target cells was assessed in 4-hour cytotoxicity assays. Data are presented as mean ± SEM, with differences between the conditions analysed by a one-way ANOVA with Dunnett's post hoc test versus untreated (UNT) group.

**(A)**




**(B)**




**(C)**

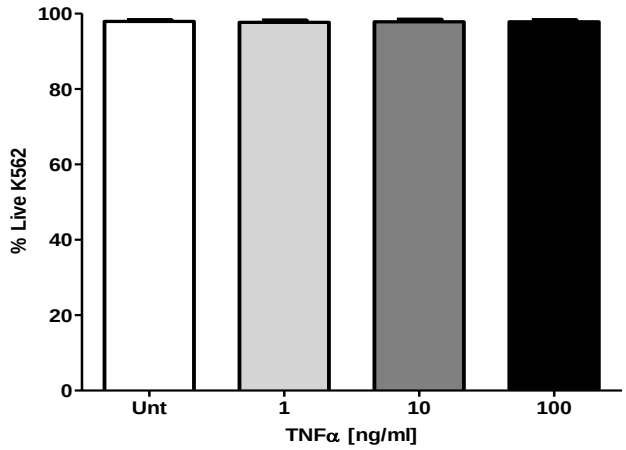
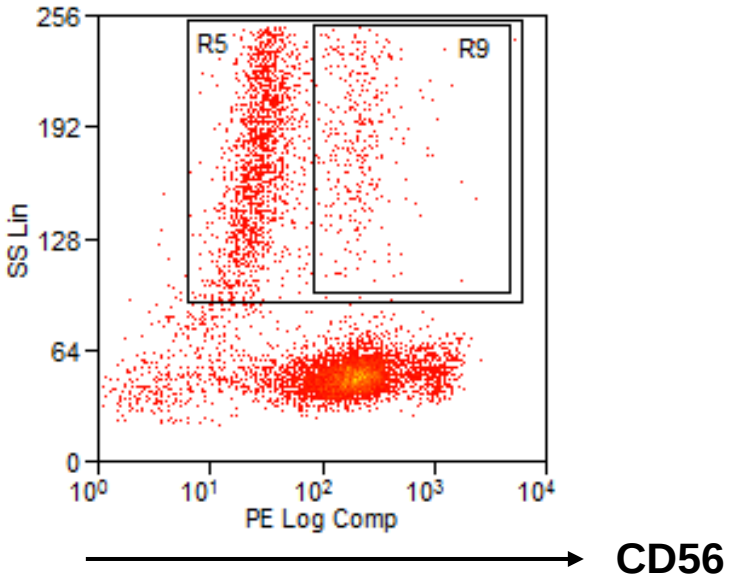



**Figure 3.5. Death receptor ligation does not induce lysis of K562 tumour cells.** K562 cells ($2x10^5$) were treated for 4 hours with increasing concentrations of sFasL **(A)**, sTRAIL **(B)** or TNF-α **(C)**, after which, the percentage of cells with perturbed membranes was examined by flow cytometry. Data are presented as the mean ± SEM of three separate experiments performed in triplicate. Differences between the groups were assessed by a Kruskal-Wallis test with Dunn's multiple comparison post hoc test.

**(A)**




**(B)**

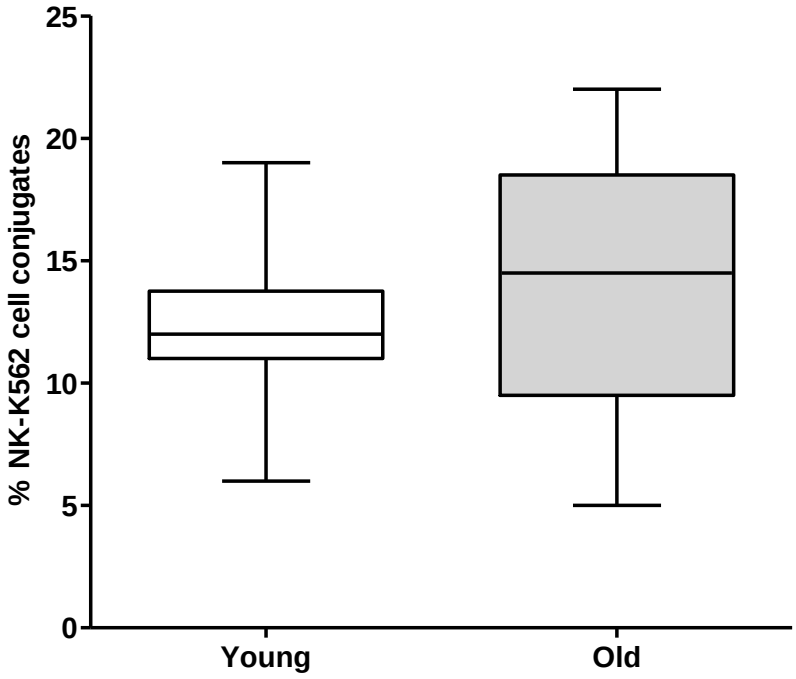



**Figure 3.6. No age-associated impairment in the formation of NK-K562 cell conjugates.** **(A)** Flow cytometry dot plot of an NK-K562 cell co-culture. By labelling NK cells with a CD56-PE antibody, NK-K562 conjugates were defined as K562 cells exhibiting positive red fluorescence (region R9). Free K562 cells are shown in region R5. **(B)** Percentage of K562 cells bound to NK cells isolated from young (n = 12) and old (n = 12) donors following 4-hour co-culture. Data is expressed as mean ± SEM, with differences between the groups assessed by an unpaired student T test.

### 3.2.4. Effect of age and CMV serostatus on NK cell receptor expression and activation

NK cell activity is regulated by signals transmitted through a multitude of germline-encoded activatory and inhibitory receptors [306]. When activatory signals dominate NKCC is induced. Thus, the possibility that an age-related reduction in activatory receptor expression was responsible for the decline observed in NKCC was considered. In particular the impact of age on the expression levels of the C-type lectin family member NKG2D and the NCR NKp30, whose ligands MICA/B and B7-H6 respectively are expressed on the K562 tumour cell line was investigated [260;501].

Both the percentage of cytotoxic $CD56^{DIM}$ NK cells expressing NKG2D and its staining intensity was comparable between young and old subjects (Table 3.3 and Figure 3.7). Similarly, no age-related changes were found in the percentage of $NKp30^{+}$ NK cells. However, a small but significant difference was noted in the surface intensity of NKp30, with NK cells from old individuals presenting with lower levels of this receptor (Table 3.3 and Figure 3.7). Other surface receptors examined included the NCR NKp46, where an age-associated reduction in the percentage of NK cells expressing this receptor was noted, and the Fc receptor CD16, whose expression when measured as either the percentage of positive cells or staining intensity did not change with age (Table 3.3 and Figure 3.7).

In addition to activatory receptors, expression of CD57, a marker of NK cell maturity and CD94, a molecule required for the surface expression of the C-type lectin family members NKG2A/B/C/E/H [502] was measured. The percentage of $CD57^{+}$ NK cells and CD57 surface density was shown to be greater in elderly subjects (Table 3.3, Figure 3.7), whilst both the frequency of $CD94^{+}$ NK cells and its staining intensity was markedly reduced with age (Table 3.3 and Figure 3.7).

**Table 3.3. Age-related changes in NK cell receptor expression.**

| | Percentage positive | | | Staining Intensity (MedFI) | | |
|---|---|---|---|---|---|---|
| | **Young** | **Old** | **P** | **Young** | **Old** | **P** |
| NKG2D | 92.10±1.50 | 91.71±1.14 | 0.512 | 42.86±2.70 | 39.62±2.80 | 0.406 |
| NKp30 | 85.17±2.63 | 81.61±3.62 | 0.601 | 16.22±1.22 | 12.89±0.96 | **0.040** |
| NKp46 | 81.95±1.90 | 67.05±4.60 | **0.005** | 10.57±0.73 | 8.52±1.18 | 0.148 |
| CD16 | 96.71±0.44 | 96.76±0.63 | 0.496 | 146.30±9.3 | 120.2±11.06 | 0.080 |
| CD94 | 62.29±3.82 | 43.52±2.91 | **0.0003** | 30.05±5.10 | 14.19±2.39 | **0.006** |
| CD57 | 61.52±2.25 | 70.05±1.92 | **0.006** | 137.9±43.74 | 214.0±35.62 | **0.014** |

Expression of NKG2D, NKp46, CD16, CD94 and CD57 was studied on the surface of 5000 $CD56^{DIM}$ NK cells isolated from 21 young and 21 old donors. Expression of NKp30 was examined on 15,000 $CD3^{-}$ $CD56^{+}$ NK cells obtained from 18 young and 18 old subjects. Data are presented as the percentage of antigen positive cells and median fluorescent intensity (MedFI) and expressed as mean ± SEM. Differences between groups were assessed by an unpaired student T test or Mann-Whitney U test with significant differences indicated in bold font.

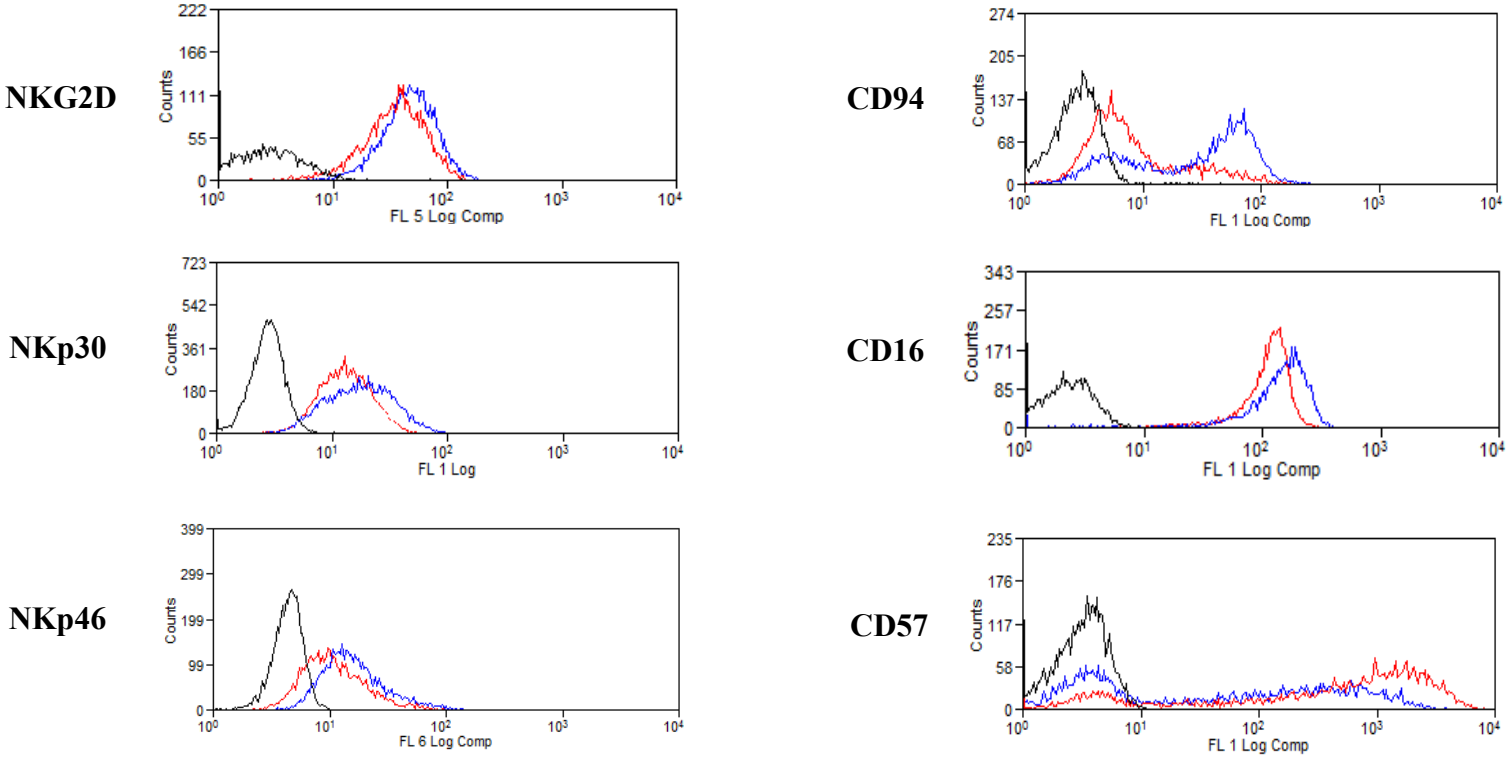



**Figure 3.7. Age-related changes in NK cell receptor expression**. Representative histograms showing the expression levels of NKG2D, NKp30, NKp46, CD94, CD16 and CD57 on the surface of resting NK cells isolated from healthy young (blue lines) and old (red lines) subjects. Black lines depict isotype controls.

Infection with the β-herpes virus CMV is known to influence NK cell receptor expression [363;364]. With this in mind, the CMV status of a subset of individuals from the above cohort was determined and its effect alongside age on the surface phenotype of circulating NK cells examined.

In this smaller group of subjects, age was associated with a significant reduction in both the percentage of $CD94^+$ NK cells and the surface density of NKG2D, whilst a trend for an age-related decline in $NKp46^+$ NK cells was also observed (Table 3.4 and Table 3.5). With regards to CMV, it was found that infection with this virus had no significant effect upon the proportions of circulating $NKG2D^+$, $CD94^+$, $NKp46^+$ or $NKp30^+$ NK cells (Table 3.4), or the surface density of these receptors (Table 3.5), with the exception of NKp30, where NK cells isolated from young $CMV^+$ donors exhibited significantly lower MedFI readings for this activatory receptor when compared to those from $CMV^-$ individuals (Table 3.5). For no receptor was a significant interaction between CMV and age found.

In addition to surface receptors, the effects of age and CMV on the expression of CD57 were also studied. Whilst a trend for an age-associated increase in the circulating frequency of $CD56^{DIM}$ $CD57^+$ NK cells was observed (Young, 65.71±2.49 Vs. Old, 73.17±2.74, $p = 0.06$), a significant effect was found for CMV ($F(1, 17) = 7.96$, $p = 0.012$, $\eta^2 = 0.319$), with $CMV^+$ individuals possessing a significantly higher proportion of this NK subset in their peripheral pool (Figure 3.8A). Post-hoc analysis revealed this effect was confined to our elderly population (Figure 3.8B). As well as influencing the frequency of $CD57^+$ NK cells, CMV elicited a strong effect upon the surface density of CD57 ($F(1, 17) = 25.19$, $p = <0.0001$, $\eta^2 = 0.597$; Figure 3.9A). It was found that NK cells isolated from both young and old seropositive donors exhibited increased surface density of CD57 when compared to their seronegative counterparts (Figure 3.9B). Interestingly, whilst aged $CMV^+$ individuals

presented with significantly higher MedFI readings for CD57 when compared to young $CMV^+$ subjects, no difference was found in CD57 surface density between young and old CMV negative donors (Figure 3.10).

The differences observed in NK cell receptor expression with age (Table 3.3 and Figure 3.7) offered no insight into whether NK cells from old donors exhibited impaired activation following target cell contact. Thus, to address this issue, the surface expression of CD69, a marker of early NK cell activation was measured both at baseline and following a 4-hour co-culture with K562 tumour cells. CD69 expression on resting NK cells was comparable between young and old subjects in terms of both the percentage of positive cells (Figure 3.11A) and surface density (Figure 3.11B), and as expected, target cell recognition led to a marked increase in both the proportions of $CD69^+$ NK cells (Young, Pre = 15.00±2.01 Vs. Post = 45.38±3.33, P = <0.0001; Old, Pre = 17.04±1.89 Vs. Post = 50.92±3.02, P = <0.0001) and its staining intensity (Young, Pre = 5.00±0.83 Vs. Post = 10.29±1.57, P = <0.01; Old, Pre = 4.92±0.81 Vs. Post = 9.77±1.13, P = <0.01). However, when the up-regulation of CD69 induced by K562 cells was compared between the two groups, no statistically significant differences were found (Figure 3.12A and 3.12B).

**Table 3.4. Effect of age and CMV status on the frequency of receptor positive NK cells**

| | Young | | Old | | Age | | CMV | |
|---|---|---|---|---|---|---|---|---|
| | CMV negative | CMV positive | CMV negative | CMV positive | F | P | F | P |
| NKG2D (n = 21) | 94.6±1.80 | 89.8±3.11 | 89.2±2.02 | 93.3±2.52 | 0.132 | 0.721 | 0.019 | 0.893 |
| CD94 (n = 18) | 65.0±4.10 | 56.3±3.40 | 43.4±3.88 | 38.7±4.74 | 6.122 | **0.027** | 0.737 | 0.405 |
| NKp30 (n = 21) | 84.7±3.55 | 77.3±2.94 | 89.0±3.22 | 74.3±4.38 | 0.014 | 0.907 | 3.938 | 0.060 |
| NKp46 (n = 22) | 82.2±2.82 | 80.3±3.10 | 61.4±4.72 | 68.8±5.35 | 3.628 | 0.073 | 0.104 | 0.751 |

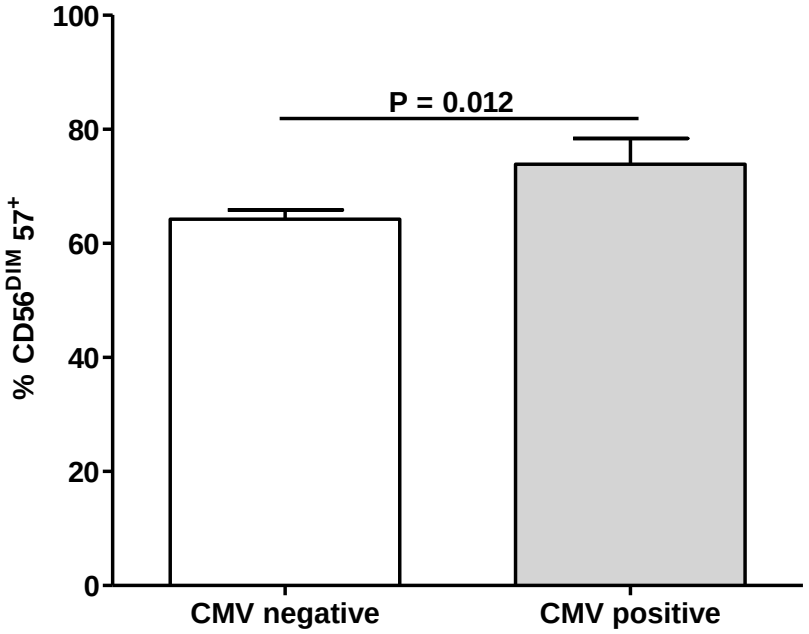
Data are expressed as mean ± SEM. Significant differences are indicated in bold font.

**Table 3.5. Effect of age and CMV status on the surface density of NK cell receptors**

| | Young | | Old | | Age | | CMV | |
|---|---|---|---|---|---|---|---|---|
| | CMV negative | CMV positive | CMV Negative | CMV positive | F | P | F | P |
| NKG2D (n = 21) | 48.3±3.62 | 40.0±3.56 | 33.2±2.33 | 32.7±2.79 | 5.004 | **0.039** | 0.762 | 0.395 |
| CD94 (n = 18) | 32.5±4.58 | 22.0±3.65 | 12.8±3.18 | 19.0±4.84 | 1.783 | 0.703 | 0.064 | 0.804 |
| NKp30 (n = 21) | 17.6±2.25 | 11.5±1.92 | 14.6±1.99 | 11.6±2.10 | 0.655 | 0.427 | 6.596 | **0.018*** |
| NKp46 (n = 22) | 10.5±1.50 | 10.8±1.82 | 7.9±2.64 | 8.8±2.22 | 1.031 | 0.323 | 0.061 | 0.808 |

Data are expressed as mean ± SEM and represents MedFI values. Significant differences are indicated in bold font. *Bonferroni post hoc analysis revealed the significant effect for CMV resided in the young cohort.

**(A)**




**(B)**

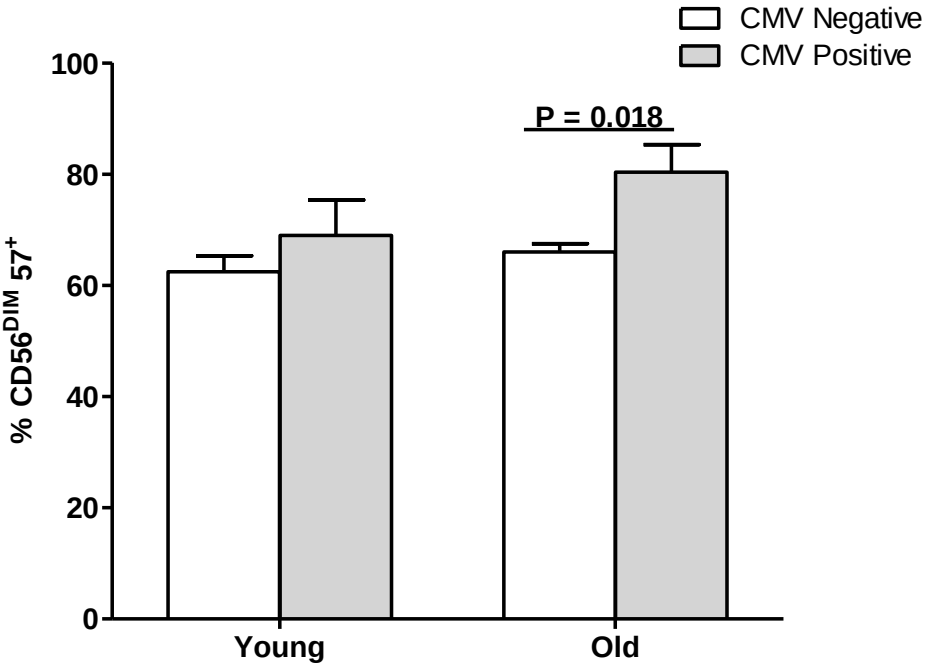



**Figure 3.8. Effect of CMV status on the frequency of circulating CD57$^+$ NK cells.** Percentage of CD56$^{DIM}$57$^+$ NK cells within the circulating NK cell pool of CMV$^+$ (n = 7) and CMV$^-$ (n = 14) subjects. **(A)** Effect of CMV status alone on the percentage of CD56$^{DIM}$57$^+$ NK cells. **(B)** Effect of CMV status on the percentage of CD56$^{DIM}$57$^+$ NK cells in young (n = 11, CMV$^+$ = 4, CMV$^-$ = 7) and old (n = 10, CMV$^+$ = 3, CMV$^-$ = 7) donors. Data are summarised as mean ± SEM and was analysed by a two-way ANOVA with Bonferroni post hoc test.

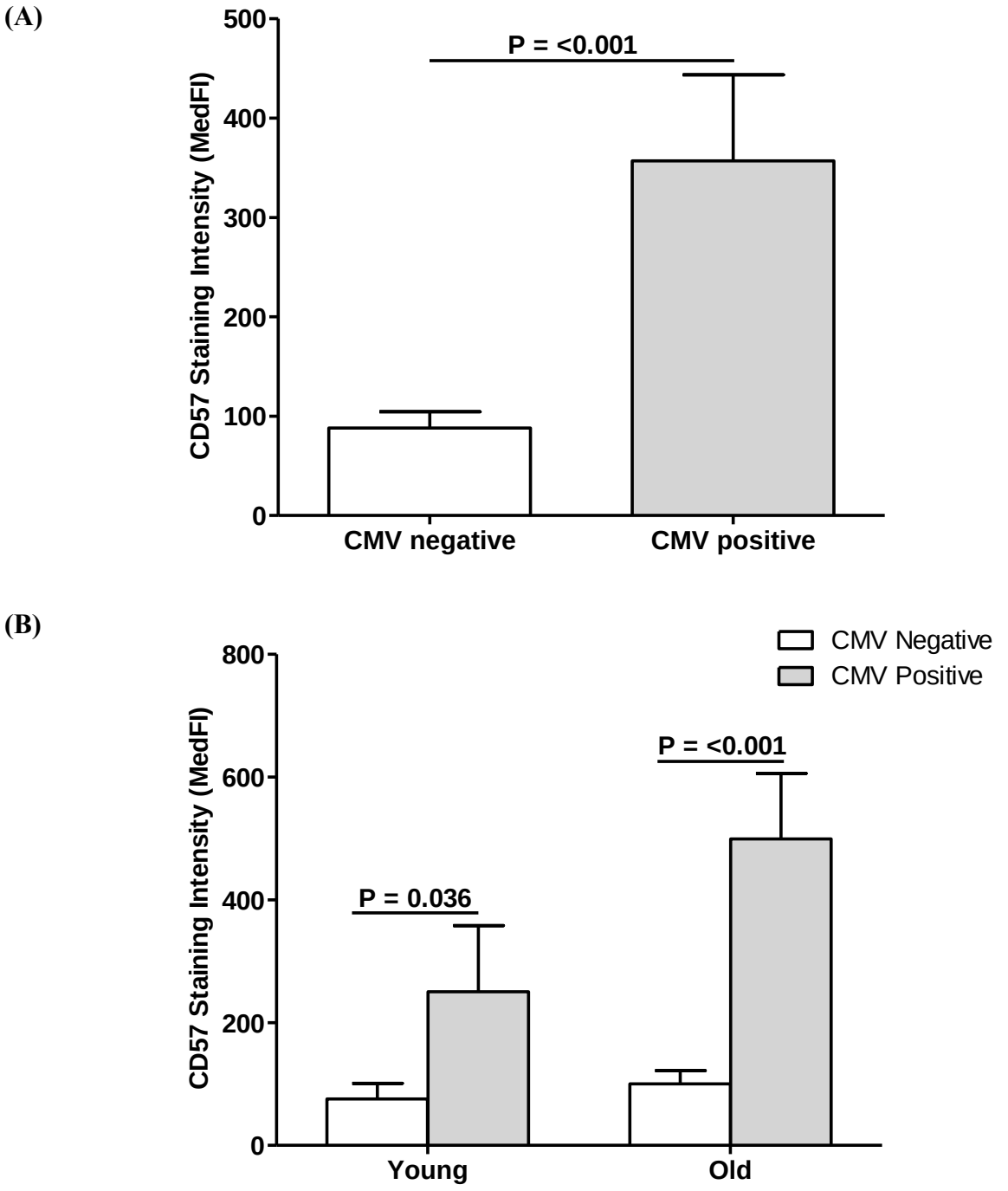



**Figure 3.9. Effect of CMV status on the surface expression of CD57 on circulating NK cells.** CD57 staining intensity on the surface of $CD56^{DIM}57^{+}$ NK cells in the circulating NK cell pool of $CMV^{+}$ (n = 7) and $CMV^{-}$ (n = 14) subjects. **(A)** Effect of CMV status alone on CD57 staining intensity. **(B)** Effect of CMV status on CD57 staining intensity in young (n = 11, $CMV^{+}$ = 4, $CMV^{-}$ = 7) and old (n = 10, $CMV^{+}$ = 3, $CMV^{-}$ = 7) donors. Data are summarised as mean ± SEM and was analysed by a two-way ANOVA with Bonferroni post hoc test.

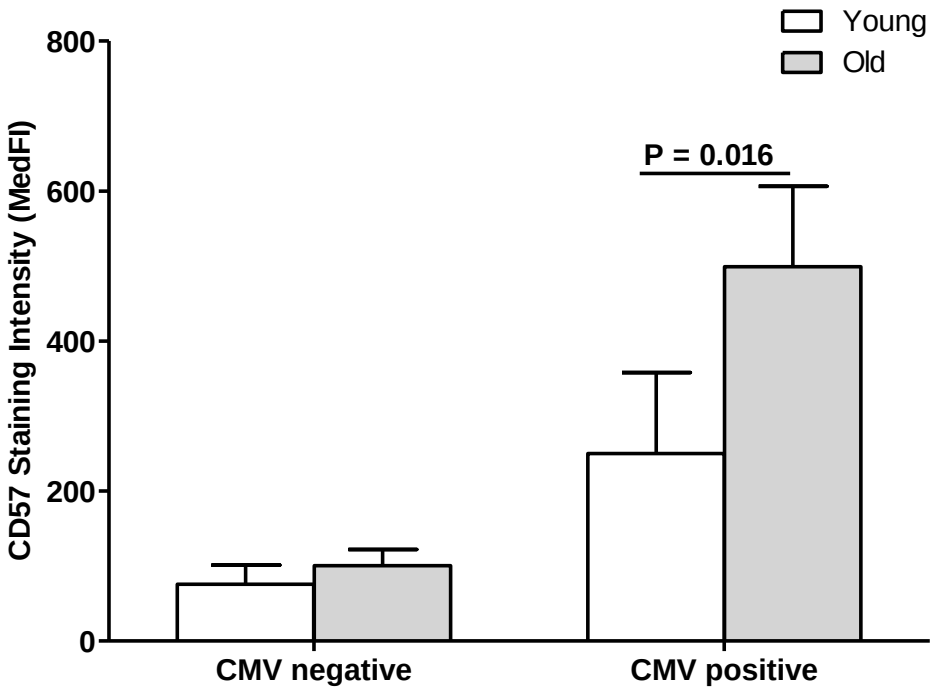
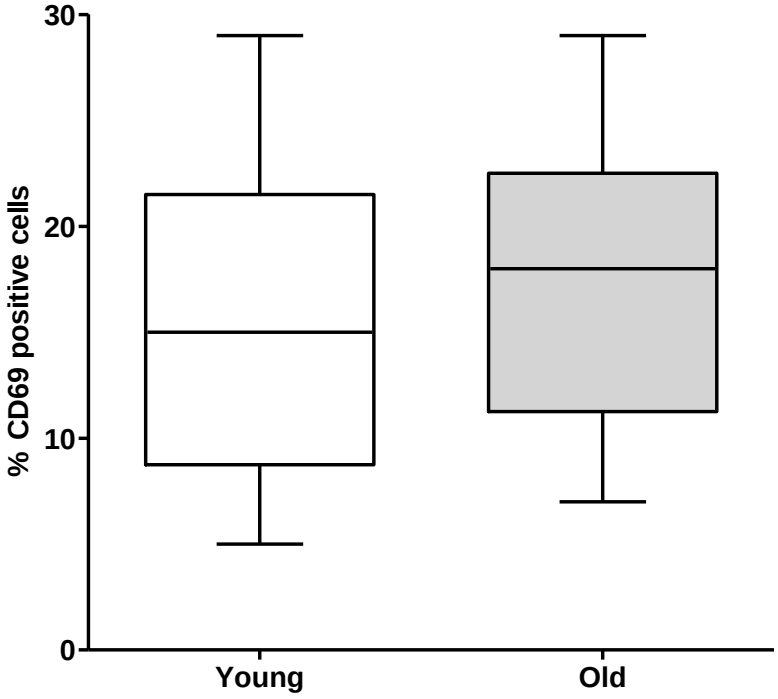



**Figure 3.10. Exposure to CMV and not age drives the increase in CD57 surface density.** CD57 staining intensity on the surface of $CD56^{DIM}57^{+}$ NK cells in the circulating lymphocyte pool of $CMV^{+}$ (young, n = 4, old, n = 3) and $CMV^{-}$ (young, n = 7, old, n = 7) subjects. Data are presented as mean ± SEM and was analysed by a two-way ANOVA with Bonferroni post hoc test.

**(A)**

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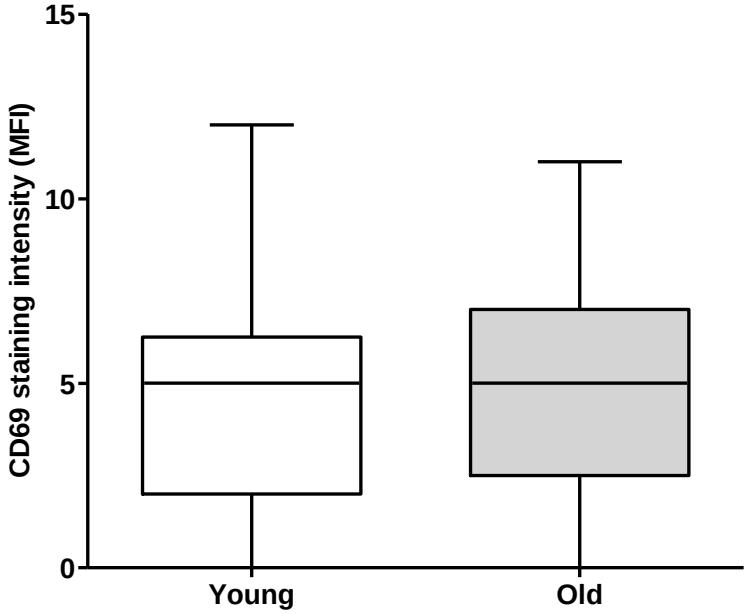
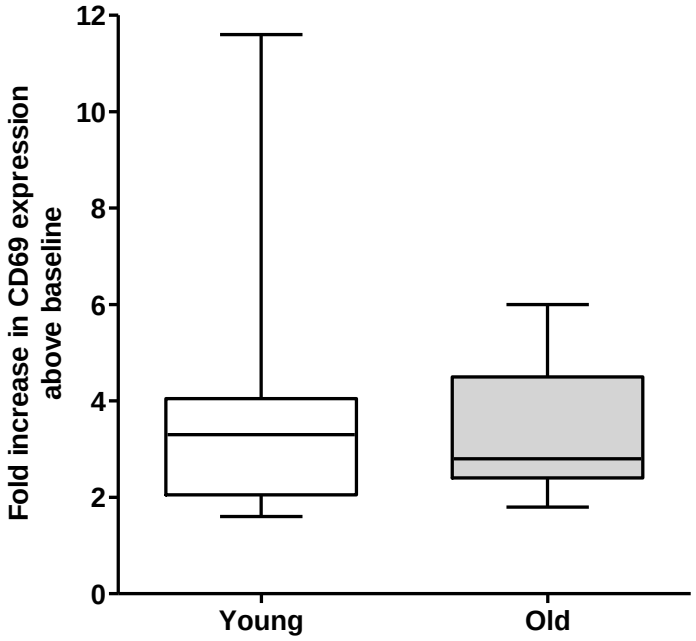



**Figure 3.11. Activation status of resting NK cells.** Expression of the early activation marker CD69 was examined on the surface of freshly isolated $CD56^+$ NK cells from young (n = 14) and old (n = 13) donors. The percentage of $CD69^+$ NK cells **(A)** and mean fluorescent intensity values (MFI) **(B)** are shown. Differences between groups were assessed by an unpaired student T test or Mann-Whitney U test.

**(A)**

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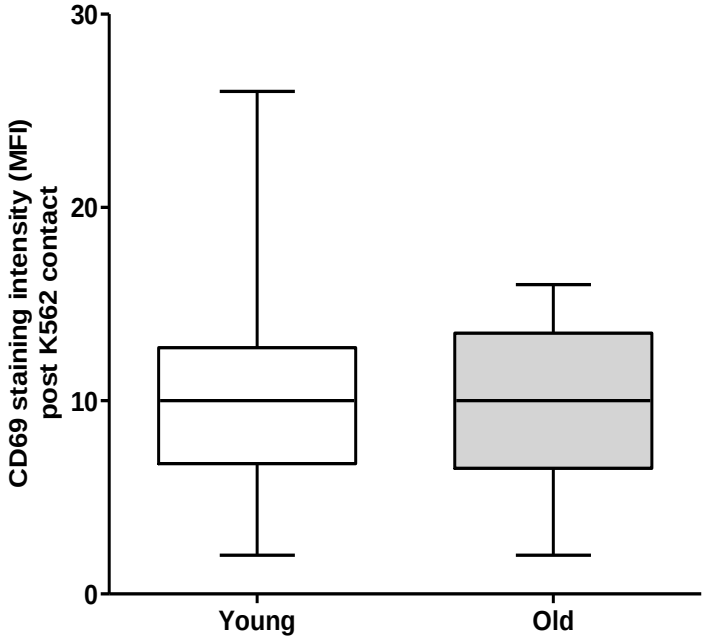



**Figure 3.12. Activation status of K562 cell stimulated NK cells.** Surface expression of the early activation marker CD69 was examined on isolated $CD3^-$ $CD56^+$ NK cells obtained from young (n = 14) and old (n = 13) subjects following a 4-hour co-culture with K562 cells at an E:T of 10:1. **(A)** Fold increase above baseline in the percentage of $CD69^+$ NK cells. **(B)** CD69 staining intensity (MFI). Data is presented after subtracting baseline CD69 expression values. Differences between groups were assessed by an unpaired student T test or Mann-Whitney U test.

### 3.2.5. Effect of age on cytotoxic effector expression and secretory lysosome fusion

In figure 3.4, it was shown that NK cells induce K562 cell lysis via the granule exocytosis pathway. Two important effector molecules of this pathway are perforin and granzyme B [206]. Thus, the possibility that the age-related decline in NKCC was borne from reduced expression of either of these two proteins was investigated.

No age-associated difference either in the percentage of NK cells positive for perforin or granzyme B (Figure 3.13A and Figure 3.13B), or in the staining intensity of these proteins (Figure 3.13C and Figure 3.13D) was found, suggesting NK cells from older subjects are armed to a similar degree with the key cytotoxic molecules needed to induce target cell lysis. Interestingly, when the absolute number of circulating perforin positive NK cells was calculated, a significant age-related decline was seen (Young (n = 10) 214.4±21.98 $x10^6$/L Vs. Old (n = 11) = 147.8±13.85 $x10^6$/L, P = 0.02). However, driving this reduction was a decreased number of absolute NK cells (Young = 243.9±22.33 $x10^6$/L Vs. Old = 166.3±13.84 $x10^6$/L, P = 0.007) rather than a change in perforin expression (% perforin positive NK cells, Young = 86.8±2.09 Vs. Old = 88.64±1.91, P = 0.523).

Since perforin and granzyme B expression was comparable between young and old NK cells, it was next investigated whether the reduction in NK cell lytic activity observed with age was the result of impaired fusion of perforin and granzyme containing secretory lysosomes with the NK cell plasma membrane. To do this, surface CD107a expression was assessed. CD107a is a glycosylated protein located on the inner membrane of lytic granules [503]. During the process of granule exocytosis, the fusion of lytic granules with the NK membrane results in expression of CD107a on the NK cell surface, where it can be detected through the use of monoclonal antibodies and its expression measured by flow cytometry [504].

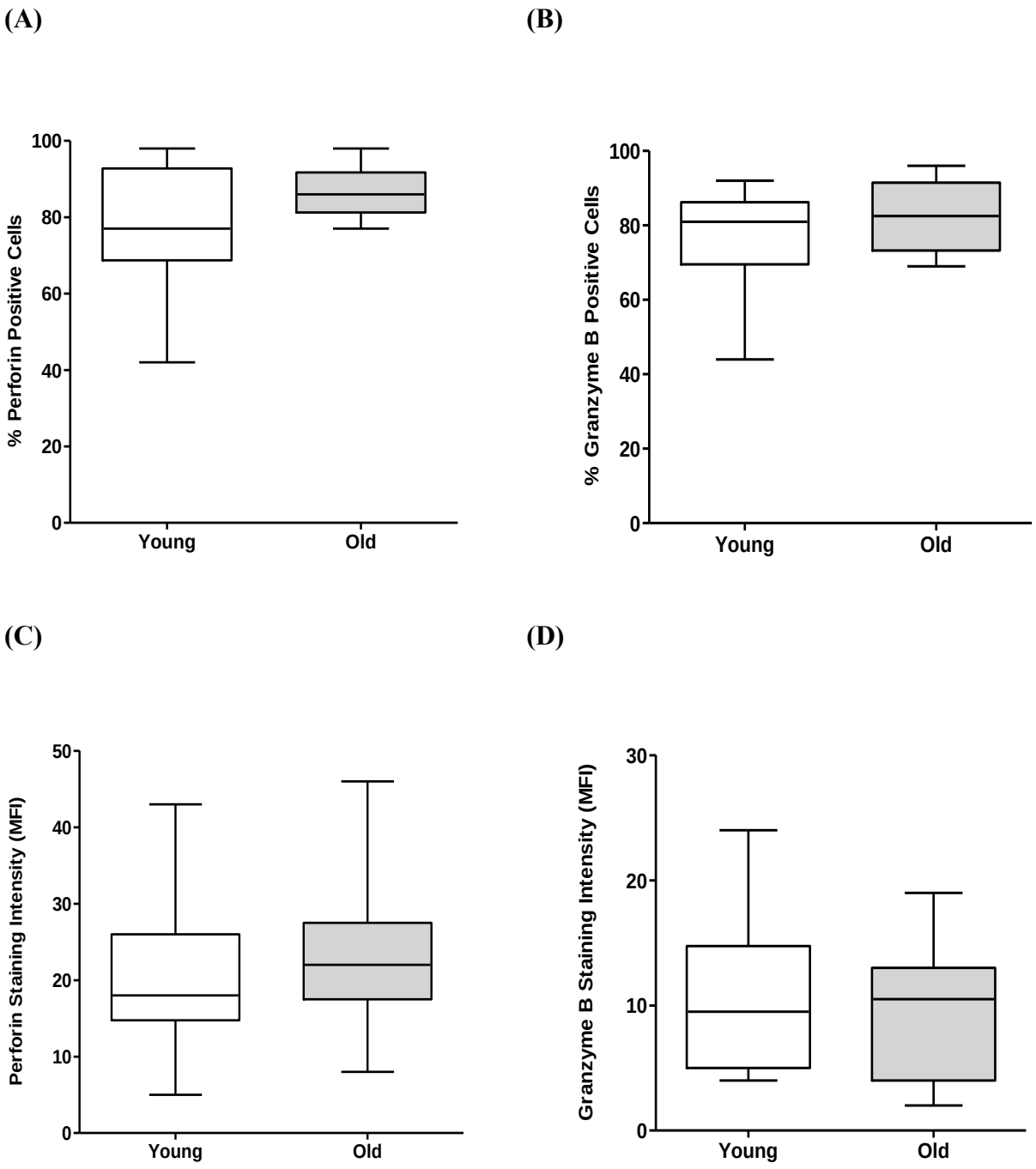



**Figure 3.13. No age-related difference in perforin or granzyme B expression in resting NK cells.** $CD3^{-}$ $56^{+}$ NK cells were obtained from 10 young and 10 old individuals and their expression of the cytotoxic effector molecules perforin and granzyme B measured by flow cytometry. **(A-B)** Percentage of NK cells positive for perforin **(A)** and granzyme B **(B)** staining. **(C-D)** MFI values for perforin **(C)** and granzyme B **(D).** Differences between the groups were analysed by an unpaired student T test.

As a sensitive marker of secretory lysosome fusion, the CD107a assay has become a popular approach for groups interested in studying granule exocytosis. Inevitably therefore, inter-study variation exists in how the assay is performed. For example, whilst some groups have measured CD107a expression on NK cells following a 6-hour co-culture of PBMCs and K562 target cells at an E:T ratio of 10:1[504], others have studied its expression after a 3-hour incubation at an E:T ratio of 1:1 [505]. Therefore, prior to examining the effect of age on lytic granule fusion, experiments were performed to determine which of the above conditions induced maximal CD107a expression. At both 3 (Figure 3.14A) and 6 (Figure 3.14B) hour time points, surface levels of CD107a were significantly higher on NK cells treated with K562 cells at an E:T ratio of 1:1 compared to those treated at a 10:1 ratio. In terms of incubation time, CD107a expression was greatest following a 6-hour incubation (Figure 3.14C). Thus, CD107a expression on NK cells was measured following a 6-hour PBMC-K562 co-culture at an E:T ratio of 1:1. As well as recording the percentage of $CD107a^+$ NK cells, the MFI of CD107a on the NK surface was determined as it has been proposed to provide information on the number of lytic granules that have fused with the NK cell membrane during the period of co-culture [506].

The results showed both the percentage of $CD107a^+$ NK cells (Figure 3.15A) and the accompanying MFI values (Figure 3.15B) to be comparable between young and old subjects, suggesting no age-associated impairment in secretory lysosome fusion. To rule out the possibility that the actions of bystander cells within the PBMC pool were masking an age-related defect in granule fusion, CD107a expression on the surface of purified NK cell populations was measured following a 4-hour co-culture with K562 target cells. These experimental conditions mirrored the NKCC assays in which an age-related decline in NK lytic activity was seen. Supporting the data obtained with PBMCs, no difference in CD107a

expression between young and old donors was found (Figure 3.16A-B).

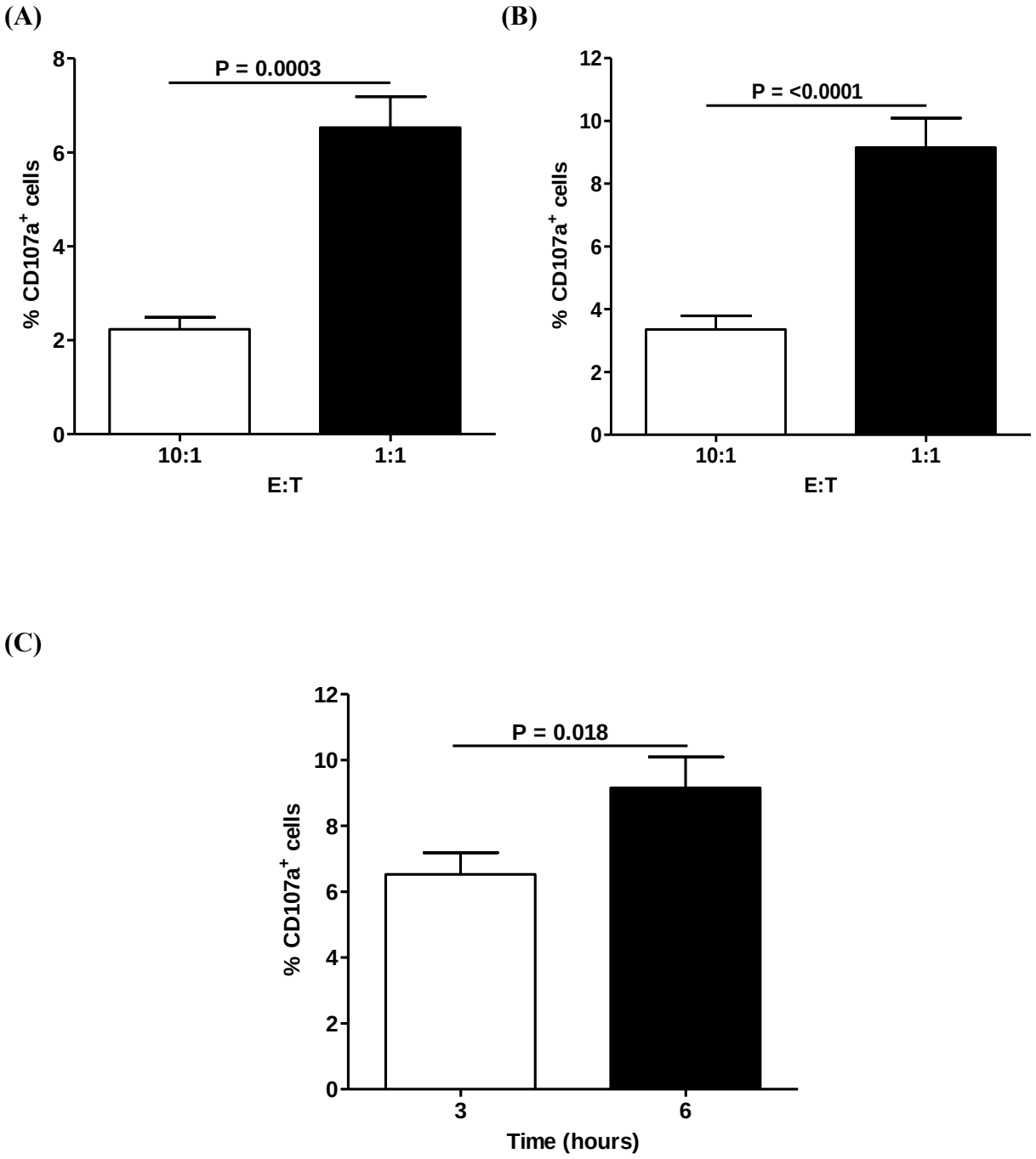
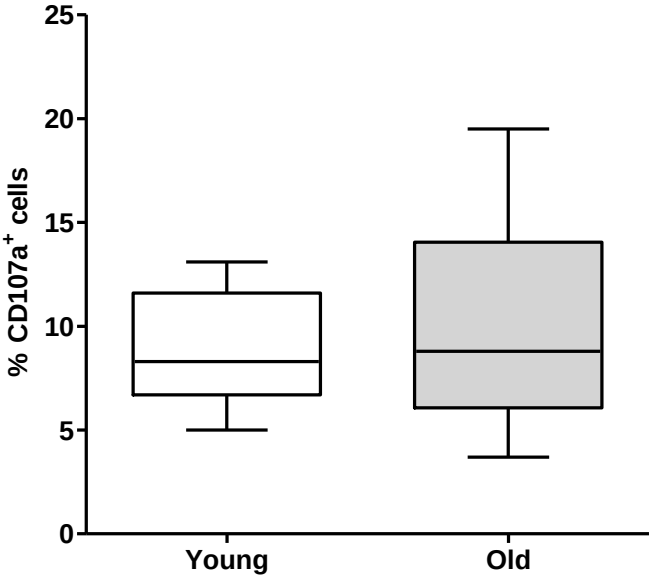



**Figure 3.14. Effect of culture time and E:T cell ratio on the surface expression of CD107a.** To determine optimal conditions for inducing CD107a expression on NK cells, PBMCs isolated from 16 young volunteers were incubated for 3 **(A)** or 6 **(B)** hours with K562 cells at an E:T ratio of 10:1 or 1:1, after which surface CD107a expression was measured on $CD3^-$ $CD56^+$ cells by flow cytometry. **(C)** CD107a expression on NK cells following a 3 and 6-hour co-incubation of PBMCs with K562 target cells at an E:T ratio of 1:1. Data are presented as mean ± SEM, with statistical significance determined by a paired student T test or Wilcoxon matched-pairs signed rank test.

**(A)**

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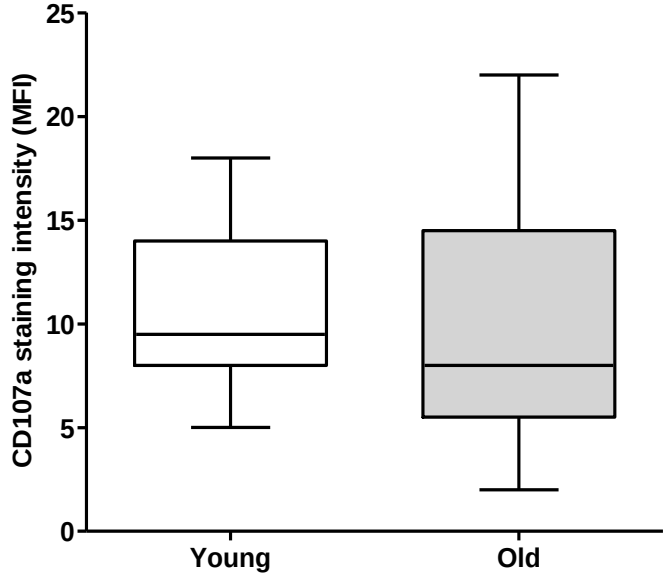
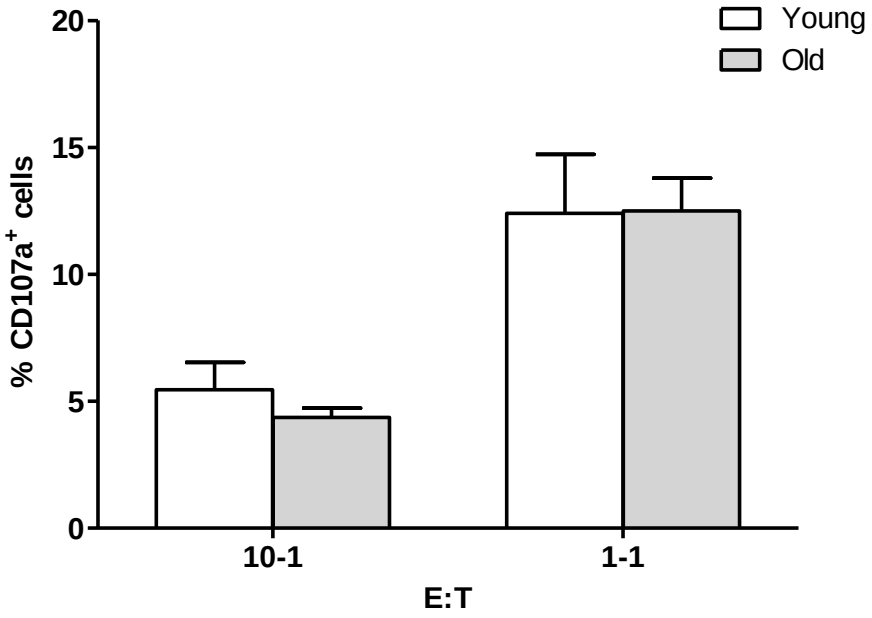



**Figure 3.15. Effect of age on secretory lysosome fusion.** Expression of CD107a on the surface of $CD3^-$ $CD56^+$ NK cells was assessed after a 6-hour co-culture of PBMC's and K562 target cells at an E:T ratio of 1:1 (young, n = 16; old, n = 12). Data are presented as the percentage of $CD107a^+$ NK cells **(A)** and the MFI of externalised CD107a **(B)**. Differences between the two groups were assessed by an unpaired student T test.

**(A)**

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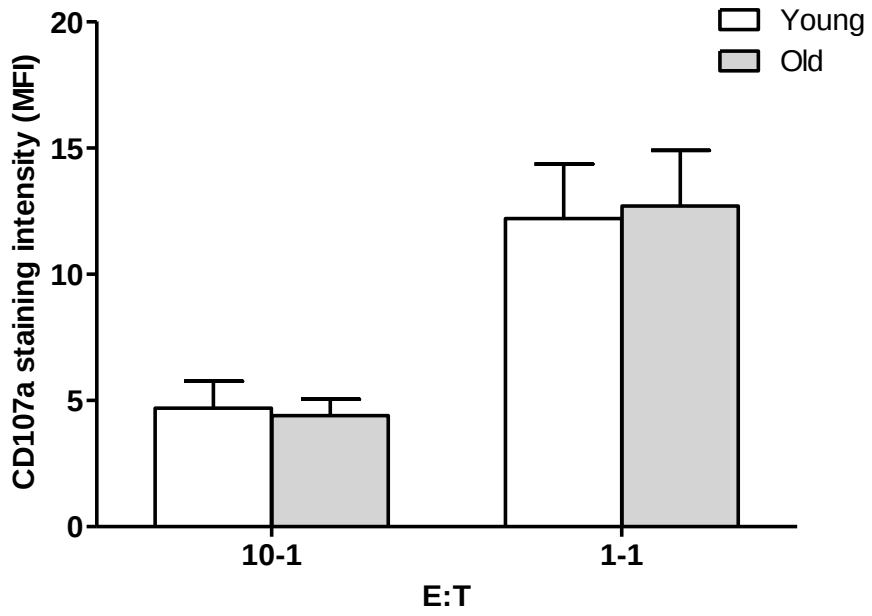



**Figure 3.16. No age-associated difference in CD107a expression on the surface of K562-stimulated NK cells.** NK cells isolated from young and old donors were cultured for 4-hours with K562 tumour cells at an E:T ratio of 10:1 (n = 12 young and 12 old) and 1:1 (n = 11 young and 11 old), after which secretory lysosome fusion was assessed by measuring surface CD107a expression by flow cytometry. Data are presented as the mean ± SEM of the percentage of $CD107a^+$ NK cells **(A)** and the MFI of externalised CD107a **(B)**. Differences between the two groups were assessed by an unpaired student T test or Mann-Whitney U test.

### 3.2.6. Effect of age on perforin binding to the target cell surface

Binding of perforin to the target cell surface is a crucial prerequisite for the induction of target cell death mediated by the granule exocytosis pathway [496]. Given the fact that no difference was seen in either perforin expression or secretory lysosome fusion with age, it was hypothesised that impaired perforin binding to the surface of K562 tumour cells could be responsible for the age-associated reduction in NKCC.

Using a FITC-conjugated anti-perforin antibody, K562 tumour cells were stained after a 4-hour culture with NK cells at an E:T ratio of 10-1. The results showed both the percentage of K562 cells exhibiting positive FITC fluorescence, and the number of perforin molecules bound to the K562 cell surface was significantly lower when these cells had been cultured with NK cells from older adults (Figure 3.17A-C). Importantly, when perforin binding and NKCC assays were performed together, a highly significant positive association between perforin binding and target cell lysis was observed (Figure 3.18), suggesting the age-associated reduction in perforin binding was related to the impairment in NKCC with age.

### 3.2.7. Effect of age on perforin secretion in to the immunological synapse (IS)

Two hypotheses could explain the age-associated decrease in perforin binding to the surface of K562 cells. The reduction in binding could be the result of either an age-related alteration in the structure and/or binding activity of perforin, or could be due to less perforin being released into the IS formed between an aged NK cell and its target. With the former option unlikely, the second proposal was tested by collecting supernatants from 4-hour NK-K562 cell co-cultures and measuring the concentration of perforin using an enzyme linked-immuno-sorbent assay (ELISA). It was found that the levels of perforin were significantly lower in supernatants collected from those co-cultures that had contained NK cells isolated from older adults (Figure 3.19).

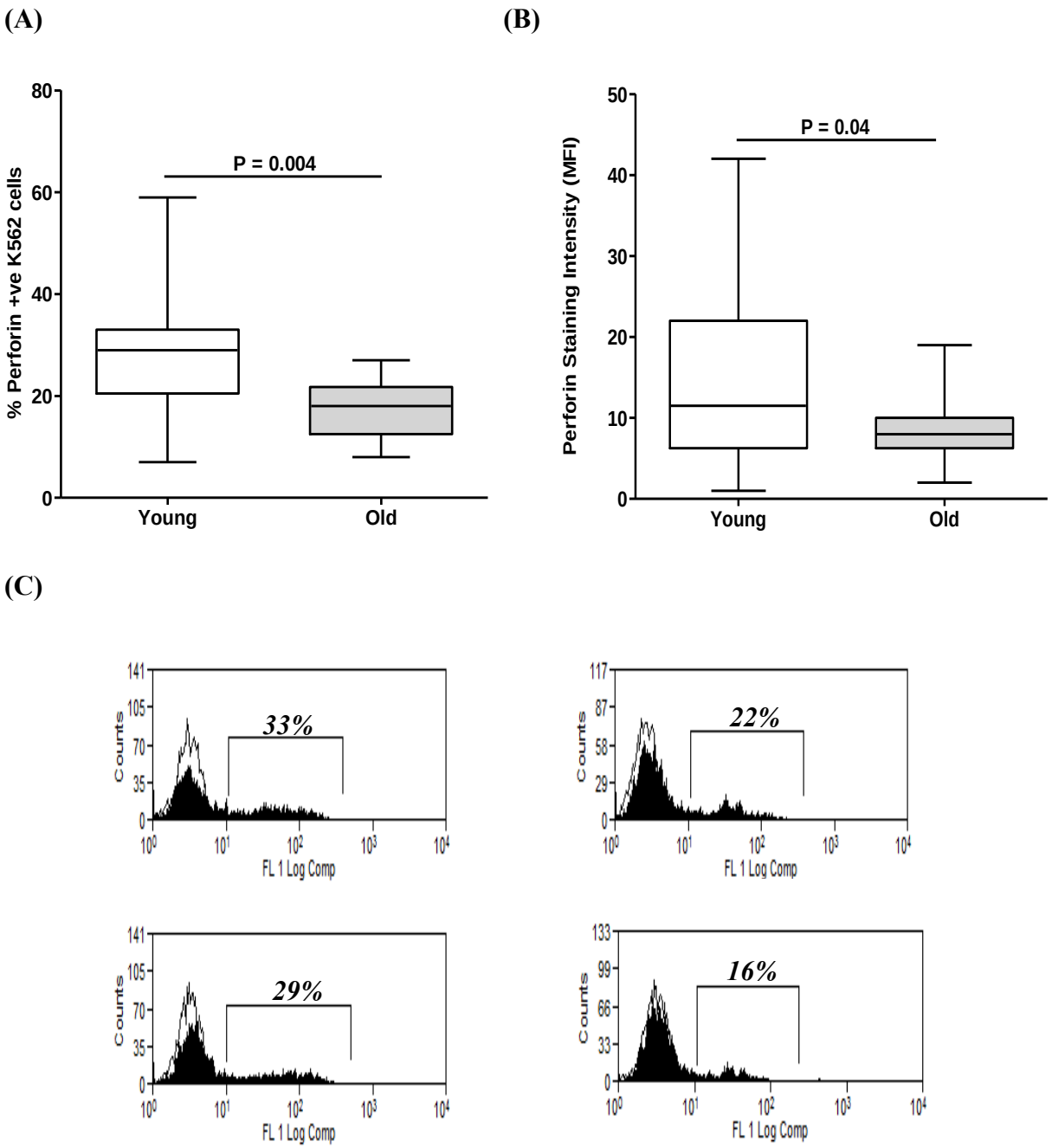



**Figure 3.17. Age-associated reduction in perforin binding to the target cell surface.** NK cells isolated from young (n = 16) and old (n = 16) subjects were co-cultured for 4 hours (E:T, 10:1) with K562 cells, after which perforin binding to the target cell surface was analysed by flow cytometry. Data are presented as the percentage of perforin positive K562 cells **(A)** and perforin staining intensity on the target cell surface **(B)**. Differences between the groups were assessed by an unpaired student T test. **(C)** Representative flow cytometry histograms showing the percentage of K562 target cells to which perforin had bound. Empty and filled histograms depict isotype and test antibody staining respectively.

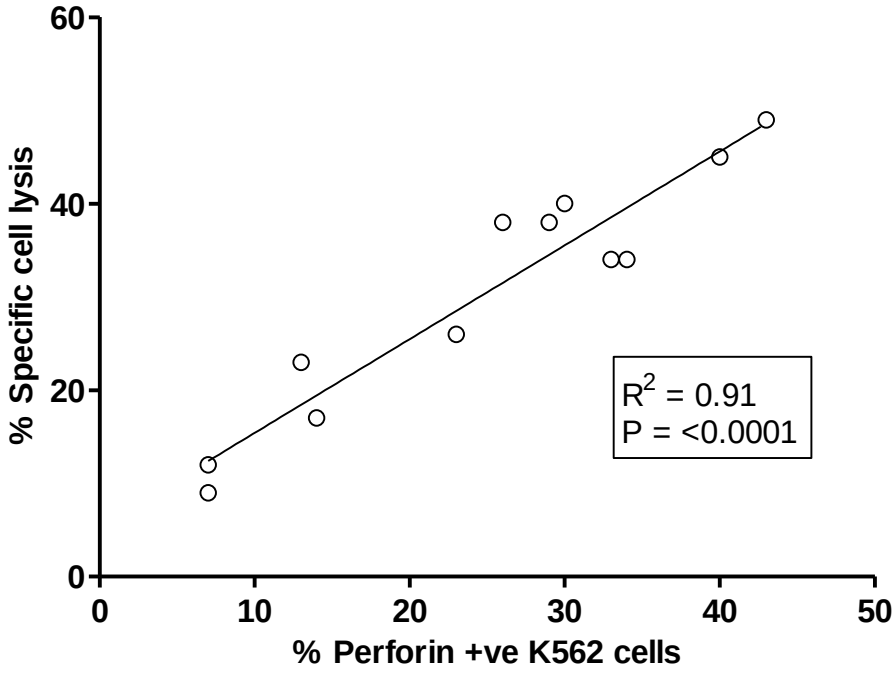



**Figure 3.18. Positive association between perforin binding and NK cell cytotoxicity.** NK cells isolated from 12 young subjects were co-cultured for 4 hours (E:T, 10:1) with K562 cells, after which perforin binding to the target cell surface and NKCC were assessed simultaneously by flow cytometry.

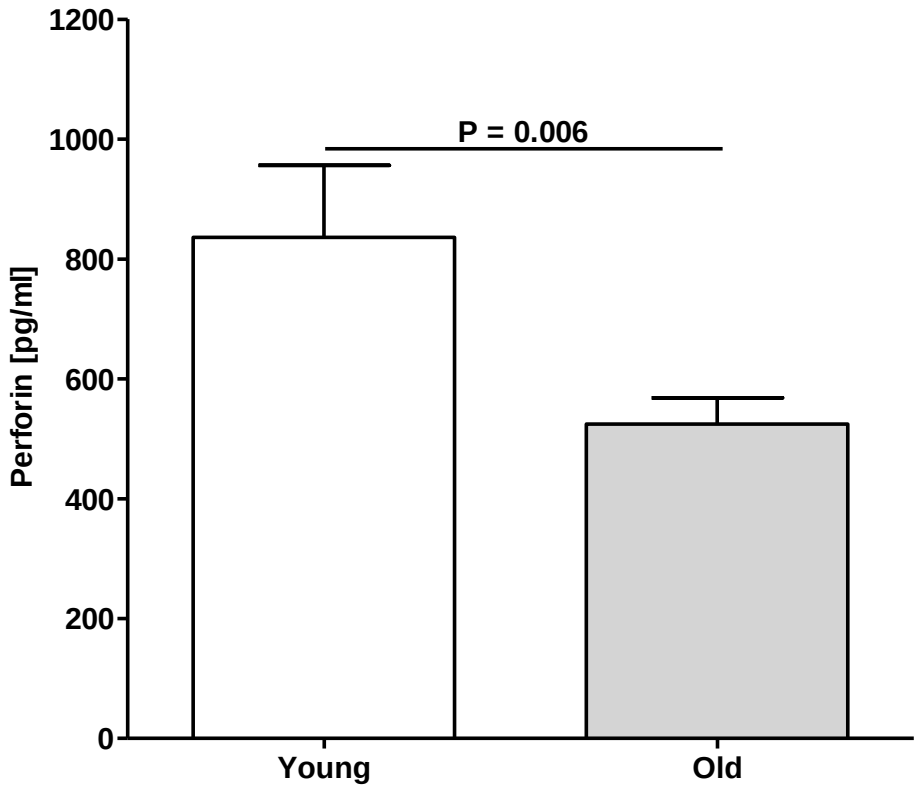



**Figure 3.19. Impaired secretion of perforin into the immunological synapse by NK cells from old individuals.** NK cells obtained from young (n = 13) and old (n = 15) subjects were co-cultured with K562 tumour cells for 4 hours at an E:T ratio of 10:1, after which cell-free supernatants were harvested and perforin concentration determined by ELISA. Data are presented as mean ± SEM, with differences between the two groups assessed by a Mann-Whitney U test.

To confirm the ELISA results, perforin secretion in response to K562 cell stimulation was studied in a separate cohort of individuals using a recently described flow cytometric protocol [507]. As expected, co-culture with K562 target cells led to a significant decrease in perforin staining intensity in NK cells from both young and old donors (Figure 3.20A). However, relative to baseline expression, the reduction in staining intensity was significantly lower for NK cells obtained from aged donors (Figure 3.20B). In contrast, no age-associated impairment in perforin secretion was seen when NK cells were treated with the phorbol ester PMA and the calcium mobilising agent ionomycin (Figure 3.20B).

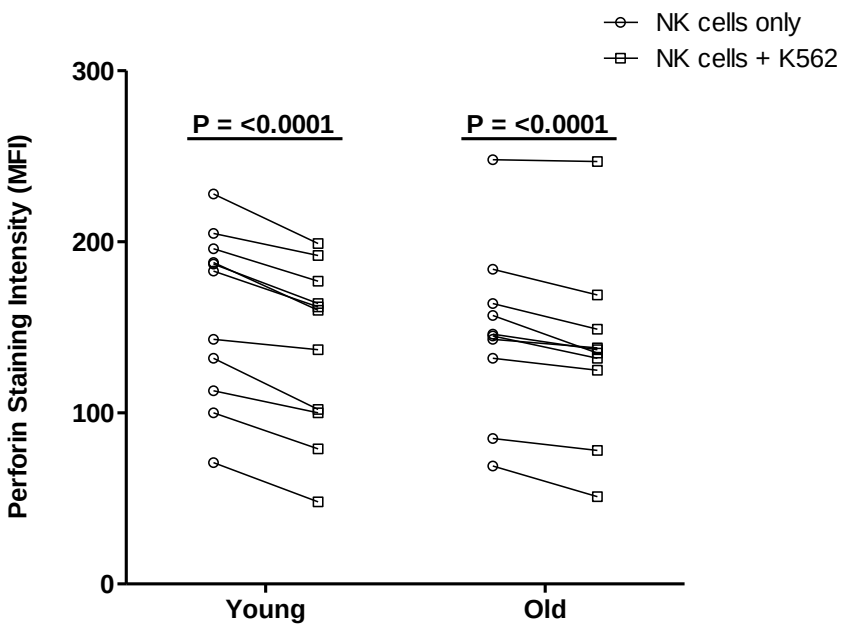
Taken together, these data suggest that the reduction observed in perforin binding to the target cell surface with age was secondary to an age-related impairment in perforin secretion.

### 3.2.8 Effect of age on NK cell apoptosis induced by K562 target cell contact

After delivering a lethal hit, NK cells have been shown to either: (i) detach from their target and eliminate additional transformed cells, a phenomenon referred to as NK cell recycling [507], (ii) become functionally anergic [508] or (iii) undergo target-cell induced apoptosis [509]. Interestingly, in a study examining the effect of age on LAK cell activity, Plett and colleagues noted that when compared to those isolated from young controls, NK cells from aged mice were more susceptible to target cell-induced apoptosis [510]. By undergoing cell death, NK cells would be unable to recycle and induce lysis of other target cells. Thus, the possibility that an age-related increase in target cell-induced apoptosis was also true for human NK cells was considered with the thinking being that the decline in perforin concentrations observed in NK-K562 co-cultures with age could have resulted from a reduced ability of NK cells from older adults to kill multiple targets.

To test this theory, NK cells from young and old donors were cultured with K562 target cells for 4 hours, after which NK cell death was studied by staining samples with Annexin V.

**(A)**




**(B)**

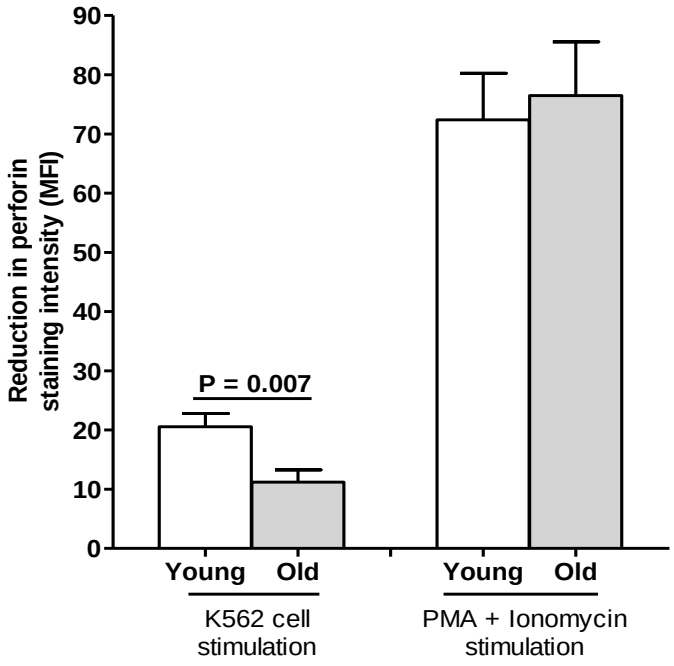



**Figure 3.20. Age-related reduction in perforin secretion by K562-stimulated NK cells. (A)** A 4-hour culture with K562 target cells resulted in a significant reduction in perforin staining intensity in NK cells from young (n = 11) and old (n = 10) subjects**. (B)** Reduction in perforin staining intensity in NK cells following a 4-hour treatment with K562 target cells or PMA (2.5 µg/ml) and ionomycin (0.5 µg/ml). For K562 treatment, data relates to the mean ± SEM for 11 young and 10 old donors. For PMA and ionomycin, data is mean ± SEM collected from 10 young and 10 old subjects.

As a protein that exhibits high affinity for phosphatidylserine, a phospholipid expressed on the outer membrane of cells undergoing apoptosis, positive Annexin V staining is considered a sensitive marker of early cell death [511]. In contrast to the findings of Plett et al [510], no age-associated difference in the percentage of annexin V positive NK cells was seen following K562 cell co-culture (Figure 3.21).

### 3.2.9. Effect of age on polarisation of perforin to the NK cell immunological synapse

NKCC mediated by the granule exocytosis pathway relies upon the mobilisation and delivery of lytic granules to the NK-target cell interface [206]. Having observed an age-related reduction in perforin release following target cell contact (Figure 3.19 and Figure 3.20), it was hypothesised that the movement of lytic granules to the NKIS was impaired with age. To test this idea, NK cells from young and old subjects were cultured either alone or with K562 tumour cells for 30 minutes, after which cells were fixed, permeabilised and stained with a FITC-conjugated monoclonal antibody specific for human perforin. The location of this pore-forming protein within NK cells was then studied by immunofluorescence microscopy.

In accordance with previous observations [263], perforin was dispersed throughout the cytoplasm of resting NK cells (Figure 3.22A), and as expected, target cell contact prompted NK cells from both young and old subjects to deliver perforin to the IS (Figure 3.22B). However, the percentage of NK cells from older adults that had polarised perforin to the NK-target cell interface was markedly lower when compared to NK cells from younger controls (Figure 3.23A-B). Whilst target cell binding triggered 63% of NK cells from young donors to deliver perforin to the NK-target cell interface, a frequency that is in line with other studies that have measured perforin polarisation in healthy young adults [512], only in 35% of NK cells from older subjects that had bound to K562 target cells was there evidence of lytic granule polarisation (Figure 3.23B).

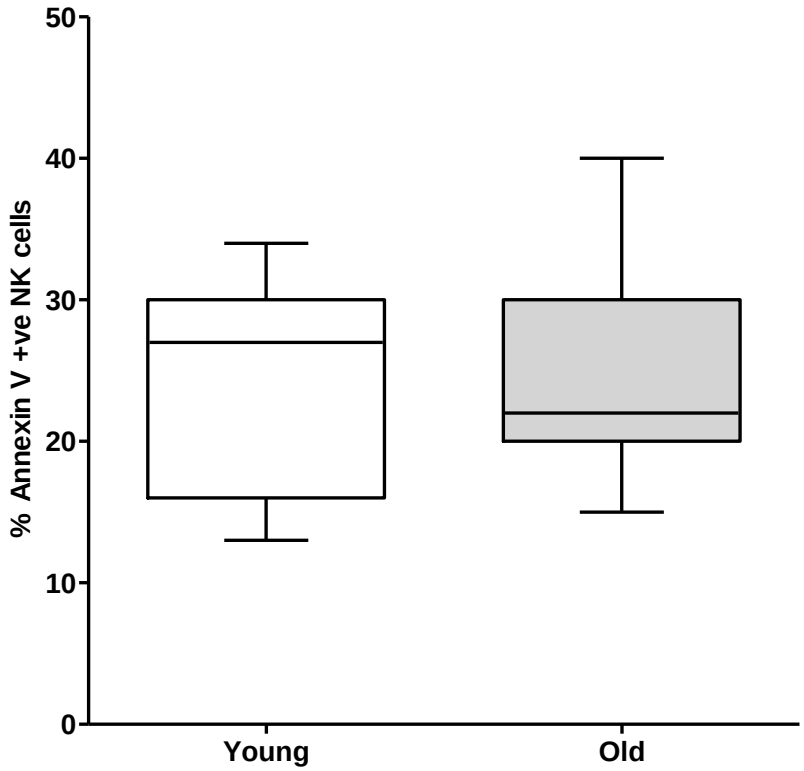



**Figure 3.21. Effect of age on NK cell apoptosis induced by target cell contact.** NK cells were co-cultured with K562 target cells at an E:T ratio of 10-1 for 4 hours, after which the percentage of NK cells staining positive for Annexin V was examined by flow cytometry. Data was collected from 12 young and 12 old donors, with differences between the groups assessed by an unpaired student T test.

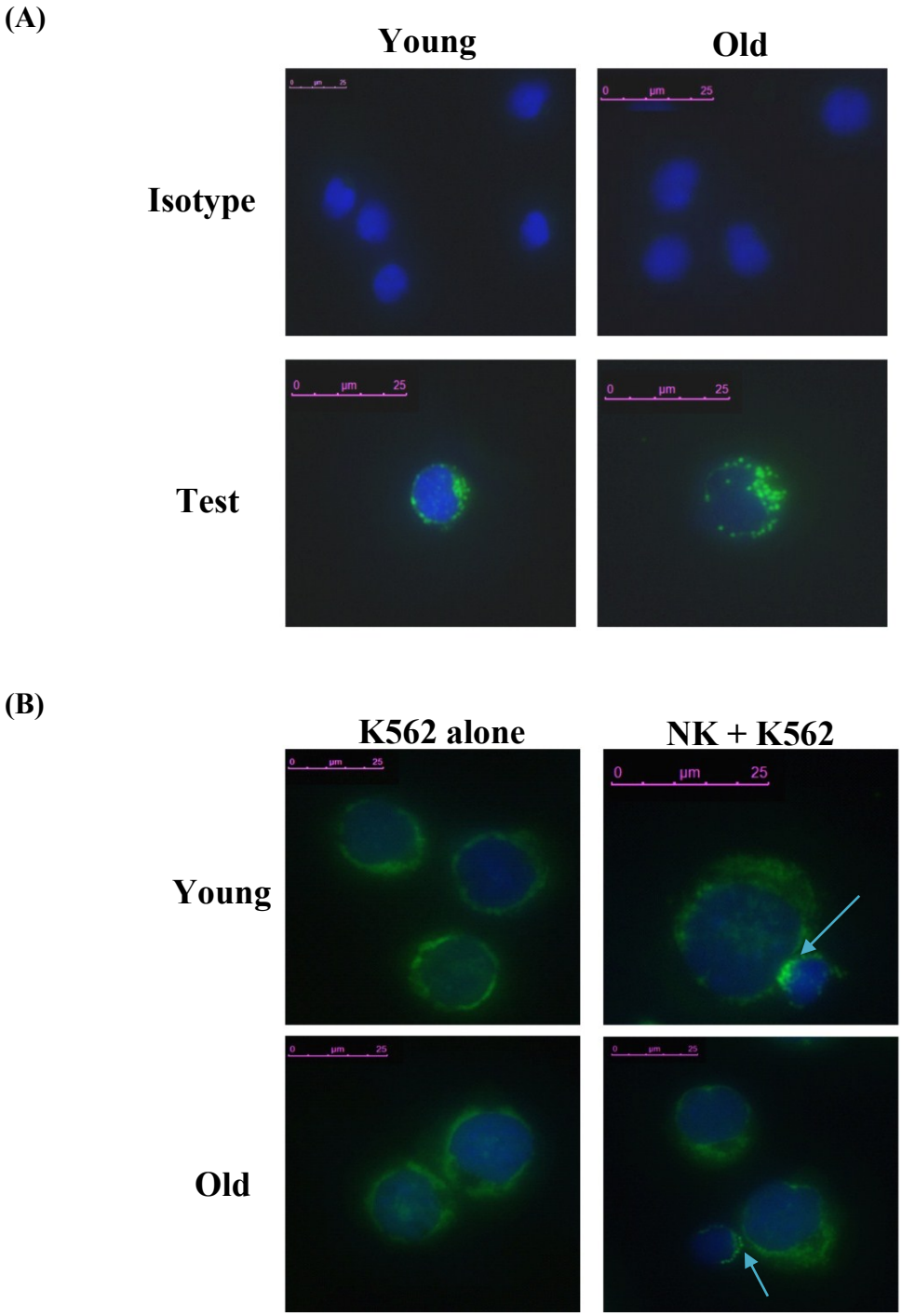



**Figure 3.22. Perforin expression in resting and K562 cell-stimulated NK cells.** Following a 30-minute culture either alone **(A)** or in the presence of K562 target cells **(B)**, NK cells were stained with a FITC-conjugated antibody against human perforin to examine the location of this pore-forming protein. Nuclei were counterstained with DAPI. Images were taken at X40 or X63 objective and are representative of experiments performed on 3 young and 3 old subjects. Blue arrows point to regions of perforin polarisation to the IS.

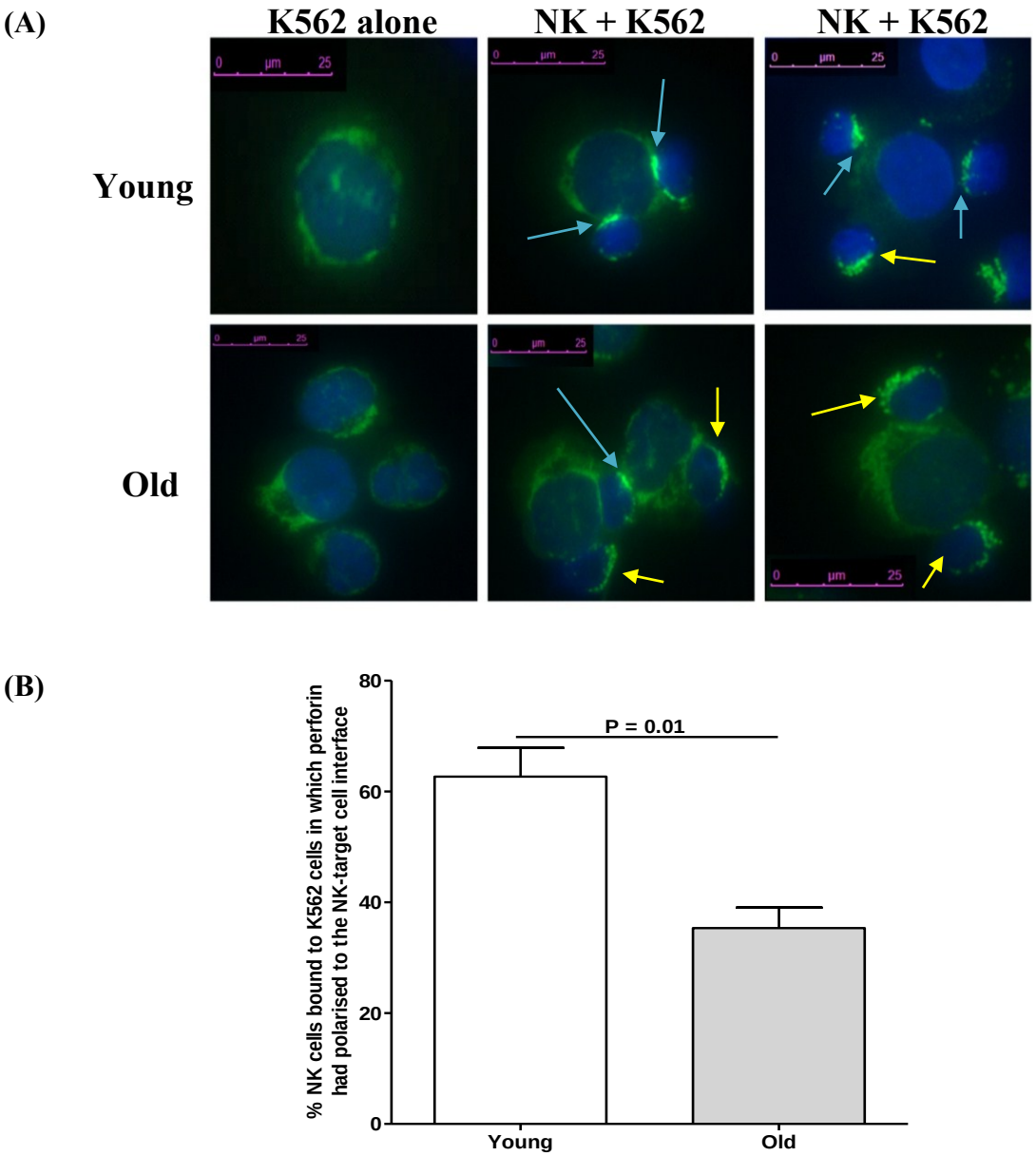



**Figure 3.23. Age-related impairment in perforin polarisation to the NKIS. (A)** NK:K562 cell conjugates prepared from young and old donors were immunostained after a 30-minute co-culture to examine perforin localisation (FITC-conjugated antibody). Nuclei were counterstained with DAPI. Images were taken at X40 or X63 objective and are representative of experiments performed on 3 young and 3 old subjects. Blue arrows point to regions of perforin polarisation to the IS, whilst yellow arrows highlight NK cells that have not delivered perforin to the NK-target cell interface. **(B)** Percentage of NK cells bound to K562 tumour cells that had polarised perforin to the NKIS. Data are presented as mean ± SEM of values provided by three individuals, who blinded to donor age, counted 50 NK-K562 cell conjugates on slides prepared from 3 young and 3 old donors.

### 3.2.10. Effect of age on F-actin polymerisation and MAPK activation

So far, the data presented above had revealed a previously unrecognised impairment in the function of NK cells from older adults: reduced release of perforin into the IS. Having identified defective mobilisation of lytic granules to the NK-target cell interface as a possible cause, the next series of experiments set out to determine the mechanism(s) behind the age-related impairment in perforin polarisation.

An early event in the formation of an activating IS is the polymerisation and accumulation of F-actin at the NK-target cell interface [513]. When this step is impaired, lytic granules fail to polarise efficiently to the NKIS and NKCC is markedly reduced [251;513]. Given that these features mirrored the behaviour of NK cells isolated from older adults, it was investigated whether this aspect of IS formation was altered with age. To do this, NK cells from young and old donors were cultured with K562 cells, and after 30 minutes, samples were stained with FITC-conjugated phalloidin. By binding to F-actin with high selectively, phalloidin is a tool often used to study the distribution and formation of F-actin networks. In conjugates formed between K562 cells and NK cells from both young and old donors, F-actin accumulated at the NK-target cell contact point (Figure 3.24), suggesting the process of actin polymerisation was not altered with age.

Actin polymerisation triggers the polarisation of perforin containing secretory lysosomes to the NK-target cell interface [514]. Important for this phase of IS formation is the activation of the MAPK family. To date, the MAPKs: ERK1/2, JNK and P38 have been proposed to be involved in delivering lytic granules to the NK-target cell interface [260;261;263;338]. Thus, it was possible that an age-related delay in the activation kinetics of these serine/threonine protein kinases was responsible for the impaired polarisation of perforin to the IS. Activation of MAPKs occurs via dual phosphorylation of threonine and tyrosine residues present within

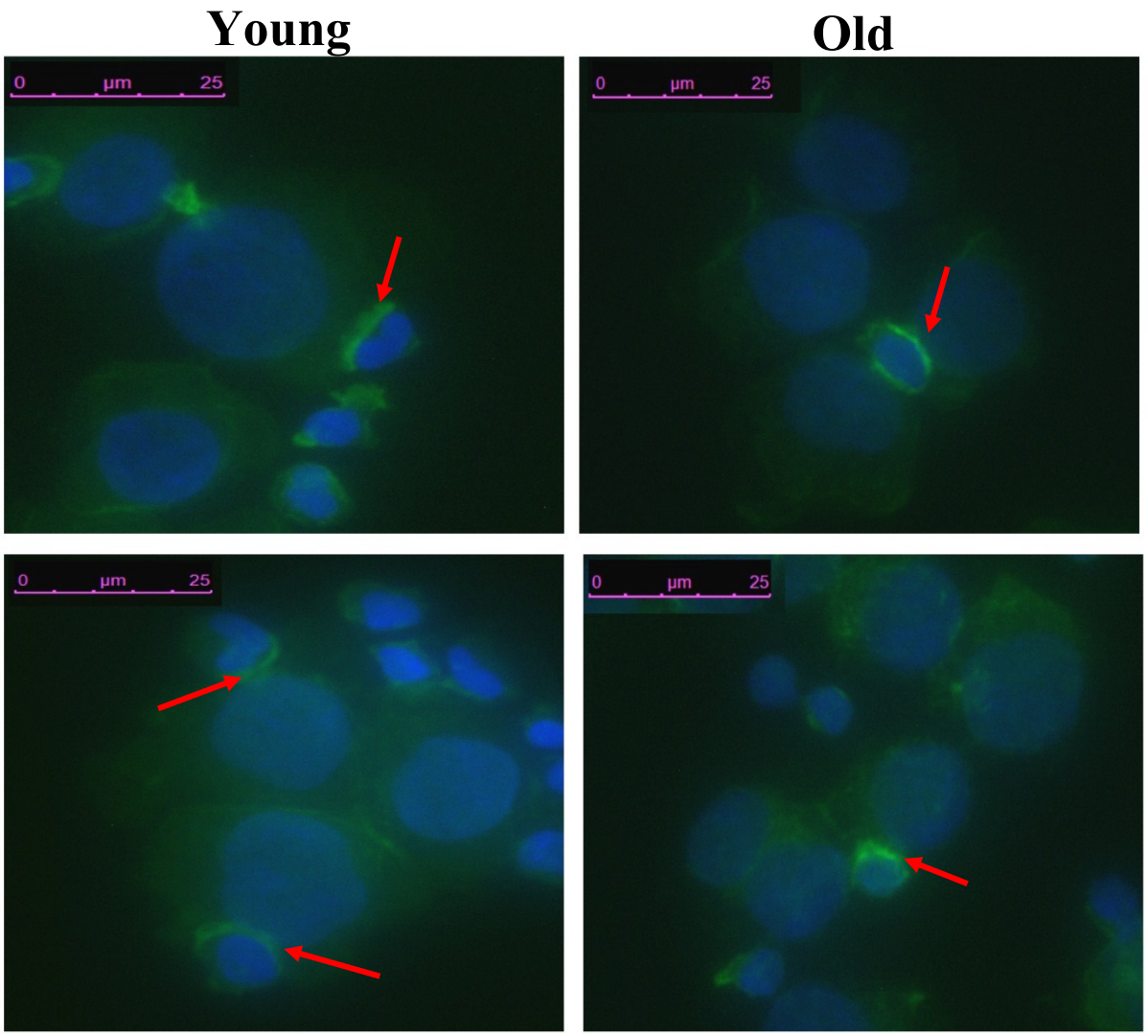



**Figure 3.24. Cellular localisation of F-actin in K562-stimulated NK cells.** NK:K562 cell conjugates prepared from young and old donors were immunostained after a 30-minute co-culture with FITC-conjugated phalloidin to study F-actin distribution. Nuclei were counterstained with DAPI. Images were taken at X40 or X63 objective and are representative of experiments performed on 3 young and 3 old subjects. Arrows point to regions of F-actin accumulation.

the activation loop of these kinases [515]. Therefore, using phospho-specific antibodies, Western blotting was used to assess the activation status of MAPKs in stimulated NK cells from young and old subjects. Due to technical difficulties with antibodies targeted against JNK, only the kinetics of ERK1/2 and P38 activation were studied.

Initial experiments revealed the presence of phosphorylated ERK1/2 and P38 in K562 cells (Figure 3.25). Given that Western blotting measures protein expression in cell lysates, this finding meant an alternative approach was required to activate NK cells since it would not have been possible for us to discriminate between NK and K562 cell-derived phosphorylated MAPKs in lysates prepared from E:T cell co-cultures. Thus, MAPK activation kinetics were studied in NK cells that had been stimulated for 0, 2, 5, 10, 30, 60 and 90 minutes with antibodies against the activatory receptors NKG2D and NKp30. These receptors were selected as their ligands have been shown to be present on the surface of K562 cells [260;501].

Figures 3.26A and 3.27A show the ERK1/2 activation kinetics in antibody stimulated NK cells from a young and old donor respectively. To quantify the up-regulation in ERK1/2 phosphorylation that occurred upon antibody stimulation, densitometry analysis was performed on Western blots from 5 young (Figure 3.26B) and 4 old (Figure 3.27B) subjects, measuring activation as a ratio of phosphorylated-ERK1/2 to total ERK1/2. For young donors, within 10 minutes of stimulation a significant increase in ERK1/2 activation was seen when compared to untreated controls (Figure 3.26B). Activation peaked at 30 minutes, but was still significantly higher than unstimulated controls at both 60 and 90 minutes (Figure 3.26B). In contrast, NK cells from older adults required 30 minutes of antibody stimulation before ERK1/2 activity was significantly greater than that observed in resting cells (Figure 3.27B). This significant increase in activation was also observed at 60 minutes

post stimulation but was no longer evident at 90 minutes, where the level of ERK1/2 activity was not significantly different from that present in control samples (Figure 3.27B). Interestingly, when stimulated with the phorbol ester PMA and the calcium ionophore ionomycin, NK cells from young and old donors showed comparable ERK1/2 activation kinetics (Figure 3.28 A-B, Figure 3.29 A-B).

In contrast to ERK1/2, there was no evidence for P38 phosphorylation upon antibody stimulation of NK cells from young subjects (Figure 3.30). For this reason, the effect of age on the activation kinetics of this MAPK was not studied further.

Taken together, these data have demonstrated an age-related delay in the activation kinetics of ERK1/2 following ligation of activatory receptors. Given the important role of this MAPK in lytic granule mobilisation and polarisation [263], this would suggest this impairment may underlie the aberrant delivery of perforin to the IS observed with age, which in turn may be responsible for the age-related reduction in perforin release.

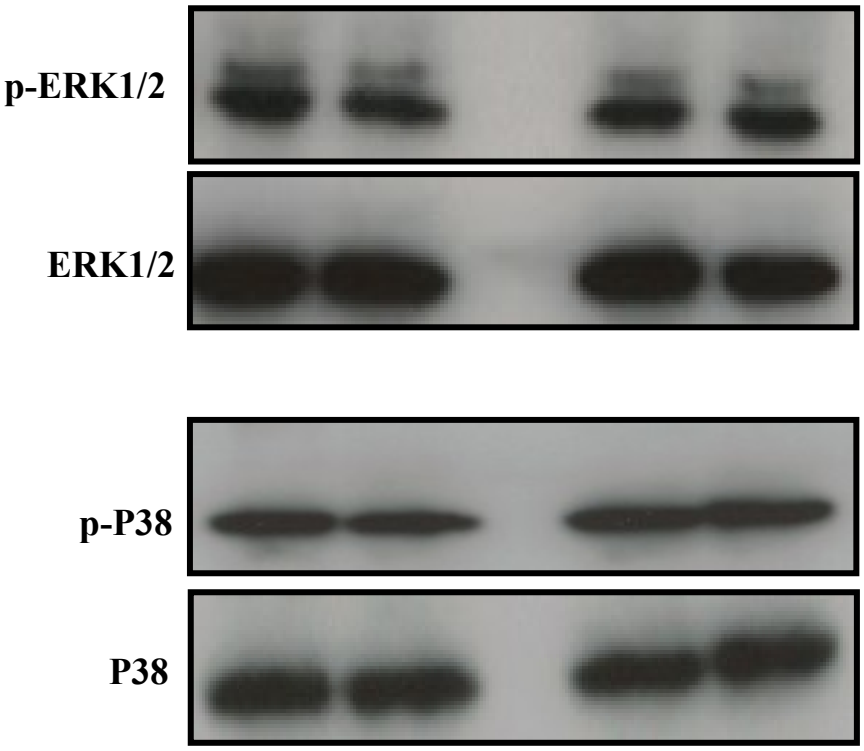
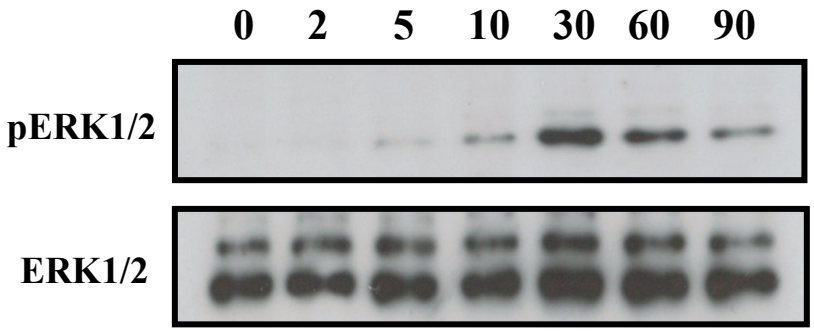



**Figure 3.25. Expression of phosphorylated MAPKs in K562 tumour cells.** Western blotting was performed on cell lysates prepared from 100,000 K562 cells to measure the expression of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated P38 (p-P38), with total ERK1/2 and total P38 serving as loading controls respectively. The results displayed are representative of four independent experiments performed on two separate K562 cell cultures.

**(A)**

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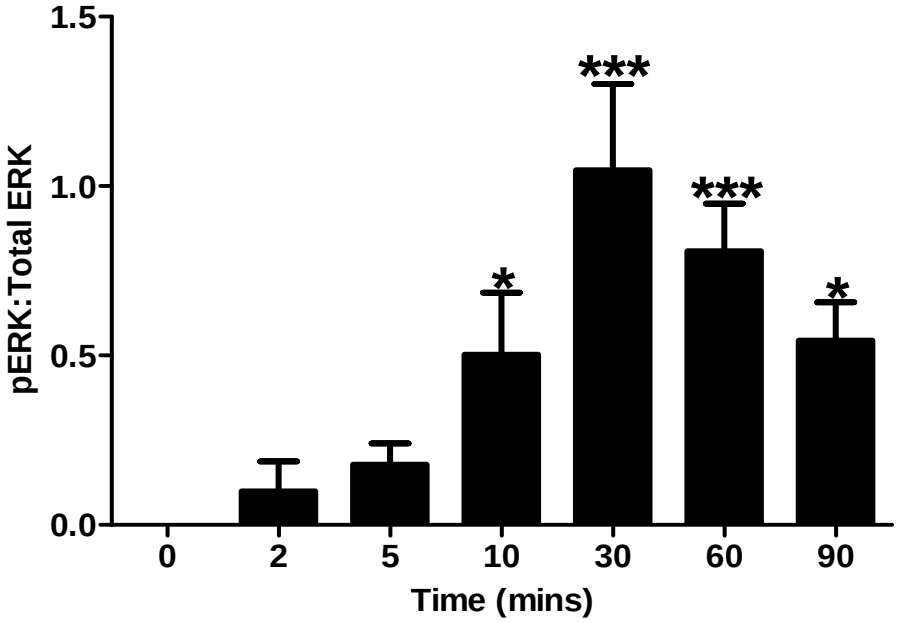
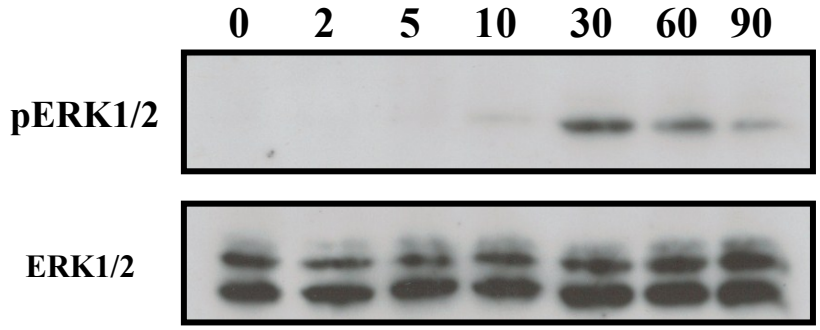



**Figure 3.26. ERK 1/2 activation kinetics in NK cells obtained from young donors following NKG2D and NKp30 receptor ligation.** Resting NK cells isolated from young subjects were stimulated over a 90-minute period with antibodies targeted against the activatory receptors NKG2D and NKp30. At the time points indicated, cell lysates were prepared and ERK1/2 activation measured by Western blotting using a phospho-specific anti-ERK1/2 antibody. Total ERK1/2 expression served as a loading control. **(A)** An example of ERK 1/2 phosphorylation kinetics in stimulated NK cells from a young donor. **(B)** Densitometry analysis of ERK1/2 activation. Data are presented as mean ± SEM of 5 independent experiments. For statistical analysis, a repeated measures ANOVA was performed with Dunnett's post hoc test. Time 0 was considered the control value to which all other time points were compared. *** indicates $p \leq 0.0001$, * indicates $p \leq 0.05$.

**(A)**

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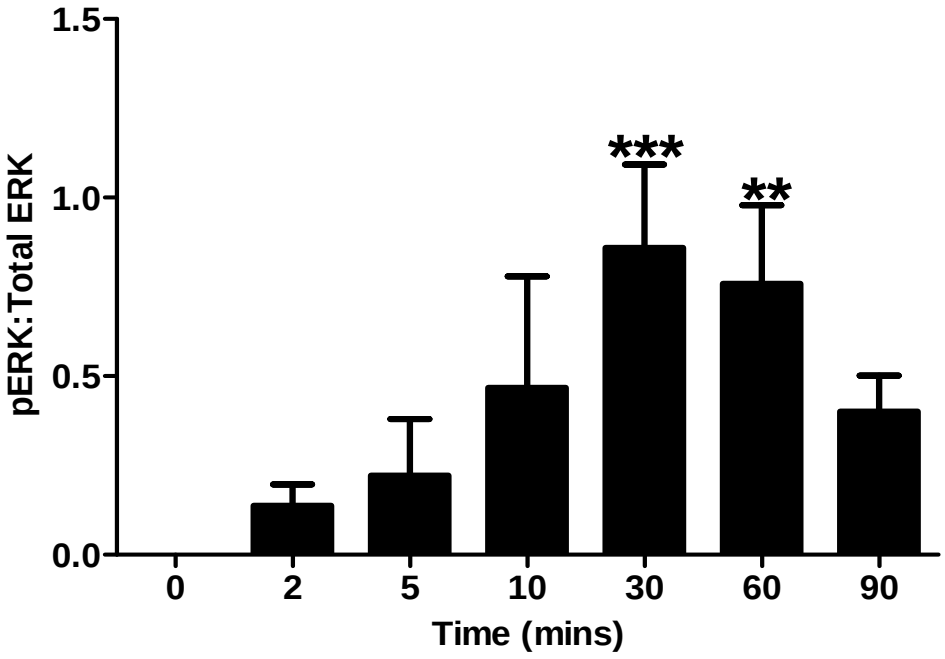



**Figure 3.27. ERK 1/2 activation kinetics in NK cells isolated from older adults in response to NKG2D and NKp30 receptor ligation**. Resting NK cells were stimulated over a 90-minute period with antibodies targeted against the activatory receptors NKG2D and NKp30. At the time points indicated, cell lysates were prepared and ERK1/2 activation measured by Western blotting using a phospho-specific anti-ERK1/2 antibody. Total ERK1/2 expression served as a loading control. **(A)** An example of ERK 1/2 phosphorylation kinetics in stimulated NK cells from an old individual. **(B)** Densitometry analysis of ERK1/2 activation. Data are presented as mean ± SEM of 4 independent experiments. For statistical analysis, a repeated measures ANOVA was performed with Dunnett's post hoc test. Time 0 was considered the control value to which all other time points were compared. ** indicates $p \leq 0.005$, * indicates $p \leq 0.05$.

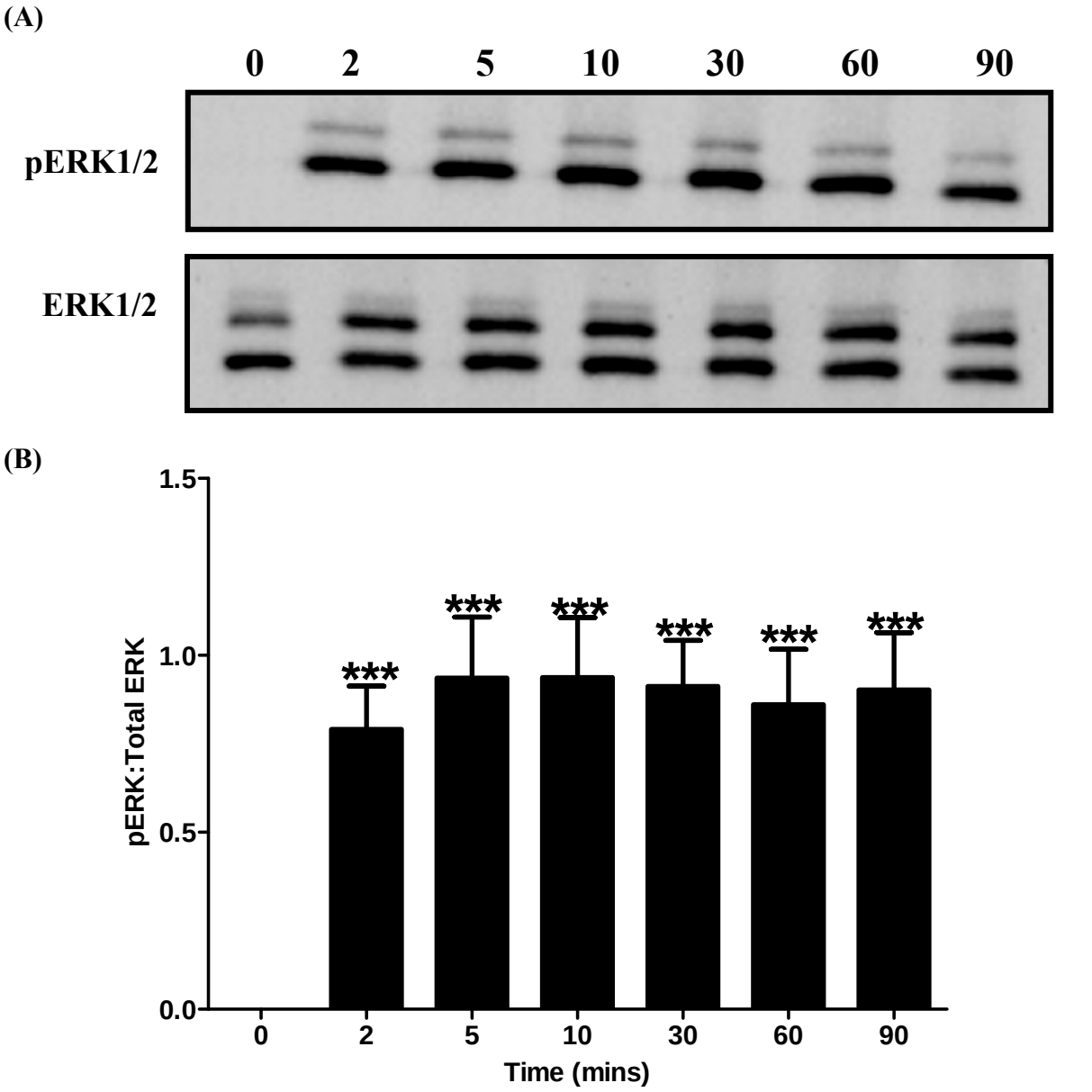



**Figure 3.28. ERK 1/2 activation kinetics in NK cells isolated from young subjects in response to PMA and ionomycin stimulation.** Resting NK cells were stimulated over a 90-minute period with 2.5 µg/ml PMA and 0.5 µg/ml ionomycin. At the time points indicated, cell lysates were prepared and ERK1/2 activation measured by Western blotting using a phospho-specific anti-ERK1/2 antibody. Total ERK1/2 expression served as a loading control. **(A)** An example of ERK 1/2 phosphorylation kinetics in stimulated NK cells from a young donor. **(B)** Densitometry analysis of ERK1/2 activation. Data are presented as mean ± SEM of 4 independent experiments. For statistical analysis, a repeated measures ANOVA was performed with Dunnett's post hoc test. Time 0 was considered the control value to which all other time points were compared. *** indicates $p \leq 0.0001$.

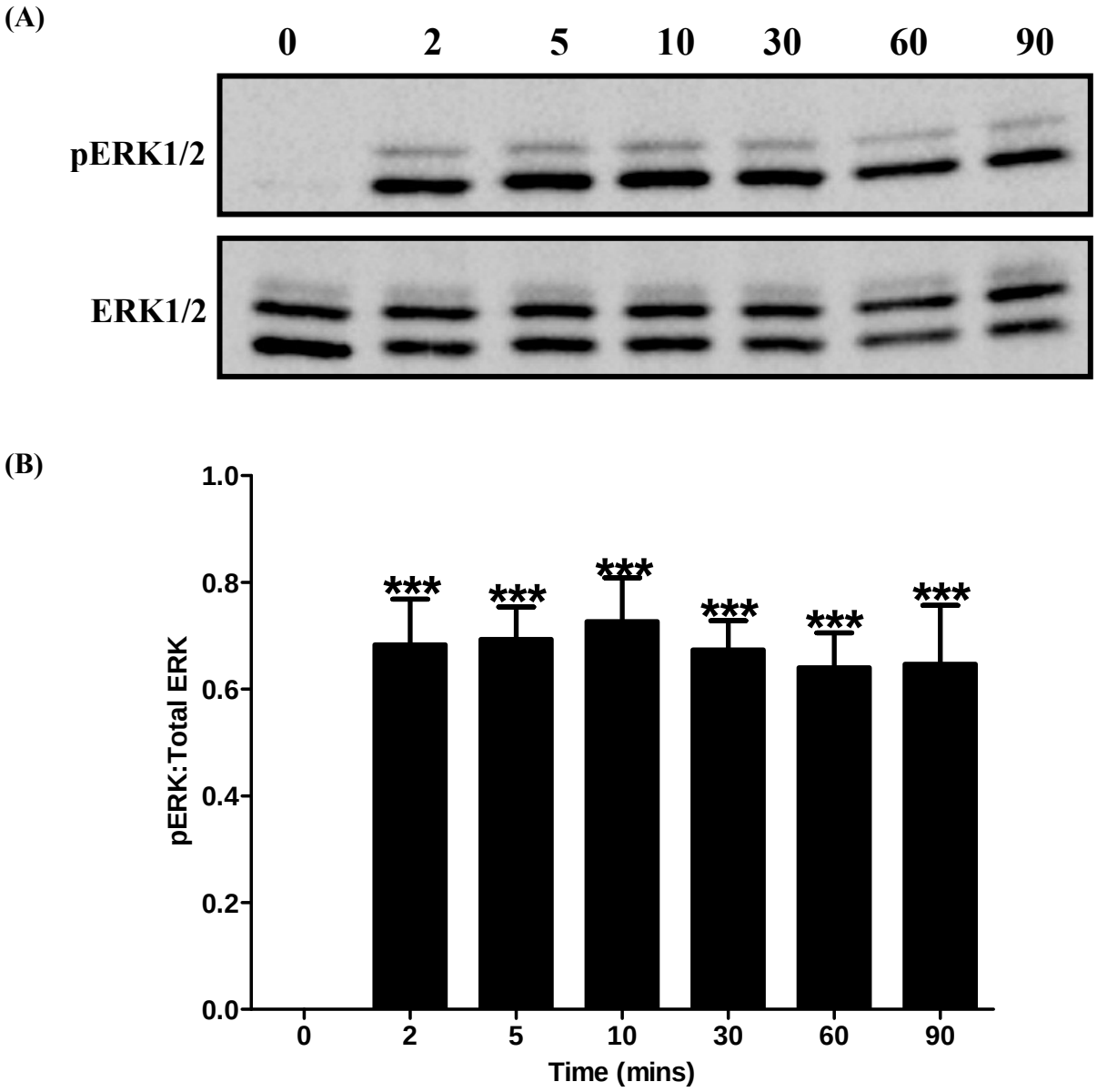



**Figure 3.29. ERK 1/2 activation kinetics in NK cells obtained from older adults in response to PMA and ionomycin stimulation**. Resting NK cells were stimulated over a 90-minute period with 2.5 µg/ml PMA and 0.5 µg/ml ionomycin. At the time points indicated, cell lysates were prepared and ERK1/2 activation measured by Western blotting using a phospho-specific anti-ERK1/2 antibody. Total ERK1/2 expression served as a loading control. **(A)** An example of ERK 1/2 phosphorylation kinetics in stimulated NK cells from an older adult. **(B)** Densitometry analysis of ERK1/2 activation. Data are presented as mean ± SEM of 3 independent experiments. For statistical analysis, a repeated measures ANOVA was performed with Dunnett's post hoc test. Time 0 was considered the control value to which all other time points were compared. *** indicates $p \leq 0.0001$.

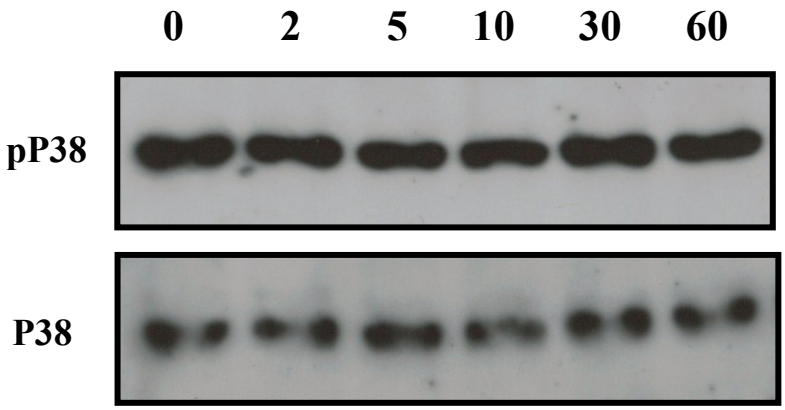



**Figure 3.30. Kinetics of P38 MAPK phosphorylation in NK cells isolated from young subjects in response to NKG2D and NKp30 receptor ligation**. Resting NK cells were stimulated over a 60-minute period with antibodies targeted against the activatory receptors NKG2D and NKp30. At the time points indicated, cell lysates were prepared and P38 activation measured by Western blotting using a phospho-specific anti-P38 antibody. Total P38 expression served as a loading control. Results displayed are representative of 2 independent experiments performed on NK cells isolated from young adults.

### 3.2.11 Effect of age on target cell-induced cytokine and chemokine production

As well as mediating direct cytotoxicity, NK cells are a rich early source of immunoregulatory cytokines and chemokines. Exposure to pro-inflammatory cytokines and interaction with target cells trigger NK cells into secreting significant amounts of these soluble mediators [277;292]. Whilst several studies have examined the effect of age on cytokine and chemokine production induced by pro-inflammatory cytokine treatment [361;378;381;382], no study to date had investigated whether target cell-induced cytokine and chemokine production was altered with age. To address this, purified NK cells from young and old donors were cultured with K562 target cells for 6 hours, after which, cell-free supernatants were harvested, and the concentrations of IFN-γ, TNF-α, IL-8 and MIP1-α measured using luminex® technology.

As expected, NK cells responded to target cell treatment by secreting cytokines and chemokines, and in line with previous publications [277], the concentrations of chemokines present in supernatants were significantly greater than that of cytokines for both young and old donors (Table 3.6). Age comparisons revealed NK cells from old donors generated significantly less MIP1-α, IL-8 and IFN-γ following interaction with K562 target cells when compared to NK cells from young subjects (Figure 3.31 A-B). In contrast, no age-associated difference was observed in TNF-α production (Figure 3.31B).

In addition to its potent chemoattractant properties, MIP1-α has been shown to increase the cytotoxic capacity of NK cells [281]. Thus, in an effort to determine whether the small but significant decrease we had observed in MIP1-α secretion with age could be of biological relevance, NK-K562 cell co-cultures were supplemented with 1 or 2 ng/ml of MIP1-α, the concentrations secreted by old and young NK cells respectively in response to target cell contact (Table 3.6), and after 4 hours NKCC was assessed. In both young (Figure 3.32A) and

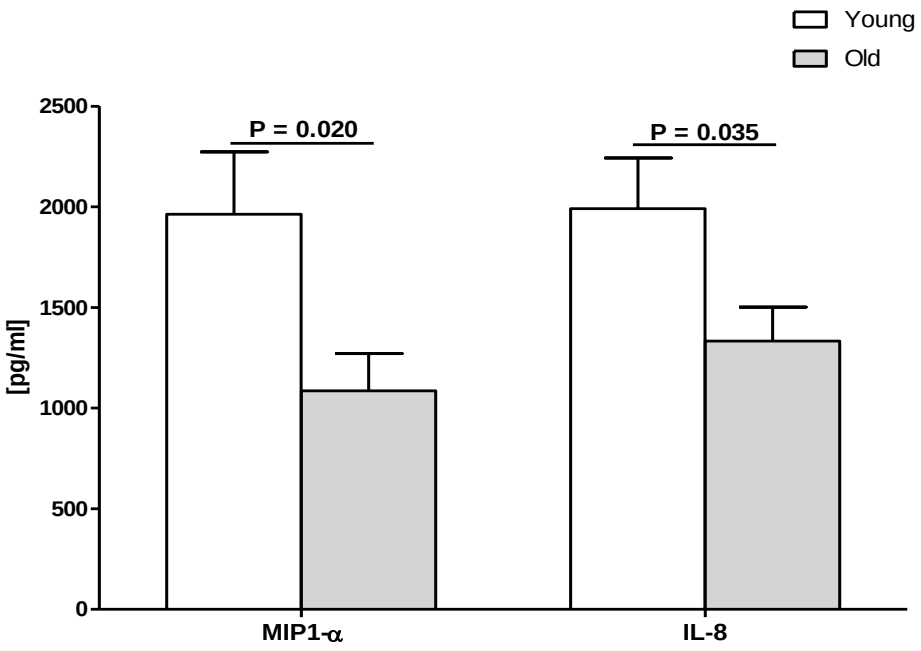
old (Figure 3.32B) cohorts, there was no significant difference in the killing activity between MIP1-α and control-treated NK cells. There was also no increase in NKCC when NK cells were treated with 5 or 10 ng/ml of MIP1-α, concentrations that have previously been shown to enhance NK cell killing [281] (Figure 3.32A-B).

**Table 3.6. Cytokine and chemokine production by target-cell stimulated NK cells**

| | Chemokine/Cytokine | Concentration (pg/ml) | P Value |
|---|---|---|---|
| **Young (n = 13)** | MIP1-α Vs. TNF-α | 1963±310.00 Vs. 155±62.14 | **<0.0001** |
| | MIP1-α Vs. IFN-γ | 1963±310.00 Vs. 93.0±25.75 | **<0.0001** |
| | IL-8 Vs. TNF-α | 1991±252.00 Vs. 155±62.14 | **<0.0001** |
| | IL-8 Vs. IFN-γ | 1991±252.00 Vs. 93.0±25.75 | **<0.0001** |
| | MIP1-α Vs. IL-8 | 1963±310.00 Vs. 1991±252.00 | n.s. |
| **Old (n = 15)** | MIP1-α Vs. TNF-α | 1086±185.70 Vs. 51.87±8.41 | **<0.0001** |
| | MIP1-α Vs. IFN-γ | 1086±185.70 Vs. 27.60±4.46 | **<0.0001** |
| | IL-8 Vs. TNF-α | 1333±168.80 Vs. 51.87±8.41 | **<0.0001** |
| | IL-8 Vs. IFN-γ | 1333±168.80 Vs. 27.60±4.46 | **<0.0001** |
| | MIP1-α Vs. IL-8 | 1086±185.70 Vs. 1333±168.80 | n.s. |

Values are presented as mean ± SEM. Differences between cytokine and chemokine concentrations were analysed by a repeated measures ANOVA. Significant differences are indicated in bold font.

**(A)**

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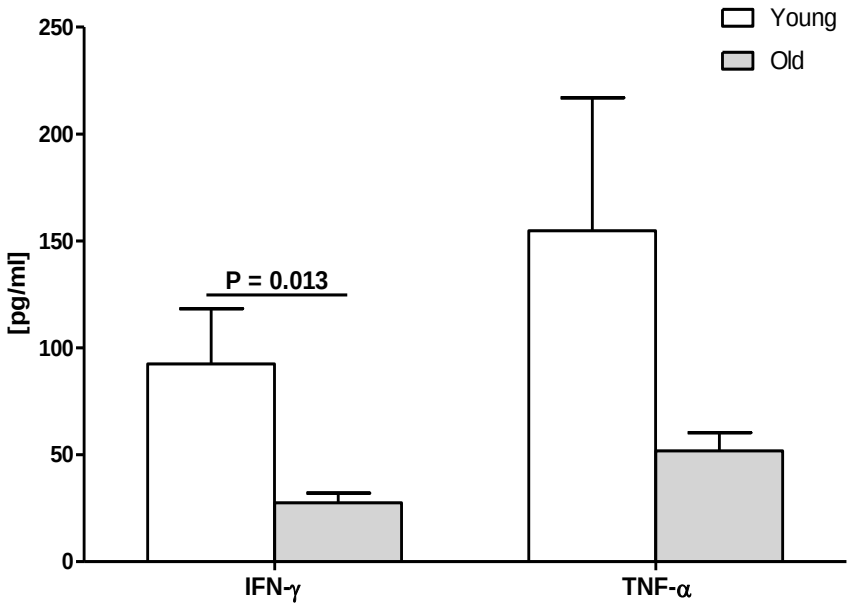
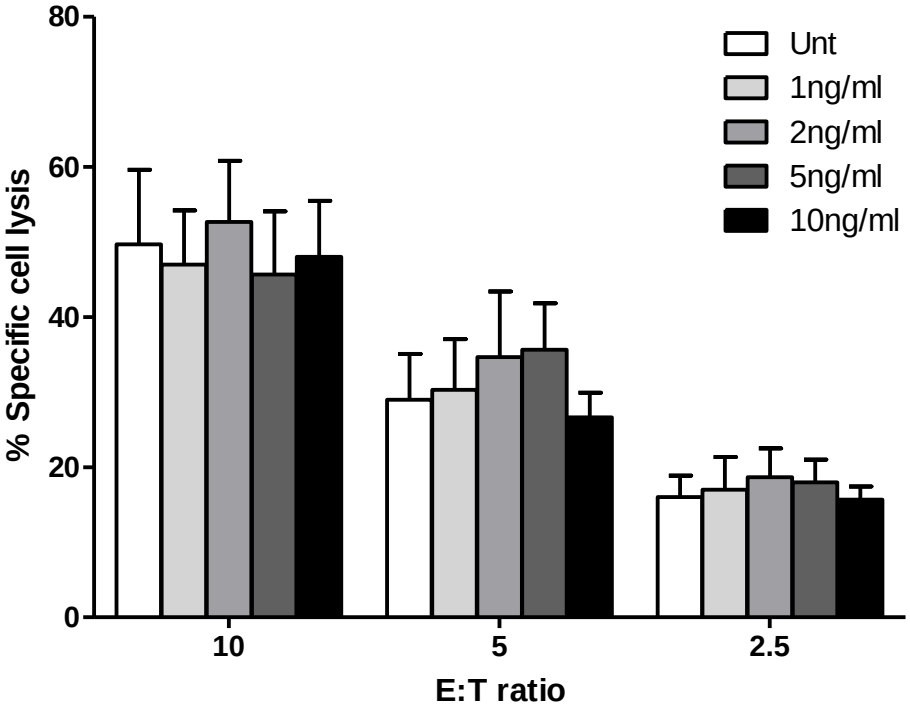



**Figure 3.31. Effect of age on target cell-induced cytokine and chemokine production**. Concentrations of MIP1-α and IL-8 **(A)**, and IFN-γ and TNF-α **(B)** in supernatants collected from 6-hour co-cultures of resting NK cells and K562 target cells (E:T, 1:1). Values represent mean ± SEM of 13 young and 15 old donors, with differences between groups assessed by either an unpaired student T test or Mann-Whitney U test.

**(A)**




**(B)**

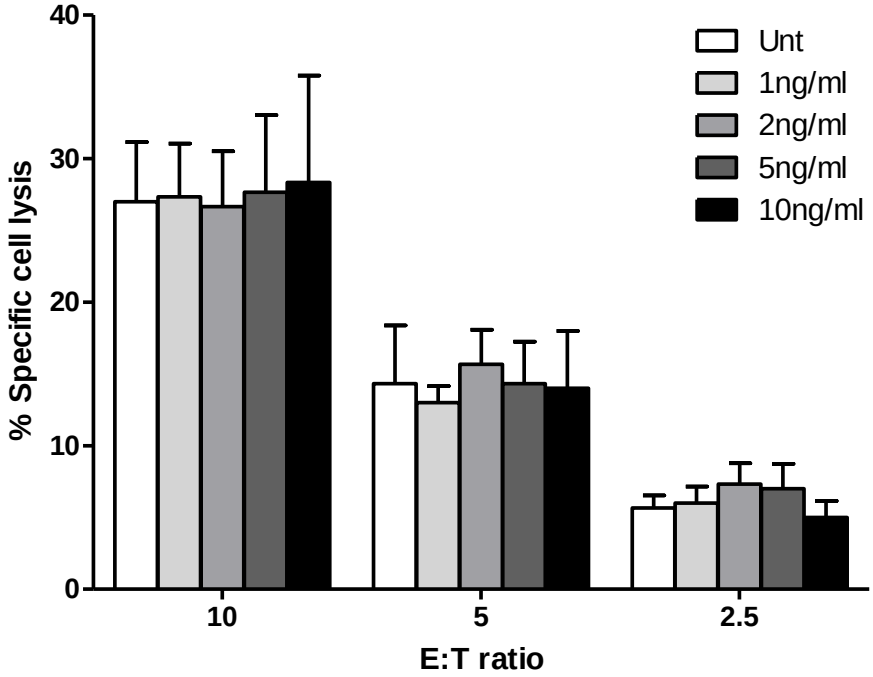



**Figure 3.32. Addition of MIP1-α to NK-K562 cell co-cultures does not enhance NKCC**. NK cells isolated from young **(A)** or old **(B)** donors were cultured for 4 hours with K562 target cells (E:T, 10:1, 5:1 and 2.5:1) in the presence or absence of MIP-1α (1-10 ng/ml). Post incubation, NKCC was measured by two-colour flow cytometry. Data from 3 young and 3 old subjects are summarised as mean ± SEM, with differences between treatment conditions assessed by a repeated measures ANOVA.

## 3.3 Discussion

NK cells are critical effector cells of the innate immune system renowned for their role in recognising and eliminating virally-infected and malignant cells. A well-documented feature of physiological ageing is a reduction in the cytotoxic capacity of NK cells at the single cell level [42;365;371], which is often proposed to contribute to the increased incidence of viral infection reported by older adults [9]. To date, only a small number of studies have addressed this theory directly [187-189]. Although limited with respect to sample size and follow-up time, these studies, which have been performed predominantly in older adults, have shown low NKCC to be associated with: (i) a past history of severe infection, (ii) an increased susceptibility to infection and (iii) death due to infection [187-189], suggesting that the age-related deterioration in NKCC is of physiological relevance. Given the significance of these data, it is surprising that no study to date has identified the mechanism(s) that underlies the reduction in NKCC with age. Thus, in addition to investigating the effect of human ageing on the composition of the circulating NK cell pool, the aim of this chapter was to elucidate the cause of the age-associated decline in NKCC.

Confirming the findings of previous studies [42;351;354-356;361;516], compared to younger adults, older individuals had a greater proportion of both $CD3^{-}$ $56^{+}$ and $CD3^{-}56^{+}57^{+}$ NK cells within their peripheral lymphocyte pool. Expression of CD57 on the surface of NK cells is considered a marker of maturity, and is associated with distinct functional properties. Compared to their negative counterparts, $CD57^{+}$ NK cells undergo fewer rounds of cell division and produce less IFN-γ in response to both target cell and cytokine stimulation [517]. As these features are reminiscent of the behaviour of NK cells isolated from old subjects [358;361;380], the accumulation of $CD57^{+}$ NK cells that this report and others [42;354] have observed in elderly donors may contribute to the changes reported in NK cell

function with age [518]. Interestingly, although impaired in their response to cytokine and target cell stimulation, $CD57^+$ NK cells exhibit greater lytic activity towards antibody-coated cells than $CD57^-$ NK cells [517]. Despite possessing a greater proportion of $CD57^+$ NK cells within their peripheral lymphocyte pool, elderly subjects perform ADCC at a level that is comparable to that of younger adults [356;376;377], suggesting that in a manner similar to natural cytotoxicity, ADCC may also be reduced with age at the single cell level [500].

What factors contribute to the accumulation of $CD57^+$ NK cells in aged individuals? Currently, the answer to this question is unknown but there are two possibilities. Firstly, with age, both the production and proliferation rates of NK cells fall [359], yet their circulating numbers have been reported to be comparable to [355;519] or higher [351;361;516] than that recorded in younger adults, suggesting the existence of a long-lived NK cell subset in elderly individuals. Given their highly mature differentiated state, $CD57^+$ NK cells may be this long-lived NK cell population [517]. An alternative explanation is that factors independent of age drive the accumulation of $CD57^+$ NK cells over time. In a recent study performed entirely on samples collected from young volunteers, Bigley and co-workers noted that seropositivity for CMV was associated with an increased proportion of circulating $KLRG1^-/CD57^+$ NK cells [364]. Based on this observation the group proposed that the elevated levels of $CD57^+$ NK cells in elderly subjects may not be the result of ageing per se but a consequence of latent viral infection [364]. Whilst no effect for CMV on the frequency of $CD57^+$ NK cells in young donors was observed in this thesis, it was found that old $CMV^+$ individuals possessed a greater percentage of this subset in their peripheral pool when compared to their $CMV^-$ counterparts. Therefore, these data suggest that long-term antigen stimulation is needed to drive the accumulation of mature NK cells. In line with this proposal, Elpek et al have recently shown that the frequency of mature NK cells increases *in vivo* following sustained

but not transient exposure to the potent NK activating cytokine IL-15 [520]. Thus, instead of being a by-product of the ageing process, the accumulation of $CD57^{+}$ NK cells in older adults may in fact result from life-long exposure to stimuli that drive NK cell activation [364]. On this note, it is of interest that when CD57 surface density was assessed, which was significantly higher in both young and old $CMV^{+}$ subjects when compared to their negative counterparts, no difference in expression levels between young and old seronegative donors was seen, a finding that lends support to the idea that factors independent of age are involved in re-modelling the circulating NK cell pool.

Based on the differential surface expression of CD56, NK cells can be divided into two major subsets: $CD56^{DIM}$ and $CD56^{BRIGHT}$. Whilst the effect of age on the absolute number of circulating $CD56^{DIM}$ NK cells is controversial [351;361;519], an age-related increase in their frequency is well documented [351;354;358;361], as is a dramatic reduction in both the proportion and total numbers of $CD56^{BRIGHT}$ NK cells [351;354;355;358;519], changes that lead to an increased $CD56^{DIM}$ to $CD56^{BRIGHT}$ ratio with age [352;358]. As well as confirming these much publicised age-associated changes in NK cell composition, this study performed a more detailed phenotypic analysis of NK cell subsets, and found the increased frequency of $CD56^{DIM}$ cells with age to be the result of an accumulation of $CD56^{DIM}CD16^{+}$ NK cells, whilst a significant decline in the percentage of $CD56^{BRIGHT}CD16^{-}$ NK cells accounted for the reduction in the $CD56^{BRIGHT}$ subset. Alongside these observations, ageing was found to be associated with an increase in the percentage of $CD56^{-}CD16^{+}$ NK cells, a subset that when compared to $CD56^{DIM}$ NK cells exhibits defective natural and antibody dependent cytotoxicity [521-523] but comparable chemokine secretion [524;525], suggesting an immunoregulatory role for $CD56^{-}CD16^{+}$ NK cells in host protection. This finding is not in agreement with the recent work of Lutz et al, who reported no effect for age on the

percentage of this NK cell subset [354]. However, in that study, samples were obtained exclusively from women, whilst the cohort used in this thesis consisted of both males and females. Possible gender-related differences in the frequency of $CD56^{-}CD16^{+}$ NK cells may therefore explain these discordant findings.

By displaying potent cytotoxic activity *in vitro,* $CD56^{DIM}$ NK cells are considered to be the NK cell subset primarily responsible for the direct elimination of transformed cells, and therefore an increased frequency of $CD56^{DIM}$ NK cells within the peripheral lymphocyte pool of older adults may represent a compensatory mechanism to counteract the age-related decrease in NKCC at the single cell level. In response to cytokine stimulation, $CD56^{BRIGHT}$ NK cells generate an array of immunoregulatory cytokines and chemokines such as IFN-γ, TNF-α, IL-8 and MIP-1α/β [275;276;292;344]. Through the production of these mediators, $CD56^{BRIGHT}$ NK cells have the capacity to activate monocytes, drive the maturation of iDCs, enhance the microbicidal capacity of local phagocytes and recruit bystander immune cells to sites of developing inflammation [526;527]. Thus, due to a lower number of blood $CD56^{BRIGHT}$ NK cells, the ability of older adults to amplify on-going immune responses may be impaired, especially as this decline in number is accompanied by a marked age-associated reduction in cytokine and chemokine production by stimulated $CD56^{BRIGHT}$ NK cells [378;380-382].

Using a two-colour flow cytometric protocol that allowed for the simultaneous assessment of NKCC and NK-target cell conjugate formation, it was found that when compared to NK cells from young volunteers, those isolated from older adults exhibited reduced lytic activity towards K562 tumour cells. This age-related impairment in NKCC was observed in both 4 and 16-hour NK-K562 co-cultures and was not accompanied by a reduction in conjugate formation, suggesting a post-binding defect was responsible. Although the findings of the

4-hour NK-K562 co-cultures confirm the work of previous studies that have shown an age-associated decline in NKCC at the single level at this time point [42;365;371], the results of long-term (16-hour) cytolytic assays are in contrast to those of Rukavina et al who reported similar levels of NKCC between young and old subjects following prolonged culture with target cells [370]. Differences in experimental design may be responsible for this variation in outcome. For instance, whilst Rukavina and co-workers cultured PBL's with K562 target cells for 18 hours before measuring NKCC [370], the 16-hour cytolytic assays presented in this thesis were performed with purified NK cells.

With the odd exception [331;332], the induction of NKCC requires both the absence of MHC class I molecules on the target cell surface and the ligation of NK cell activatory receptors. Thus, it was conceivable that the reduced lytic activity exhibited by aged NK cells towards the MHC class I deficient K562 cell line was the result of an age-associated reduction in activatory receptor expression. In accordance with previous studies [351;362], a significant age-related decline in the percentage of $NKp46^+$ NK cells and the surface density of NKp30 was observed here. However, these changes in phenotype are unlikely to explain the impaired lytic activity elicited by aged NK cells towards K562 cells as this cell line does not express ligands for NKp46 [528], and the addition of blocking antibodies against NKp30 and/or NKp46 to NK-K562 cell co-cultures does not alter the rate of K562 cell lysis [528;529]. In contrast, numerous studies have shown that blocking antibodies against NKG2D, whose ligands MICA/B and ULBP2 are expressed on the surface of K562 cells [260;529;530], significantly reduces NK-mediated target cell lysis [529;530]. However, in line with the published literature [351;355], this study observed no age-related difference in the percentage of $NKG2D^+$ NK cells or in its surface density. Thus, changes in receptor expression cannot

be put forward as an explanation for the reduced lytic activity of aged NK cells towards the K562 cell line.

Influenza virus hemagglutinin is a recently identified ligand for NKp46 [335]. In addition to inducing direct NK-mediated lysis of flu-infected cells [335], recognition of HA by NKp46 enhances IFN-γ production by NK cells, an outcome that has been proposed to aid the early developmental phases of an influenza specific adaptive immune response by promoting the maturation of influenza-infected DCs [531]. As well as protecting against influenza infection, NKp46 is important for the recognition and elimination of monocytes and macrophages infected with M. tuberculosis by NK cells [532;533]. Thus, whilst not responsible for the age-related decline in NKCC towards K562 cells, a reduced frequency of $NKp46^+$ NK cells may contribute to the increased incidence and severity of influenza infection reported by older adults [9;16;19;20], and possibly to the reactivation of latent TB, for which age is a major risk factor [21]. Away from infection, Thoren and colleagues have recently shown NK cells directly induce neutrophil apoptosis in an NKp46 dependent manner [534]. Given the reduced percentage of $NKp46^+$ NK cells in older adults, it would be interesting to compare the survival rates of neutrophils cultured with purified NK cells from young and old donors. An age-related reduction in NK-mediated neutrophil apoptosis could be one factor that contributes to the delayed resolution of inflammatory responses in elderly individuals [535].

In addition to examining to the effect of age on activatory receptor expression, the impact on the expression of CD94, a protein that interacts with both activatory (CD94-NKG2C/E/H) and inhibitory (CD94-NKG2A/B) receptors of the C-type lectin family [502] was considered. Confirming the work of other groups [351;352], an age-associated reduction in the percentage of circulating $CD94^+$ NK cells was observed, which was accompanied by a marked reduction in its staining intensity. It is known that the inhibitory receptor

CD94-NKG2A has a higher binding affinity than its activatory counterpart CD94-NKG2C for their shared ligand HLA-E, and can override the activatory signals that emanate from this complex [536;537]. Recently, Hayhoe and colleagues [352] speculated that the loss of CD94 with age may lead to unregulated NK cell lysis, presumably as a result of reduced CD94-NKG2A expression. However, CD94 shows no preference in terms of its binding partner. Thus, one would assume that a loss of CD94 would impact upon the expression of both activatory and inhibitory complexes, making it difficult to suggest what functional consequences if any the decline in CD94 expression with age has on NK cell function.

Granule exocytosis and death receptor ligation are the two mechanisms through which NK cells directly eliminate transformed cells [206]. In cytotoxicity assays that employ K562 cells as the target, killing is thought to be mediated exclusively by the granule exocytosis pathway. Indeed, it was shown in this report that pre-treating NK cells with CMA, a potent inhibitor of perforin-based cytotoxicity [494;495], significantly reduced their lytic activity towards K562 cells in subsequent 4-hour cytotoxicity assays, whereas culturing K562 cells with death receptor ligands had no effect on their viability. Based on these observations it was hypothesised that a defect in the granule exocytosis pathway was responsible for the age-related decline in NKCC and this issue was addressed by examining the effect of age on events fundamental to this form of NKCC.

The pore-forming protein perforin and the serine protease granzyme B are critical effector molecules of the granule exocytosis pathway, which work together to induce caspase-dependent and independent cell death. When both the percentage of perforin and granzyme B positive NK cells, as well the staining intensity of these proteins, were compared between young and old subjects no age-related differences were seen, suggesting that NK cells from older adults are suitably armed with the effector molecules needed to induce target cell lysis.

Whilst in agreement with the previous work of Mariani and co-workers [375], these data relating to perforin expression contrast with that of Rukavina et al, who reported an age-related decline in both the percentage of perforin positive NK cells and the number of perforin molecules expressed per cell [370]. A possible explanation for these opposing observations is inter-study differences in subject inclusion criteria. As well as being small in number (n = 7), the elderly cohort studied by Rukavina et al consisted entirely of care home residents, who may have been a more frail group when compared to the community dwelling elderly subjects that were enrolled into both this study and that performed by Mariani and colleagues [370;375]. In a separate group of young and old individuals, full blood counts were performed alongside flow cytometric analysis in order to calculate the absolute number of perforin positive NK cells, and the results showed a significant age-related decline. However, since this was driven by a significant fall in the total number of NK cells rather than a reduction in the percentage of perforin positive cells, which was again comparable between young and old subjects, this cannot explain the age-associated decline in NKCC reported in this thesis since purified NK cells at a fixed concentration were used in cytolytic assays.

Fusion of secretory lysosomes with the NK cell plasma membrane precedes the release of perforin and granzymes into the IS. In contrast to the findings of previous murine [538] and human [355] studies that had shown this aspect of NKCC to be impaired with age, the data here showed lysosome fusion to be comparable between NK cells from young and old donors. Using the expression of CD107a on the outer surface of NK cells as a marker of lysosome fusion, this study found no significant age-associated difference in the percentage of $CD107a^{+}$ cells or its staining intensity when either whole PBMC's or purified NK cells were cultured with K562 cells. In the abovementioned human-based study, the impairment in

lysosome fusion was observed only when the response of young donors was compared to those subjects considered "very old" (≥80 years, n = 30). Very few individuals within the elderly cohort used here met this criterion (n = 8), which may explain why no effect of age on lysosome fusion was seen.

Two major models exist that attempt to explain how perforin delivers granzymes into the target cell cytosol. The first suggests that perforin acts entirely at the plasma membrane, where it forms channels through which granzymes passively diffuse [211;539]. In the second model it is proposed that perforin works first on the plasma membrane to trigger perforin and granzyme uptake by the target cell and then on the endosomal membrane to facilitate granzyme entry into the cytoplasm [207;208;218]. Whilst it remains to be determined which of these models is correct, both agree that interaction of perforin with the target cell surface is critical for granzyme-mediated apoptosis. When perforin binding to the surface of K562 cells was determined, it was found that the percentage of target cells to which perforin had bound, as well as the number of molecules present on the cell surface, was significantly lower when these cells had been cultured with NK cells from older adults. Importantly, cytotoxicity assays performed alongside perforin binding experiments revealed a strong positive association between these two events suggesting that reduced perforin binding contributes to the age-related decline in NKCC. Underlying this reduction in perforin binding was reduced availability. ELISA analysis of supernatants collected from 4-hour NK-K562 cell co-cultures revealed the concentration of perforin was significantly lower in supernatants harvested from cultures that had contained NK cells from older individuals. This finding revealed a previously undescribed age-related impairment in perforin release into the IS and was confirmed at the single cell level when perforin secretion in response to K562 cell contact was measured by flow cytometry. Interestingly, when NK cells were stimulated with PMA

and ionomycin, no age-associated difference in perforin release was observed. As PMA and ionomycin bypass the cell membrane to activate cells, this suggested that either a defect in target cell recognition or aberrant intracellular signalling downstream of NK cell receptors was responsible for the age-associated decline in perforin secretion. Given the previously described similarity observed in conjugate formation, combined with comparable expression of the early activation marker CD69 on the surface of young and old NK cells following target cell recognition, the latter hypothesis seemed more likely to be correct.

In the absence of overriding inhibitory signals, target cell recognition leads to the formation of an activating IS, which is essential for the directed secretion of cytolytic effector molecules onto the target cell surface. An important early step in IS formation is the reorganisation of the actin cytoskeleton, for which actin polymerisation is required [513]. When NK-K562 cell conjugates were assessed by immunofluorescence, an accumulation of F-actin at the interface formed between K562 cells and NK cells was seen with both young and old donors, suggesting that signalling events leading to actin polymerisation were not impaired with age. Formation of F-actin networks serves as a trigger for the movement of the MTOC to the developing IS, which is essential for the delivery of lytic granules to the NK-target cell interface. To test whether this stage of IS formation was impaired with age, NK cells cultured alone or with K562 target cells were assessed for the location of lytic granules by staining samples with an antibody against perforin. As expected, lytic granules were dispersed throughout the cytoplasm of resting NK cells and polarised to the IS that was formed upon target cell recognition. However, from the images it was evident that the delivery of lytic granules to the NK-target cell interface was not as efficient in NK cells from older adults. Moreover, when the proportion of NK cells that had polarised granules to the IS was compared between young and old subjects, a significant age-related reduction was found.

Thus, these findings suggest that defective polarisation of lytic granules to the IS underlies the reduction in perforin secretion observed with age.

The mobilisation and delivery of lytic granules to the NK-target interface requires activation of the MAPK ERK1/2 [259;540]. Having observed a defect in granule polarisation in NK cells of older adults, the kinetics of ERK 1/2 activation was investigated. To do this, NK cells were stimulated with antibodies targeted against the activating receptors NKG2D and NKp30, and by Western blot analysis the expression of phosphorylated ERK1/2 was determined. Within 10 minutes of treatment, a significant increase in the phosphorylation status of ERK1/2 within NK cells from young individuals was seen, which was maintained for the remainder of the time-course that terminated after 90 minutes. In contrast, only after 30 minutes did a significant increase in ERK1/2 phosphorylation occur in NK cells isolated from older adults, which although maintained at 60 minutes was no longer evident 90 minutes post stimulation. Thus, it would seem from these data that NK cells from aged donors display slower kinetics of ERK1/2 activation and fail to sustain this signalling pathway as long as their younger counterparts. However, it must be stressed that whilst the ligands for NKG2D and NKp30 are expressed on the surface of K562 cells [260;501;528-530], the antibody stimulation technique used does not reflect the vast array of receptor-ligand interactions that occur upon NK-K562 contact. Thus, the subtle age-related changes observed in the kinetics of ERK1/2 phosphorylation may not have been evident had NK cells been stimulated with K562 cells. Due to difficulties in establishing a method that would have allowed us to differentiate between phosphorylated ERK1/2 expressed by activated NK cells and that endogenously expressed in K562 cells, it was not possible to perform this experiment. However, ERK1/2 activation kinetics in NK cells from young and old donors following PMA and ionomycin stimulation were compared and no age-related

difference was seen. As these agents bypass the cell membrane, these data suggest that aberrant intracellular signalling proximal to the NK cell membrane was responsible for the age-associated delay observed in ERK1/2 activation.

From the data presented in this chapter, it is proposed that reduced perforin secretion underlies the reduction in NKCC that accompanies physiological ageing. Furthermore, this impaired secretion is attributed to defective polarisation of lytic granules to the IS. However, there may be an alternative mechanism by which to explain the age-associated decline in perforin release. In a recent study, Herz et al [541] found that despite no difference in perforin expression or lytic granule fusion, cytotoxic $CD8^+$ T cells from acid sphingomyelinase (ASMase) deficient mice released fewer cytolytic effector molecules into the IS upon target cell recognition and exhibited reduced perforin dependent cytotoxicity when compared to $CD8^+$ T cells from their wild type counterparts [541]. Through a series of elegant experiments, the group attributed the impaired cytolytic activity of ASMase-deficient $CD8^+$ T cells to the failure of cytotoxic granules that had fused with plasma membrane to contract and expel their contents into the IS [541]. Thus, could it be that the activity of ASMase is reduced in NK cells of older adults? Currently, the answer to this question is unknown but it is certainly a plausible explanation for the significantly lower perforin concentrations we found in supernatants harvested from NK-K562 co-cultures that had contained NK cells from older individuals, especially since despite observing a marked age-associated impairment in perforin polarisation to the IS, no difference in lytic granule fusion was found with age. Furthermore, it is known that lytic granule contraction and exocytosis are sensitive to the lipid composition of the plasma cell membrane [542].Whilst no study has examined the effect of age on the lipid composition of the NK cell membrane, an age-related increase in cholesterol content has been reported in the plasma membrane of T cells [65].

Interestingly, Roozemond and colleagues [543] found that decreasing the fluidity of the NK cell membrane significantly reduced their lytic activity towards K562 cells, a defect that they proposed resulted from reduced secretion of cytotoxic effector molecules [543]. Should ageing be associated with an increase in the cholesterol content of the NK cell membrane then the resultant increase in membrane rigidity may impede the contraction of lytic granules and/or the ejection of their contents into the IS.

In response to cytokine or target cell stimulation, NK cells secrete an array of immunoregulatory cytokines and chemokines, such as TNF-α, IFN-γ, MIP-1α and IL-8 [275;277;278]. Whilst a number of studies had examined the effect of age on the production of these soluble mediators by cytokine-stimulated NK cells [378;380-382], no group had investigated whether target cell-induced cytokine and chemokine production was altered with age. To address this, purified NK cells from young and old subjects were cultured with K562 target cells for 6 hours, after which the concentrations of TNF-α, IFN-γ, IL-8 and MIP-1α in cell-free supernatants were measured. In line with previous work [277], NK cells from both young and old donors secreted significantly greater amounts of chemokines than cytokines upon target cell contact, suggesting chemokine production is initiated at a lower activatory threshold. Comparison of cytokine and chemokine production between our young and old cohort showed a significant age-associated decline in the production of IFN-γ, IL-8 and MIP-1α, but no difference in TNF-α secretion. Based on these findings and those of other studies that have reported an age-related reduction in both cytokine and chemokine production by cytokine-stimulated NK cells [378;380-382], it would be tempting to suggest that the immunoregulatory capacity of NK cells wanes with age. However, although statistically significant, the differences that this study and others have observed in the concentrations of cytokines and chemokines secreted by NK cells from young and old donors are small and

may not be of biological relevance. To address this issue, it would be useful to compare the functional responses of immune cells treated with the concentrations of cytokines and chemokines secreted by target cell or cytokine stimulated NK cells from young and old subjects. This was attempted in this thesis, where MIP-1α, which had previously been shown to augment the lytic activity of NK cells [281], was added to NK-K562 co-cultures at the concentrations secreted by young or old NK cells in response to target cell stimulation. However, no enhancing effect of this chemokine on NKCC was observed.

In summary, as well as confirming previously described age-associated alterations in the composition of the circulating NK cell pool, studies presented in this chapter have shown for the first time that NK cells from older adults exhibited impaired perforin secretion upon target cell contact, a defect we propose mediates the reduction in NKCC that accompanies physiological ageing. This impairment in perforin secretion is attributed at least in part to defective polarisation of lytic granules to the IS. Furthermore, target cell-induced cytokine and chemokine production was reduced with age, although the biological relevance of this finding remains to be determined.

# CHAPTER 4

## Effect of human ageing on the generation of neutrophil extracellular traps (NETs)

## 4.1 Introduction

Neutrophils are a critical component of the innate immune system, providing frontline protection against infection with rapidly dividing bacteria, fungi and yeast. Upon arrival at a site of infection, neutrophils unleash an array of anti-microbial mechanisms in an effort to directly contain and eliminate the invading pathogen. For many years, these mechanisms were thought to be limited to phagocytosis, production of ROS and degranulation [387]. However, in 2004, a fourth defensive strategy was discovered: the generation of neutrophil extracellular traps (NETs) [435]. Consisting of a DNA backbone decorated with a multitude of granule-derived anti-microbial peptides and enzymes, NETs are ejected from dying neutrophils and are thought to play an important role in the entrapment of bacteria, parasites and fungi [465].

Compared to younger subjects, older adults report an increased incidence of bacterial and fungal infection. As well as being more frequent, these infectious episodes are more severe and are associated with increased morbidity and mortality rates [544-547]. As neutrophils represent the first line of defence against bacteria and fungi, this data implies that the protective capacity of neutrophils wanes with age. Indeed, numerous *in vitro* studies have documented an age-associated reduction in neutrophil phagocytosis [404;409;417], ROS production [412;415;420;421] and degranulation [410;427]. However, no study to date had examined whether NET formation was altered with age.

Whilst data are emerging from murine-based studies that suggest NETs play an important role in pathogen containment during the early phases of infection [478;479], no direct *in vivo* evidence exists in humans to suggest a role for these structures in conferring host protection. However, a role has been proposed based on the results of studies that have examined NET production by neutrophils isolated from patients who exhibit an increased risk of infection.

For instance, Yost and colleagues showed neutrophils from newborn children, in whom bacterial infection is a major cause of death and morbidity [548], failed to generate NETs when exposed to inflammatory agonists [471]. Conversely, Bianchi et al restored NET formation in a CGD patient via gene therapy, and showed that this coincided with the clearance of a pre-existing fungal infection *in vivo* [463].

Based on these observations and the increased susceptibility older adults exhibit towards both fungal and bacterial infection [544-547], the aim of this chapter was to study the effect of human ageing on the generation of NETs. To achieve this, the following objectives were set:

1. Quantify NET formation in response to PMA stimulation by unprimed neutrophils from young and old donors.
2. Determine the effect of neutrophil priming on NET formation.
3. Examine the effect of age on NET generation by primed neutrophils in response to IL-8 and LPS stimulation.
4. Study the impact of age on ROS generation: a key event in NET formation.

## 4.2 Results

### 4.2.1 Neutrophil isolation by dextran-Percoll® density centrifugation does not result in spontaneous NET production

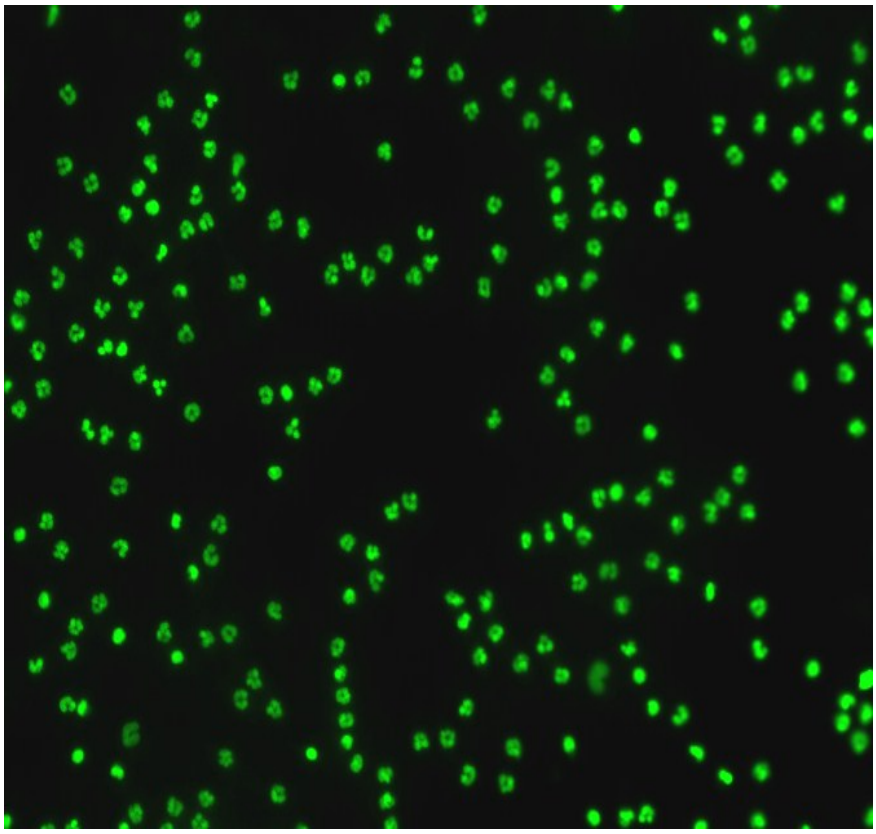
Dextran-Percoll® density centrifugation is a common method used to isolate neutrophils from whole blood. Recently, it was reported that neutrophils obtained by this procedure generated NETs in the absence of additional stimulation, culminating in high background fluorescence values in untreated control samples [443]. Given that dextran-Percoll® density centrifugation is the routine method by which neutrophils are isolated in this laboratory, it was important to investigate this finding. To do this, neutrophils that had been obtained by dextran-Percoll density centrifugation and cultured for 3 hours at $37^0$C were stained with the nucleic acid binding dye SYTOX® Green and studied by immunofluorescence microscopy.

In contrast to the aforementioned study [443], no evidence of NET formation was seen in cultures of resting neutrophils (Figure 4.1A). The cells retained their distinctive multi-lobed nucleus and showed no signs of chromatin decondensation, an early event in NET formation (Figure 4.1B).

### 4.2.2 Assessment of NET formation

Two techniques that are commonly used to study NET formation are their visualisation by immunofluorescence microscopy and the quantification of extracellular DNA in supernatants taken from cultures of stimulated neutrophils. Thus, prior to studying the effect of age on NET generation, it was first necessary to establish these methods within the laboratory. To do this, neutrophils were stimulated with the phorbol ester PMA, a potent inducer of NET formation.

**(A)**



**(B)**

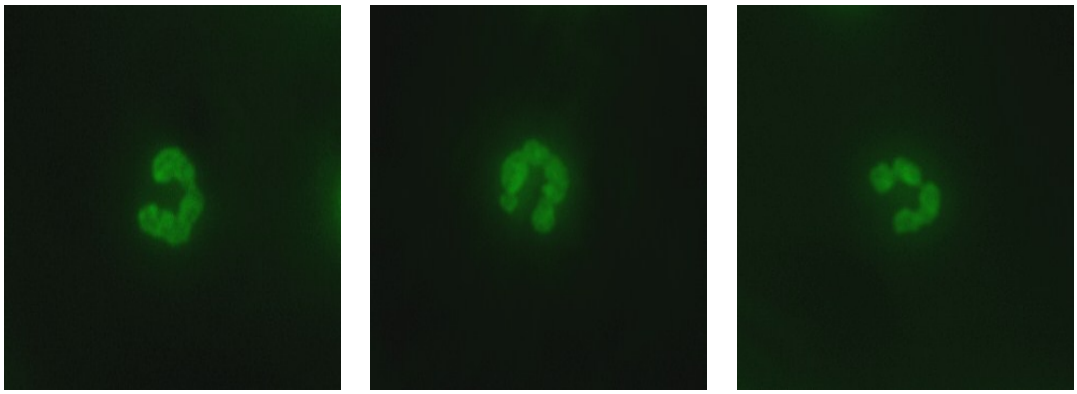
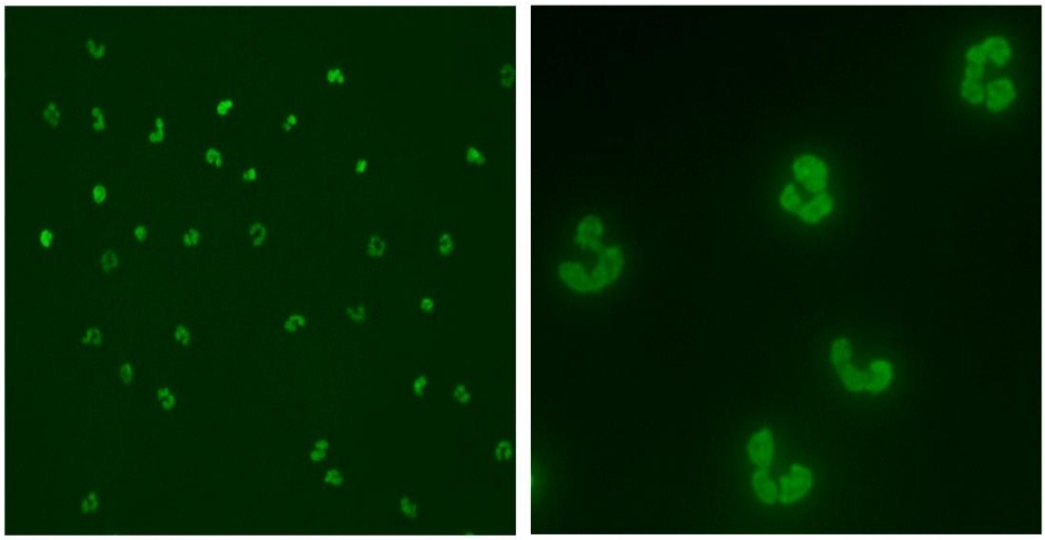


**Figure 4.1. Neutrophil isolation by dextran-Percoll® density centrifugation does not induce spontaneous NET formation.** Neutrophils isolated by dextran-Percoll® density centrifugation were cultured for 3 hours at $37^0$C. Post incubation, samples were stained with the nucleic acid binding dye SYTOX® Green, and their nuclear morphology visualised by fluorescence microscopy. Images were taken at X10 **(A)** and X63 **(B)** objective and are representative of 5 separate experiments.

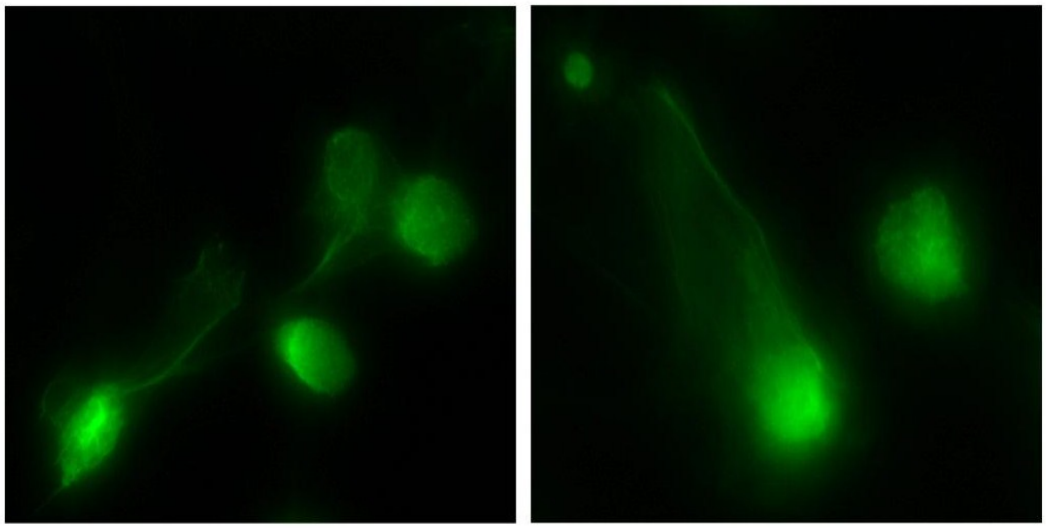
For immunofluorescence microscopy, the protocol recently described by Brinkmann et al [549] was used. Following a 3-hour incubation in the presence or absence of 25 nM PMA, neutrophils were fixed in 2% paraformaldehyde and stained with SYTOX® Green. As shown in figure 4.2A, there was no evidence of NET formation by neutrophils that had been cultured in media alone. In contrast, those stimulated with PMA had formed NETs, as shown by the presence of extracellular material that stained positive for SYTOX® Green (Figure 4.2B).

To quantify NET formation, cultures of PMA-stimulated neutrophils were treated with SYTOX® Green and micrococcal nuclease, an endo-exonuclease that digests double-stranded nucleic acids. Cell-free supernatants were then collected and their DNA content measured by fluorometry. Using this approach, the generation of NETs by PMA-treated neutrophils was consistently observed (Figure 4.3), which as reported by other groups [443;486] occurred in a dose and time-dependent manner (Figure 4.4A-B).

**(A)**



**(B)**



**4.2. Assessment of NET formation by immunofluorescence microscopy.** Neutrophils isolated from young adults were cultured for 3 hours in the absence **(A)** or presence **(B)** of 25 nM PMA. Post incubation, samples were stained with the nucleic acid binding dye SYTOX® Green, and visualised by fluorescence microscopy. Images are representative of two separate experiments performed and were taken at X20 and X63 objective.

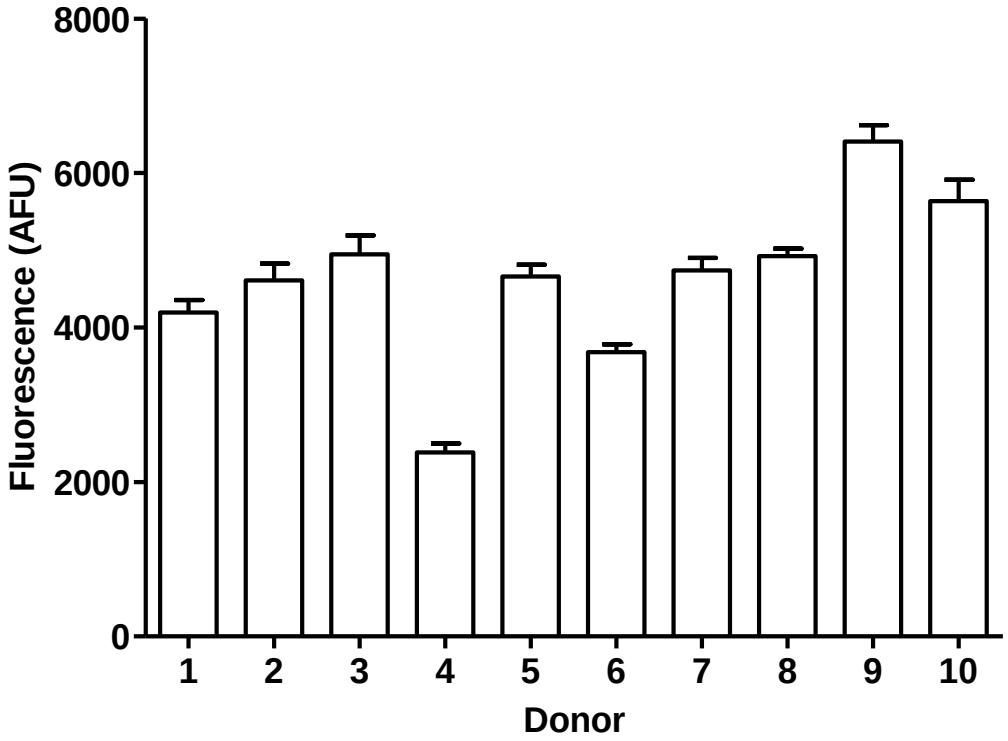
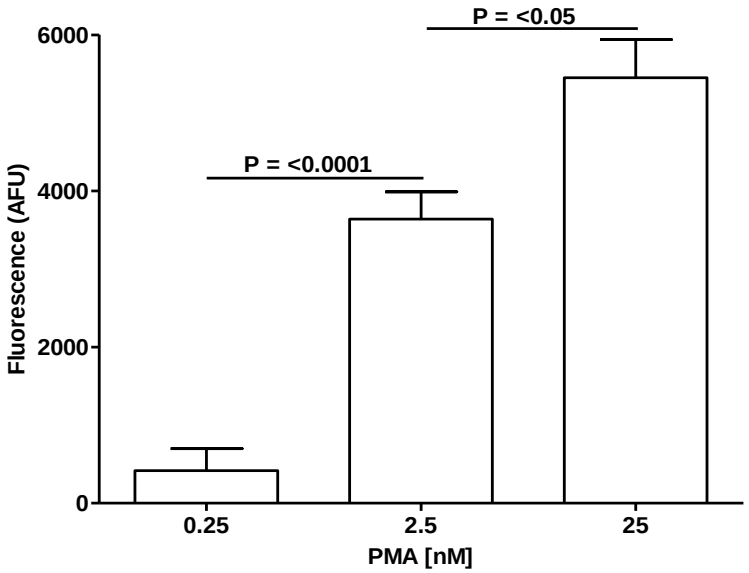



**Figure 4.3. PMA is a potent inducer of NET formation.** Neutrophils isolated from young donors (n = 10) were stimulated for 3 hours with 25 nM PMA, after which the DNA content of cell-free supernatants was assessed by fluorometry. Data are presented as mean ± SEM of experiments performed in quadruplicate after subtracting the background fluorescence of untreated controls. AFU, Arbitrary fluorescence units.

**(A)**




**(B)**

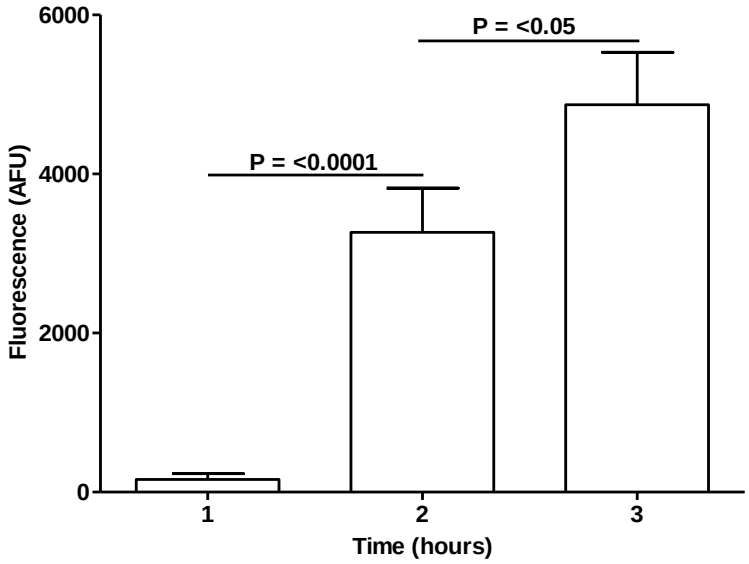



**Figure 4.4. NET formation induced by PMA is dose and time-dependent. (A)** Neutrophils isolated from 5 young donors were cultured with the indicated doses of PMA for 3 hours, after which the DNA content of cell-free supernatants was assessed by fluorometry. **(B)** Neutrophils obtained from 5 young subjects were treated for 1, 2 and 3 hours with 25 nM PMA, after which the DNA content of cell-free supernatants was measured by fluorometry. For both figures, data are presented as mean ± SEM after subtracting the background fluorescence of untreated controls. Differences between treatment conditions were assessed using a repeated measures ANOVA with Tukey's multiple comparison post hoc test. AFU, Arbitrary fluorescence units.

### 4.2.3 Effect of age on PMA-induced NET formation

To date, the majority of studies that have examined NET production by human neutrophils have used PMA as their stimulating agent. Having confirmed that PMA was a potent inducer of NET formation, it was investigated whether the generation of NETs triggered by PMA was altered with age. Quantification of extracellular DNA in cell-free supernatants (Figure 4.5A), and the visualisation of NETs by immunofluorescence microscopy (Figure 4.5B) revealed no age-associated difference in PMA-induced NET formation.

A prerequisite for the generation of NETs is the production of ROS. As expected, given that no age-associated difference in NET formation was seen, ROS production in response to PMA stimulation was also found to be comparable between neutrophils obtained from young and old donors (Figure 4.6).

### 4.2.4 Neutrophil priming enhances NET formation

Although PMA is a potent inducer of NET formation, it is a pharmacological agent with no physiological relevance. Therefore, the next set of experiments focused on NET production by neutrophils treated with the pro-inflammatory cytokine IL-8 and the bacterial product LPS.

Whether IL-8 and LPS trigger NET formation is an area of debate within the literature, with those who fail to report their production suggesting it to reflect the inability of these inflammatory agonists to activate NADPH oxidase [468]. To determine whether IL-8 or LPS could induce NET production, neutrophils were treated with 100 ng/ml LPS or 10 ng/ml IL-8, and after 3 hours the DNA content of cell-free supernatants was measured. With the exception of two individuals who failed to generate NETs following treatment with IL-8, all subjects produced NETs in response to both stimuli (Figure 4.7A-B).

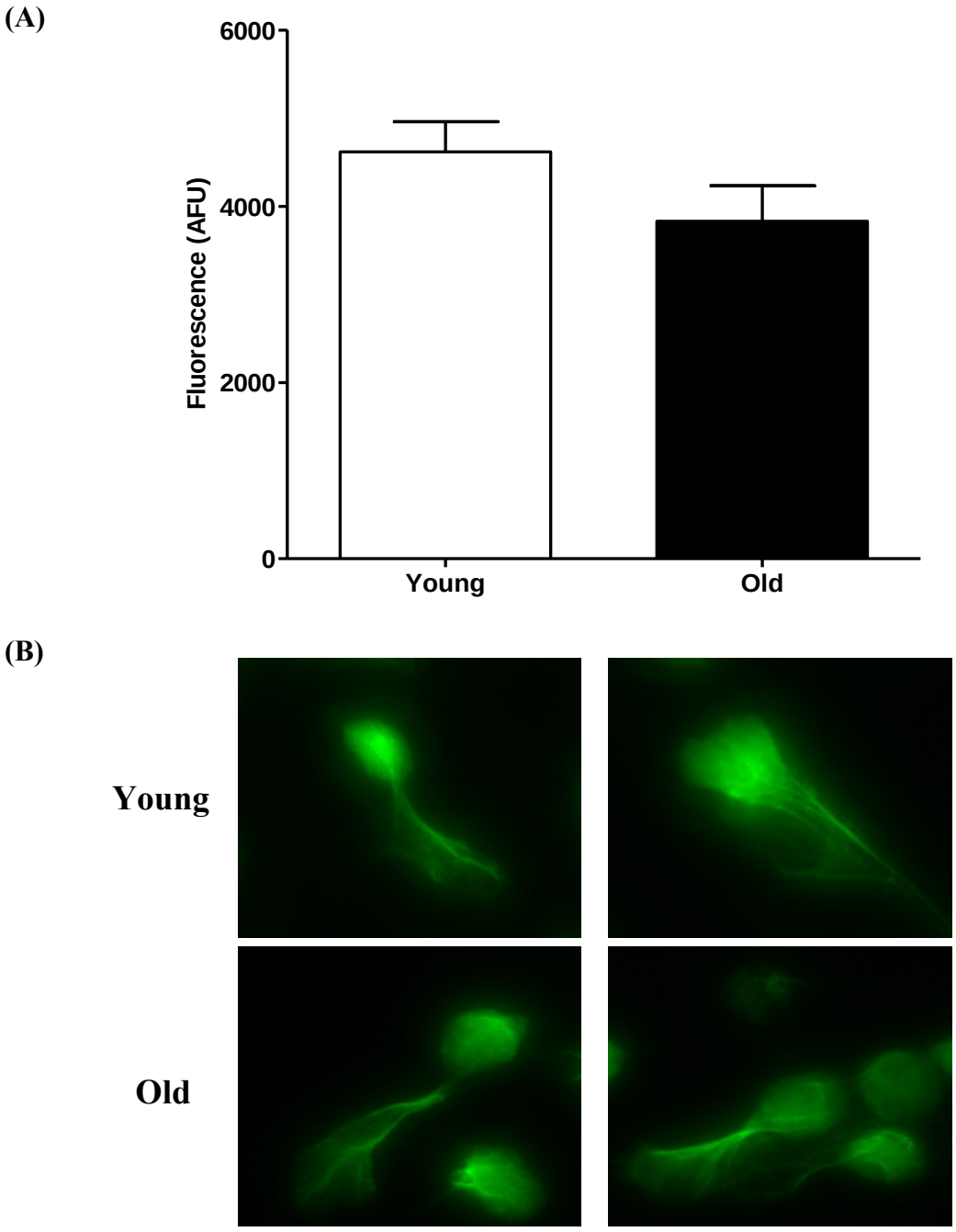



**Figure 4.5. Effect of age on PMA-induced NET formation. (A)** NET formation by neutrophils following 3-hour stimulation with 25 nM PMA. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, and represents the mean ± SEM of 10 and 8 experiments performed on neutrophils from young and old donors respectively. **(B)** Immunofluorescence images of NET production by neutrophils from young and old donors in response to 3-hour stimulation with 25 nM PMA. Images were taken at X63 objective and are representative of 2 separate experiments.

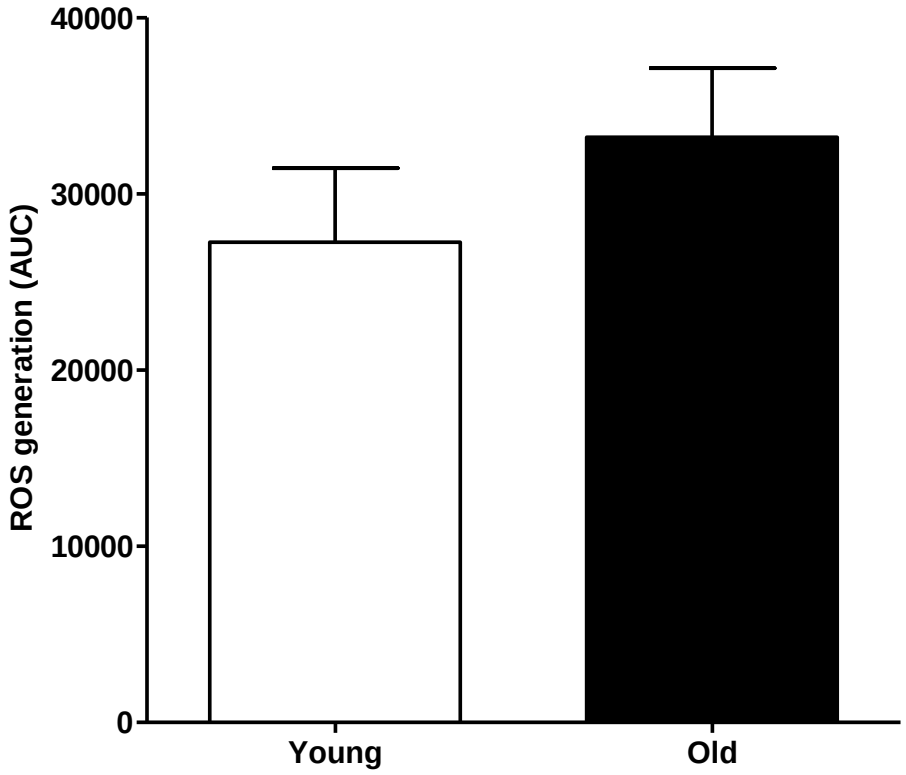
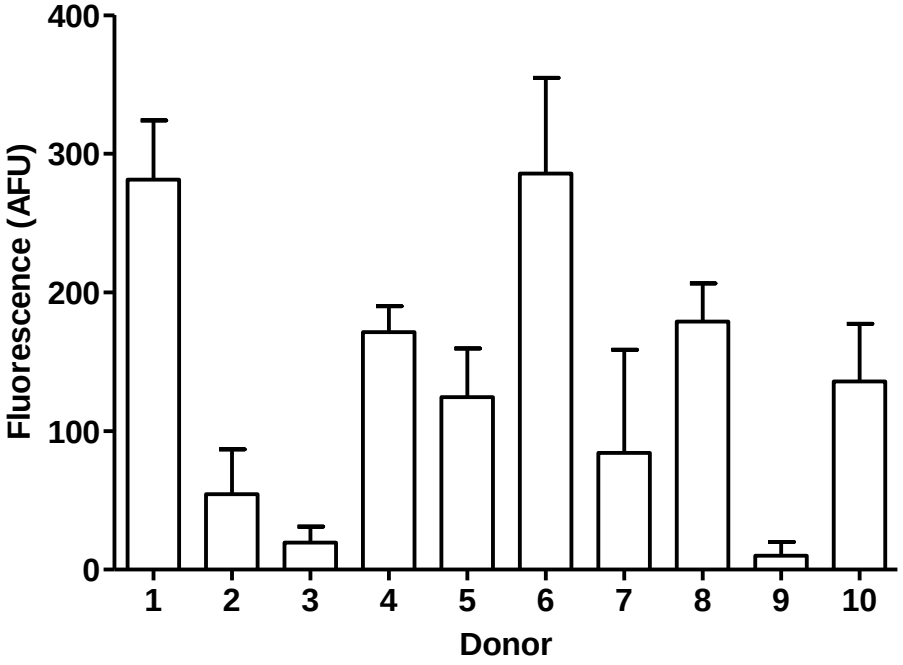



**Figure 4.6. Effect of age on ROS generation by PMA treated neutrophils.** ROS generation by freshly isolated neutrophils in response to stimulation with 25 nM PMA was measured over a 60-minute period by luminol-amplified chemiluminescence. Data are presented as area under the curve (AUC) and represents the mean ± SEM of experiments performed on 10 young and 8 old subjects.

**(A)**




**(B)**

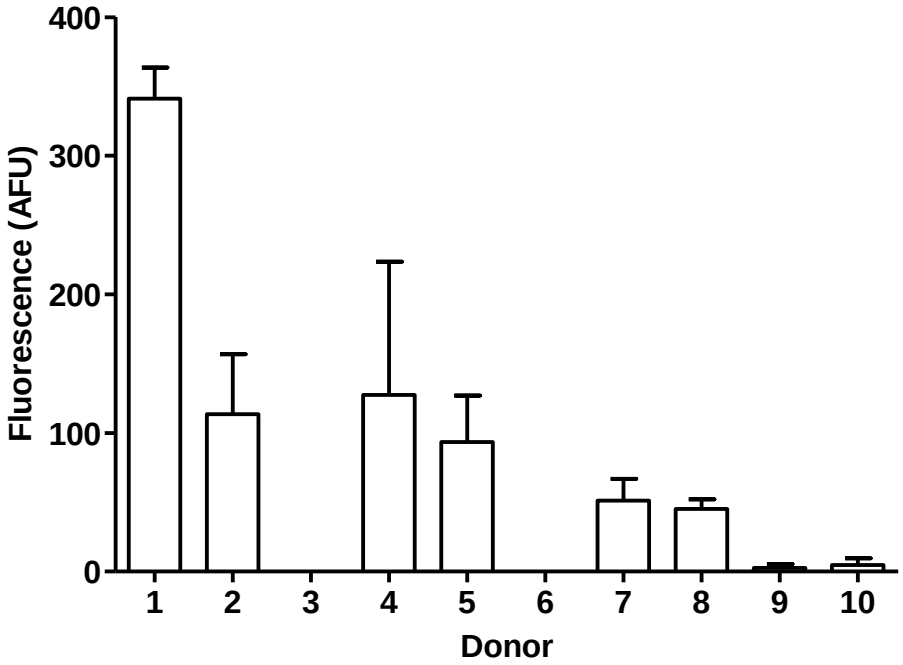


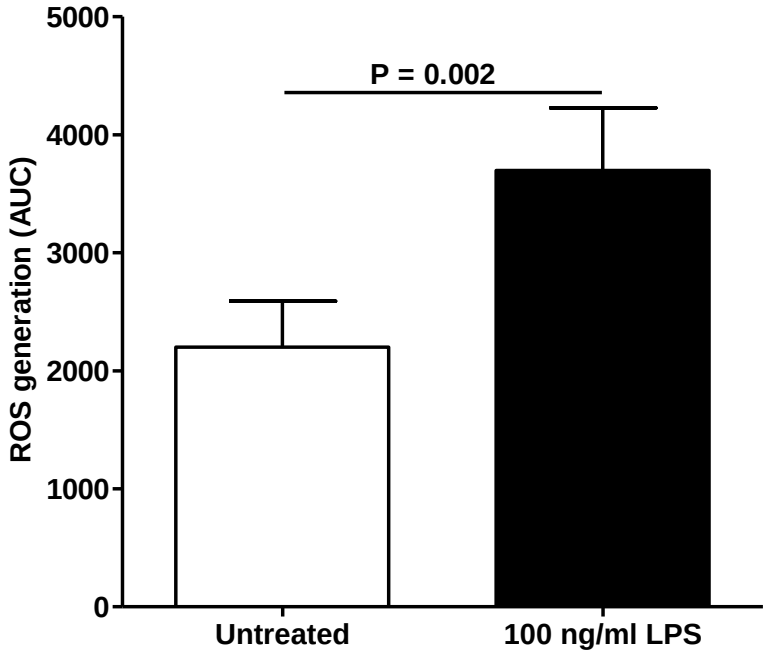

**Figure 4.7. NET generation by unprimed neutrophils in response to LPS and IL-8 stimulation.** Neutrophils isolated from young donors (n = 10) were stimulated for 3 hours with 100 ng/ml LPS **(A)** or 10 ng/ml IL-8 **(B)**, after which the DNA content of cell-free supernatants was assessed by fluorometry. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, with each column representing the mean ± SEM of experiments performed in quadruplicate.

Simultaneous assessment of ROS production revealed that both LPS and IL-8 stimulation significantly increased the generation of free radicals above the levels produced by untreated controls (Figure 4.8A-B).

Although both IL-8 and LPS were able to drive NET formation, experiments were performed on neutrophils that were in a resting state prior to stimulation. En route to sites of infection, neutrophils are exposed to pro-inflammatory cytokines such as TNF-α and GM-CSF, which through a process referred to as "priming" heighten the sensitivity of neutrophils to subsequent stimulation. Thus, in an effort to mimic the conditions that neutrophils are subjected to in times of pathogenic challenge, neutrophils were primed with 10 ng/ml TNF-α prior to assessing NET formation. The DNA content of cell-free supernatants taken from cultures of primed neutrophils following LPS (Figure 4.9A) and IL-8 (Figure 4.10A) treatment was significantly higher than that present in supernatants obtained from cultures that consisted of unprimed cells, suggesting that priming had enhanced NET production. Indeed, when assessing NET formation via immunofluorescence microscopy, it was apparent that compared to resting cells, primed neutrophils had extruded a greater amount of DNA into the extracellular environment upon LPS (Figure 4.9B) and IL-8 (Figure 4.10B) stimulation. Furthermore, it appeared based on nuclear morphology, that a greater proportion of primed neutrophils, which had not yet generated NETs, were undergoing the process of NETosis as very few of these cells possessed a multi-lobed nucleus (Figures 4.9B and 4.10B).

As well as augmenting NET formation, priming enhanced the production of free radicals. Compared to resting cells, ROS generation by primed neutrophils was significantly greater in response to both LPS (Figure 4.11A) and IL-8 (Figure 4.11B) challenge.

**(A)**




**(B)**

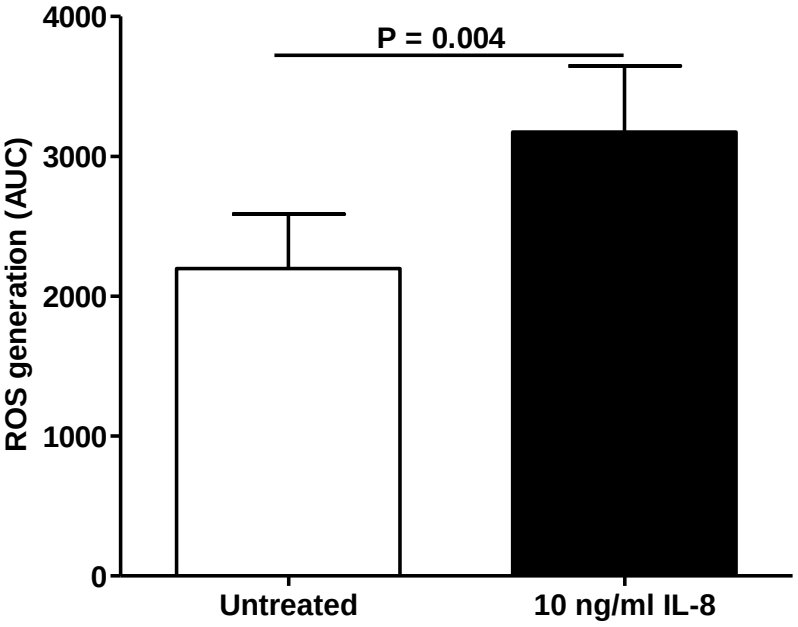
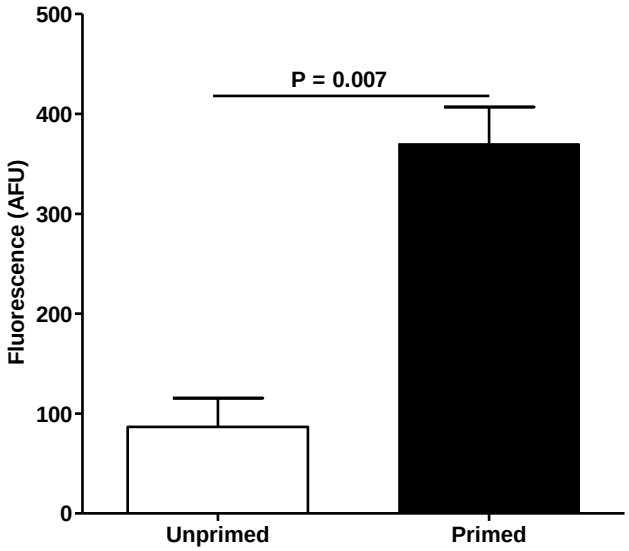



**Figure 4.8. ROS production by unprimed neutrophils in response to LPS and IL-8 stimulation.** Generation of ROS by freshly isolated neutrophils in response to stimulation with 100 ng/ml LPS **(A)** or 10 ng/ml IL-8 **(B)** was measured over a 60-minute period by luminol-amplified chemiluminescence. Data are presented as area under the curve (AUC) and represents the mean ± SEM of 10 experiments performed on neutrophils obtained from young subjects. Group differences were assessed using a Wilcoxon matched-pairs signed rank test.

**(A)**

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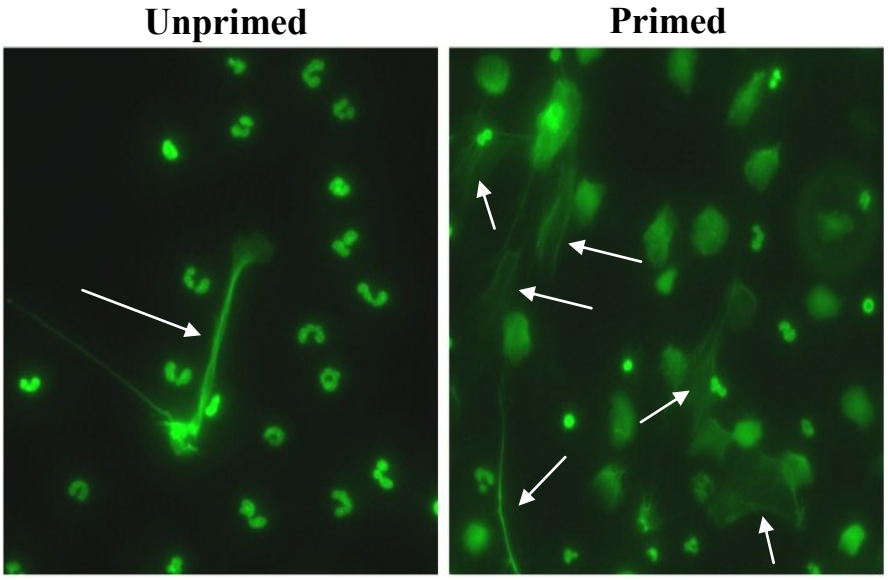
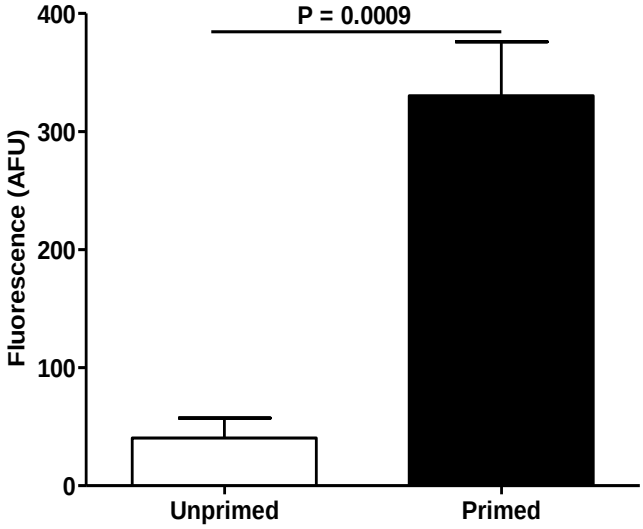



**Figure 4.9. Neutrophil priming significantly enhances LPS-induced NET formation.** **(A)** Quantification of extracellular DNA present in supernatants taken from cultures of unprimed or TNF-α primed neutrophils following a 3-hour stimulation with 100 ng/ml LPS. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, and represents the mean ± SEM of experiments performed on neutrophils obtained from 5 young donors. Differences between groups were analysed using a paired student T test. **(B)** Immunofluorescence images of NETs generated by unprimed and primed neutrophils following a 3-hour stimulation with 100 ng/ml LPS. Arrows point to regions of extracellular DNA. Images are representative of 2 independent experiments and were taken at X20 objective.

**(A)**




**(B)**

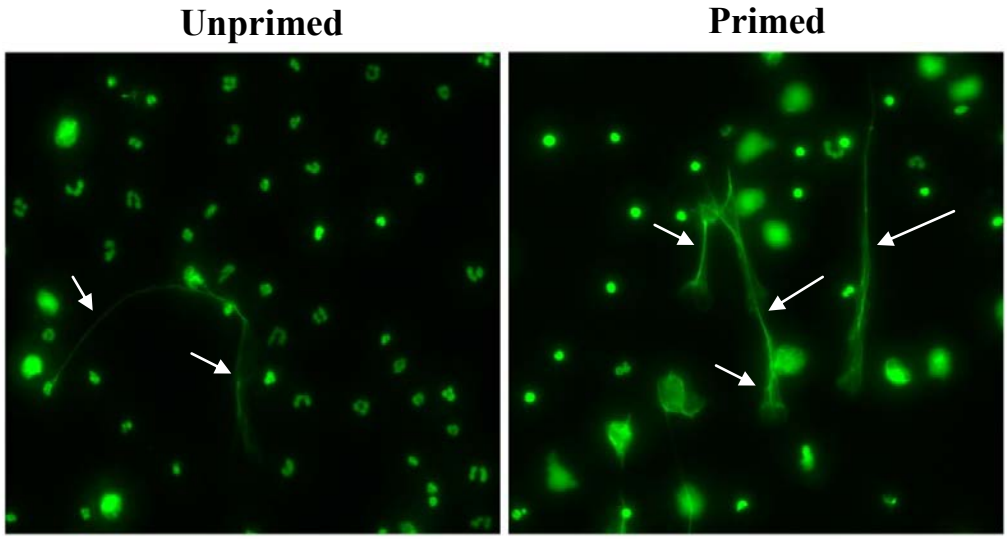
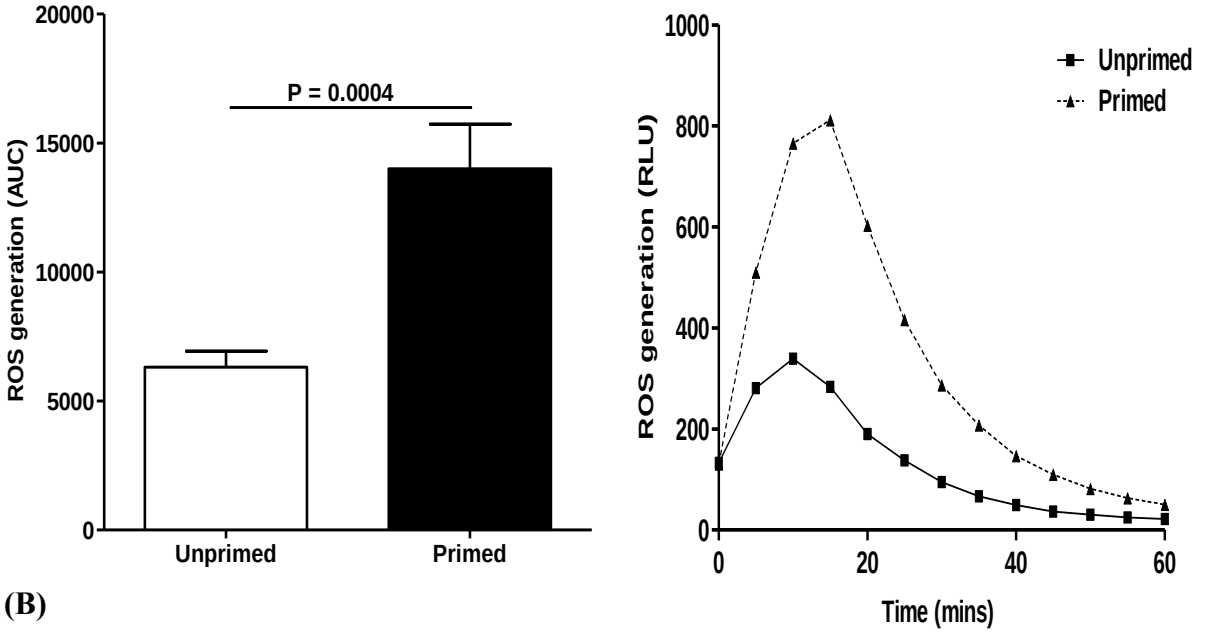



**Figure 4.10. Neutrophil priming significantly enhances IL-8-induced NET formation.** **(A)** Quantification of extracellular DNA present in supernatants taken from cultures of unprimed or TNF-α primed neutrophils following a 3-hour stimulation with 10 ng/ml IL-8. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, and represents the mean ± SEM of experiments performed on neutrophils obtained from 5 young donors. Differences between groups were analysed using a paired student T test. **(B)** Immunofluorescence images of NETs generated by unprimed and primed neutrophils following a 3-hour stimulation with 100 ng/ml IL-8. Arrows point to regions of extracellular DNA. Images are representative of 2 independent experiments and were taken at X20 objective.

**(A)**

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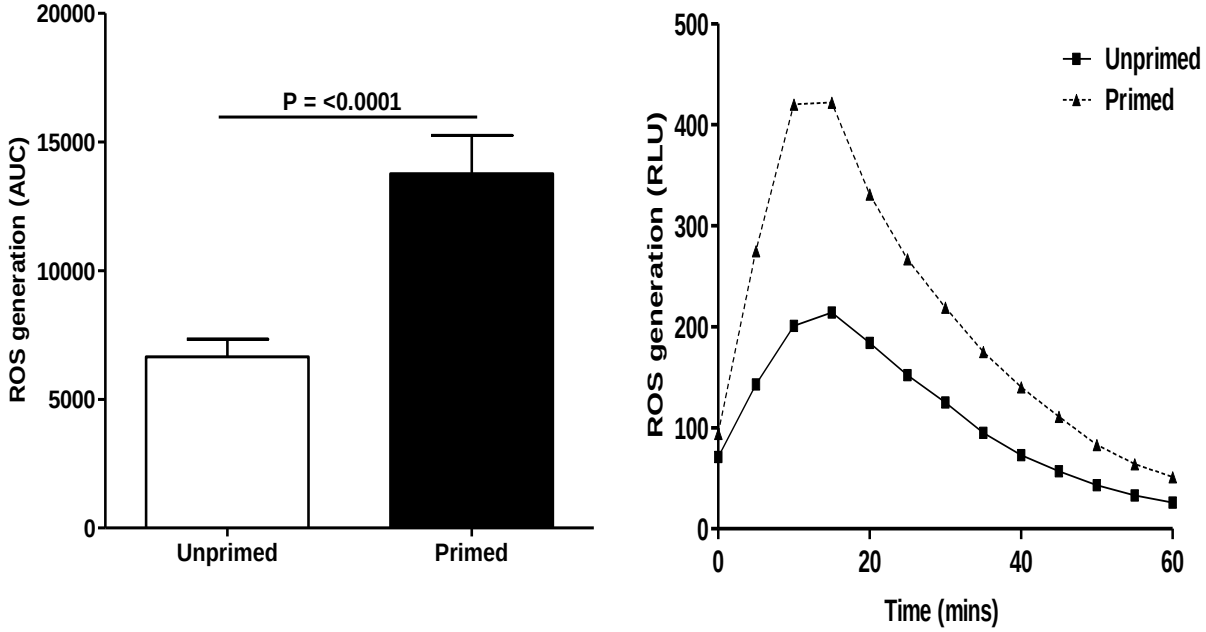


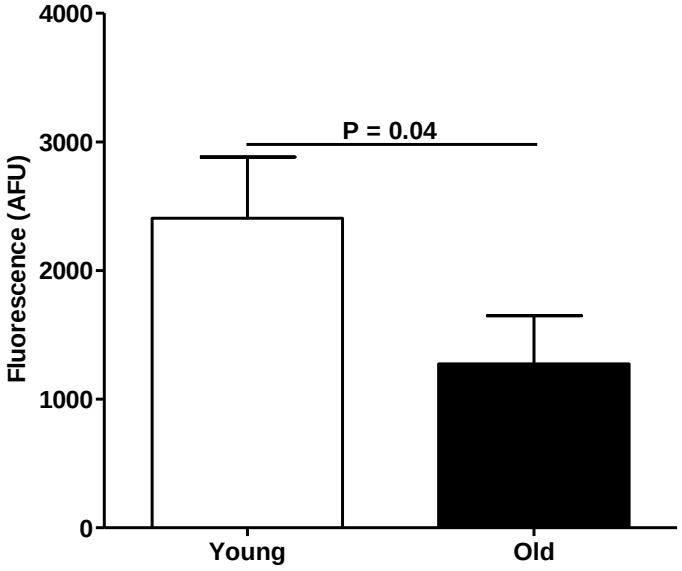

**Figure 4.11. Neutrophil priming significantly enhances ROS production.** ROS generation by unprimed or TNF-α primed neutrophils in response to 100 ng/ml LPS **(A)** or 10 ng/ml IL-8 **(B)** was measured over a 60-minute period by luminol-amplified chemiluminescence. In the left panel, ROS generation is presented as area under the curve (AUC) and represents the mean ± SEM of 8 experiments performed on neutrophils from young subjects. In the right panel, representative plots of ROS generation are shown. Group differences were assessed using a paired student T test. RLU, relative light units.

### 4.2.5 Effect of age on NET production and ROS generation by primed neutrophils

Having observed that when compared to their resting counterparts, primed neutrophils elicited a more robust NET response upon LPS and IL-8 treatment, it was decided that primed neutrophils would be used in the assays that set out to determine whether NET production induced by these two inflammatory agonists was altered with age.

Quantification of extracellular DNA in cell-free supernatants revealed a significant age-associated decline in NET production induced by LPS (Figure 4.12A) and IL-8 (Figure 4.13A) stimulation, findings confirmed when NET formation was visualised by immunofluorescence microscopy (Figures 4.12B and 4.13B). Accompanying this impairment in NET formation was an age-related reduction in the generation of free radicals. After priming with TNF-α, neutrophils were challenged with LPS (Figure 4.14A) or IL-8 (Figure 4.14B) and neutrophils from older adults generated significantly less ROS when compared to the levels produced by neutrophils from younger controls (Figure 4.14A-B).

**(A)**




**(B)**

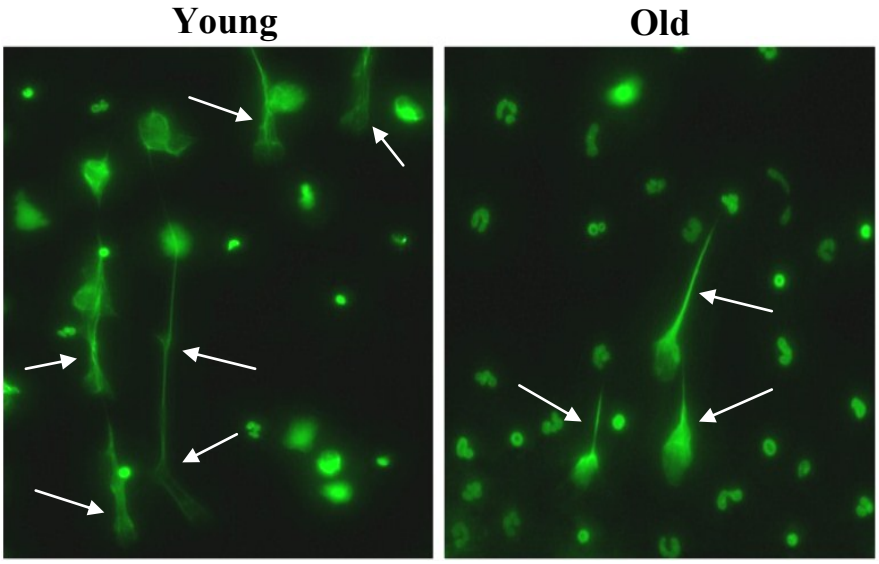
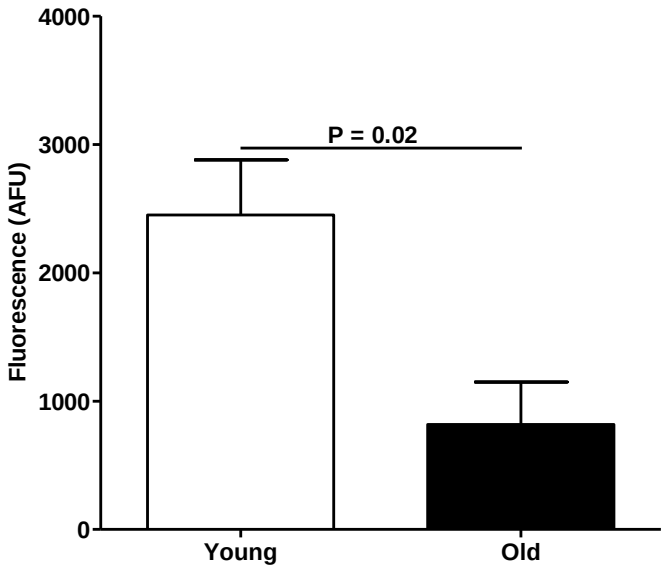



**Figure 4.12. Age-related impairment in LPS-induced NET formation. (A)** Neutrophils obtained from young (n = 8) and old (n = 11) donors were primed with 10 ng/ml TNF-α prior to a 3-hour treatment with 100 ng/ml LPS. Post stimulation, the DNA content of cell-free supernatants was assessed by fluorometry. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, and represents the mean ± SEM. **(B)** Immunofluorescence images of LPS-induced NET production by primed neutrophils from young and old donors. Images were taken at X20 objective and are representative of 2 independent experiments. Arrows point to regions of extracellular DNA.

**(A)**




**(B)**

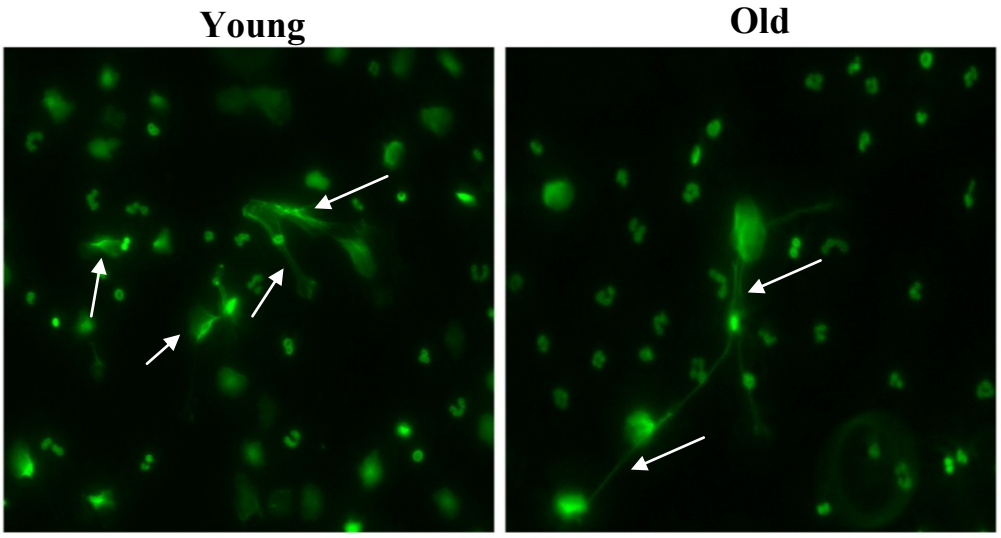
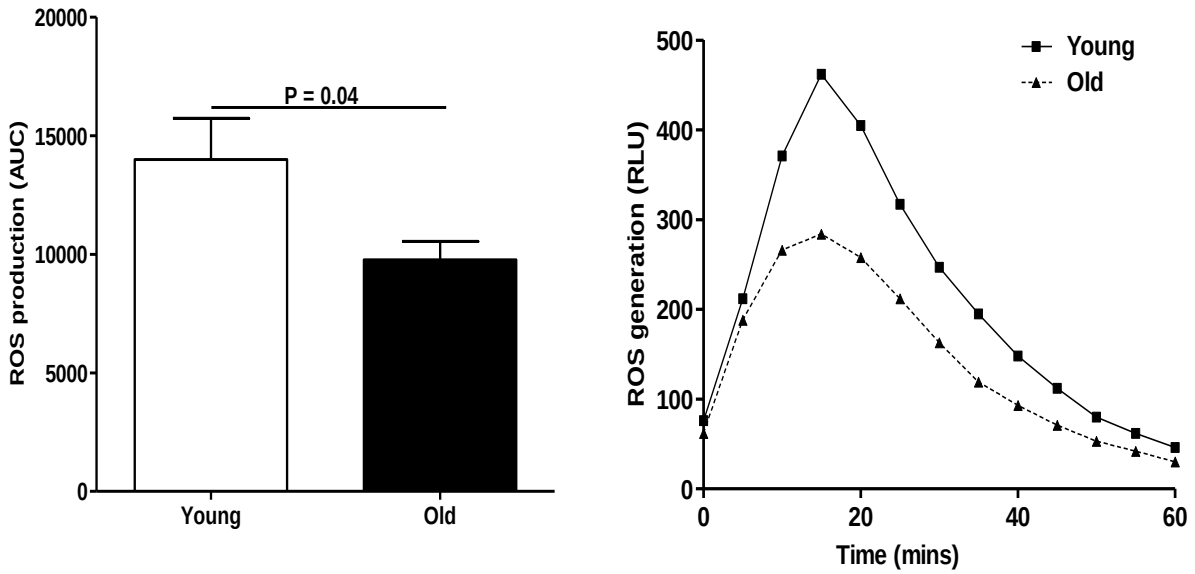



**Figure 4.13. Age-associated reduction in IL-8-induced NET formation by primed neutrophils.** Neutrophils obtained from young (n = 9) and old (n = 10) donors were primed with 10 ng/ml TNF-α prior to a 3-hour treatment with 10 ng/ml IL-8. Post stimulation, the DNA content of cell-free supernatants was assessed by fluorometry. Data are presented as arbitrary fluorescence units (AFU) after subtracting the background fluorescence of untreated controls, and represents the mean ± SEM. Group differences were assessed by a Mann Whitney U test. **(B)** Immunofluorescence images of IL-8-induced NET production by primed neutrophils from young and old donors. Images were taken at X20 objective and are representative of 2 independent experiments. Arrows point to regions of extracellular DNA.

**(A)**

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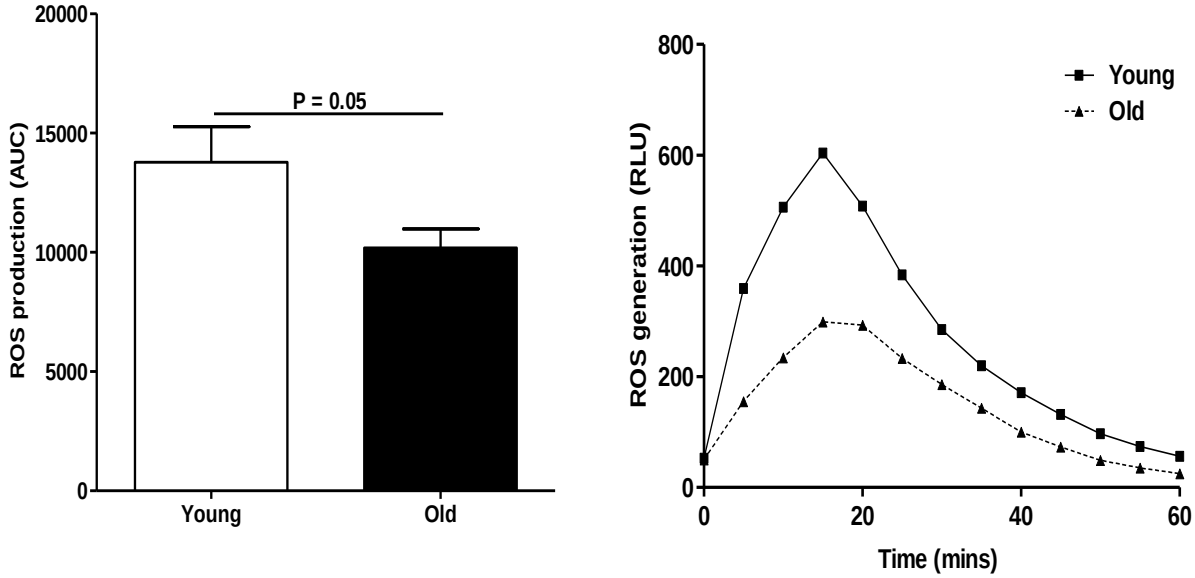



**Figure 4.14. Age-related decline in ROS production by primed neutrophils.** ROS generation by TNF-α primed neutrophils in response to 100 ng/ml LPS **(A)** or 10 ng/ml IL-8 **(B)** was measured over a 60-minute period by luminol-amplified chemiluminescence. In the left panel, ROS generation is presented as area under the curve (AUC) and represents the mean ± SEM of experiments performed on neutrophils isolated from 8 young and 8 old subjects. In the right panel, representative plots of ROS generation are shown. Group differences were assessed using an unpaired student T test. RLU, relative light units.

### 4.2.6 Simvastatin increases IL-8 induced ROS production by neutrophils from older adults but has no effect upon NET formation

It has recently been demonstrated that pre-treating neutrophils with simvastatin, an inhibitor of 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, significantly increases NET formation triggered by PMA [550]. As the only treatment regimen known to date to enhance NET generation, it was examined whether pre-treatment with simvastatin could boost IL-8-induced NET production by primed neutrophils from older adults.

Quantification of extracellular DNA in cell-free supernatants revealed statin pre-treatment had no significant effect upon NET generation by IL-8 stimulated neutrophils (Figure 4.15). However, simvastatin-treated neutrophils did produce a modest but significant increase in ROS following IL-8 challenge when compared to those that had been treated with vehicle control (Figure 4.16 A-B).

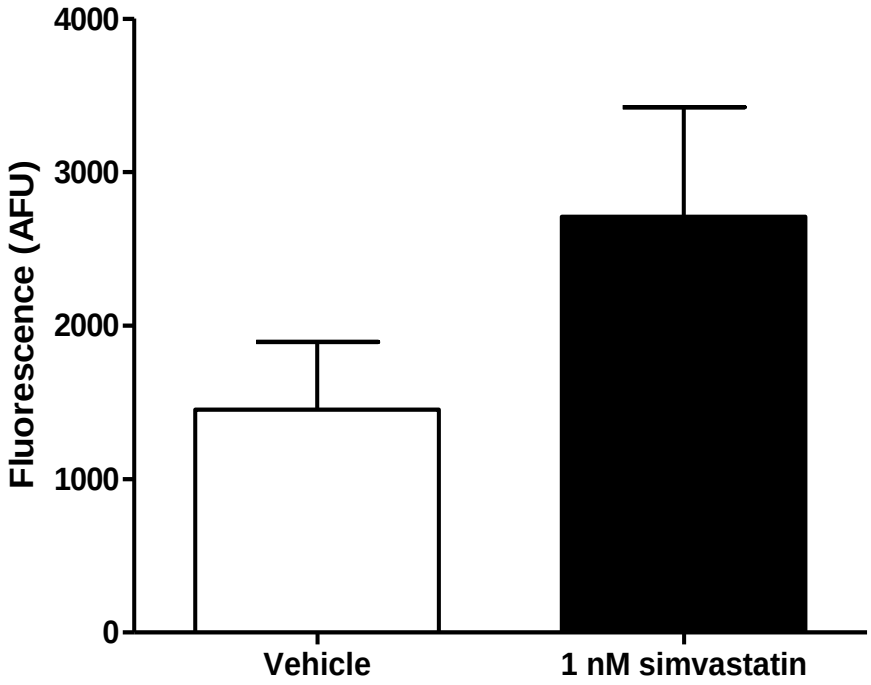
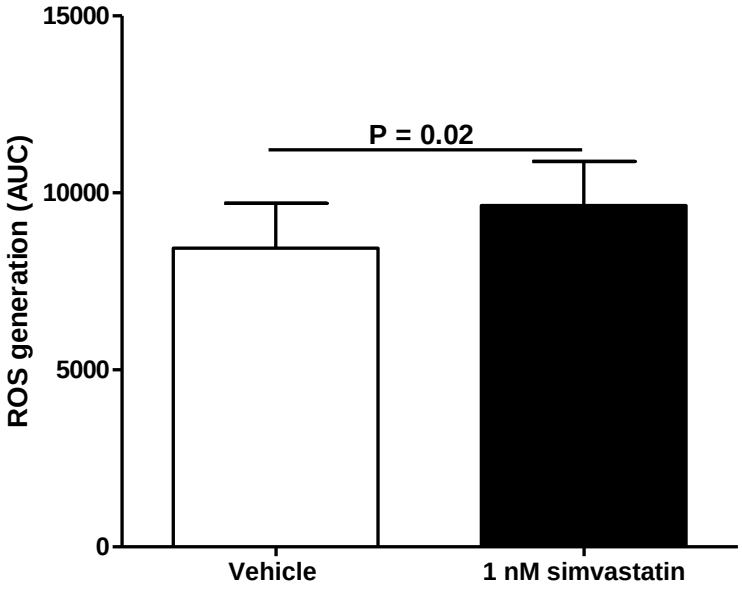



**Figure 4.15. Simvastatin treatment does not alter IL-8 induced NET production by primed neutrophils from older adults.** After a 40-minute treatment with 1 nM simvastatin or vehicle control, TNF-α primed neutrophils were stimulated for 3 hours with 10 ng/ml IL-8, after which the DNA content of cell-free supernatants was quantified by fluorometry. Data are presented as mean ± SEM of 7 independent experiments after subtracting the background fluorescence of untreated samples. Differences between conditions were assessed by a paired student T test. AFU, Arbitrary fluorescence units.

**(A)**




**(B)**

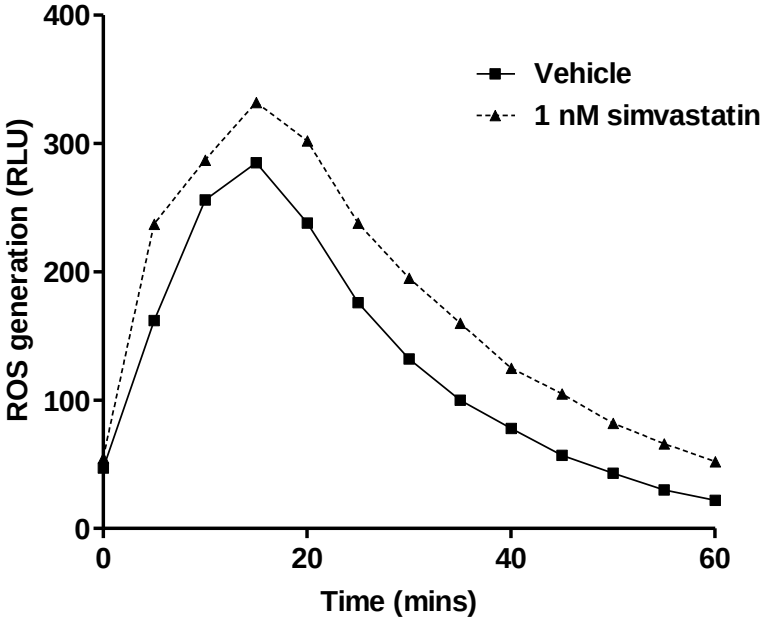



**Figure 4.16. Simvastatin enhances IL-8 induced ROS generation by primed neutrophils from older adults.** Following a 40-minute treatment with 1 nM simvastatin, TNF-α primed neutrophils were challenged with 10 ng/ml IL-8 and ROS production measured over a 60-minute period by luminol-amplified chemiluminescence. **(A)** ROS generation presented as area under the curve (AUC). Data represents mean ± SEM of 6 experiments performed on neutrophils isolated from older adults. Differences between conditions were assessed by a paired student T test. **(B)** Representative plot illustrating the increased ROS generation by simvastatin-treated neutrophils. RLU, relative light units.

## 4.3 Discussion

Consisting of a DNA backbone decorated with granule-derived peptides and enzymes, NETs are a recently identified defensive strategy of neutrophils [435;436]. Generated in response to bacterial, fungal and parasitic challenge [435;440;472;473;475], NETs, as their name suggests, are thought to play an important role in pathogen entrapment [478]. Compared to younger individuals, older adults succumb more readily to bacterial and fungal infections, which are more severe in nature and associated with greater morbidity and mortality rates [544-547]. As neutrophils represent the first-line of defence against these two types of pathogen, which are potent inducers of NET formation, the aim of this chapter was to determine whether like other forms of neutrophil defence, NET formation was altered with age.

When challenged with PMA, the most potent inducer of NET formation, neutrophils isolated from young and old donors generated comparable levels of NETs. As a stimulus that bypasses the cell membrane, this observation indicates that downstream of activation of protein kinase C (PKC), signalling molecules involved in NET formation are not inherently altered by the ageing process. Indeed, ROS generation, which is a prerequisite for NET formation was comparable between neutrophils from young and old donors in response to PMA stimulation.

In this thesis, it was shown that upon IL-8 or LPS stimulation, resting neutrophils generated NETs. This observation is in agreement with some [435;471;486] but not all [470;551] studies that have previously investigated NET production by neutrophils treated with these inflammatory agonists. However, although NETs were generated, the response of resting neutrophils was weak. In a recent study, Demers and colleagues [552] challenged control and G-CSF-treated mice with low-dose LPS and measured levels of plasma DNA, which they

considered to be a marker of NET generation *in vivo*. Whilst an increase in plasma DNA was observed in control mice, the levels recorded in G-CSF-treated mice at both 1 and 24-hours post challenge were significantly greater, suggesting that G-CSF had sensitised neutrophils for NET formation [552]. This finding supported earlier *in vitro* work that had shown upon stimulation with the complement protein C5a, primed neutrophils generated significantly more NETs than their resting counterparts [553]. Thus, both sets of data suggest priming neutrophils strongly enhances NET production upon secondary stimulation. Indeed, in this report it was shown that short-term exposure to TNF-α prior to IL-8 or LPS treatment, significantly increased NET formation above the levels generated by their unprimed counterparts. What this and other studies have not yet addressed however is the mechanism(s) by which this occurs, although it is likely to be the result of two factors, which will now be briefly discussed.

ROS production by neutrophils is dependent upon activation of NADPH oxidase, a multicomponent enzyme system that consists of the trans-membrane protein cytochrome $b_{558}$, a cytosolic complex comprising of three proteins, namely $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, and the G protein Rac2 [554]. In resting cells, the distribution of these proteins between the membrane and cytosol ensures that NADPH oxidase resides in an inactive state. Through inducing partial phosphorylation of the cytosolic proteins $p47^{phox}$ and $p67^{phox}$ [555;556], which triggers their translocation to the cell membrane, and increasing the expression of cytochrome $b_{558}$within the plasma membrane [557], priming agents such as TNF-α promote assembly of the NADPH oxidase complex at the neutrophil cell membrane, leading to enhanced ROS generation when these cells are exposed to a second stimulus. Indeed, when compared to their resting counterparts, a 2.2 and 2.1-fold increase in ROS generation by TNF-α primed neutrophils following LPS and IL-8 challenge respectively was seen here. As

ROS production is a key event in NET generation [443;462;463], this priming-induced increase in ROS generation may be one mechanism by which to explain the greater NET formation by primed neutrophils. Another possibility is that priming initiates the process of NET formation. In a recent paper, Wang and co-workers reported that TNF-α stimulation of human neutrophils for 15 minutes, the time-frame we used for neutrophil priming, resulted in the hypercitrullination of histone H4, a modification that is thought to drive chromatin decondensation [458;459]. Therefore, it would appear that short-term stimulation with TNF-α is sufficient to activate PAD4, the enzyme that catalyses histone citrullination and whose activity is essential for NET production [458]. Thus, it is reasonable to suggest that the process of NET formation may have already been underway in TNF-α primed neutrophils prior to their stimulation with LPS or IL-8.

When the effect of age on NET production by TNF-α primed neutrophils was investigated, neutrophils from older adults generated significantly fewer NETs when stimulated with either LPS or IL-8 when compared to cells from young donors. Accompanying this defect in NET production was an age-associated reduction in the generation of ROS. Whilst an age-related impairment in NET formation is a novel observation, the decline in ROS generation has been reported previously. In these studies, it was shown that ROS production by GM-CSF-primed neutrophils upon fMLP stimulation was markedly reduced with age [412;433]. Thus, these findings along with the observations shown here suggest that neutrophils from elderly subjects are not primed as efficiently as those from younger adults.

As the generation of ROS is fundamental for NET formation [443;462;463], a decline in ROS production is a possible mechanism by which to explain the age-related impairment in NET generation by TNF-α primed neutrophils. However, although necessary, ROS by themselves cannot induce NET formation [458;470]. Therefore, other factors in addition to the decline

we observed in ROS production may have contributed to the age-associated impairment in NET generation. These include: defective intracellular signalling, aberrant induction of autophagy and/or histone citrullination and age-related alterations in the composition of the circulating neutrophil pool. Each of these possibilities will now be discussed.

*(i) Defective intracellular signalling*

Although neutrophils from elderly individuals failed to generate NETs as effectively as those from young controls in response to IL-8 or LPS treatment, no age-associated difference in PMA-induced NET formation was seen. Since PMA activates cells independently of membrane receptors via direct activation of PKC, this finding suggests that defective intracellular signalling proximal to the neutrophil membrane and upstream of PKC may underlie the age-related impairment observed in both ROS and NET production following IL-8 and LPS stimulation.

Neutrophils respond directly to IL-8 and LPS challenge as a result of their expression of the G protein coupled receptors CXCR1 and CXCR2, and the pathogen recognition receptor TLR4 respectively. Results of a single study have shown no changes with age in either the proportion of neutrophils expressing TLR4 or in the surface density of this receptor [412], suggesting no age-related impairment in the ability of neutrophils to detect LPS. In contrast, studies performed within our group have shown ageing to be accompanied by a significant reduction in the percentage of $CXCR1^+$ neutrophils, although no change was observed in CXCR2 expression (H. Greenwood, unpublished observations). As it is signalling through CXCR1 and not CXCR2 that triggers activation of NADPH oxidase [558], this reduced frequency of $CXCR1^+$ neutrophils may be responsible in part not only for the age-related impairment we observed in IL-8-induced ROS generation, but also NET formation given that ROS production is necessary for the generation of these structures.

The signalling pathway downstream of TLR4 consists of adaptor molecules such as MyD88 and a number of serine/threonine protein kinases [559]. When studying the response of neutrophils from young and old subjects to LPS stimulation, Fulop and colleagues noted that the quantity of MyD88 was significantly lower in the membranes of neutrophils that had been obtained from older adults [412]. Furthermore, aged neutrophils failed to recruit the protein kinase IRAK-1 into lipid rafts, a defect the group assigned to previously described age-associated alterations in the physicochemical status of the neutrophil membrane [412;425]. IRAK-1 is an upstream activator of MAPKs, which have recently been shown to play an important role in NET formation [559;560]. Thus, an age-related impairment in MAPK signalling resulting from inefficient recruitment of IRAK-1 to lipid rafts could underlie the reduction in LPS-induced NET formation with age. That said, it should be noted that TLR4-induced activation of MAPK can also occur in a MyD88 and IRAK-1 independent manner [559]. Whether this pathway is also altered in neutrophils with age is unknown.

To date, no group has investigated whether the recruitment and/or activation of signalling elements downstream of CXCR1 and/or CXCR2 are also affected by age. However, based on the concept of Fulop et al [412], which suggests age-associated changes in the composition of the neutrophil membrane would lead to impaired signalling for all surface receptors, then it is likely that IL-8 induced signalling pathways would be altered with age.

*(ii) Autophagy and histone hypercitrullination*

Based on their observation that PMA-induced NET formation was preceded by massive vacuolization and the generation of vesicles with double membranes, Remijsen et al [470] investigated whether autophagy was involved in NET production. Using the PI3-kinase inhibitor wortmannin, the group showed autophagy induction was essential for both the generation of these vesicles and NET formation [470]. Several forms of autophagy exist, and

whilst not stated by the group, the generation of double membrane vesicles suggests it is the induction of macroautophagy that is important for NET formation.

Whilst it is currently unknown as to whether IL-8 can induce autophagy in neutrophils, a recent study has shown LPS to be a potent inducer of autophagy in these cells [561]. Thus, did an age-related impairment in autophagy induction contribute to the decline in NET production observed with age following LPS stimulation? To answer this question it first has to be determined whether macroautophagy is required for LPS-induced NET formation since Remijsen and co-workers only studied this phenomenon in the context of PMA treatment [470]. If it is, then interestingly there is evidence, albeit in non-immune cells, that has shown the rate of macroautophagy declines with increasing age [562;563]. Since this thesis found no age-related difference in PMA-induced NET production, this would suggest that the machinery that drives autophagy is not inherently defective in neutrophils from older adults. Therefore, any impairment in autophagy induction with age would most likely be borne from aberrant intracellular signalling proximal to the neutrophil membrane. On this note, the activation of signalling molecules that regulate macroautophagy such as MAPKs have been shown to be impaired in neutrophils from older adults following ligation of cell surface receptors [432;564], which suggests the induction of macroautophagy may not be as efficient in neutrophils from aged donors when compared to that of their younger counterparts.

Alongside ROS generation and the induction of autophagy, hypercitrullination of histones H3 and H4 is a critical event in NET generation. Catalysed by the calcium-dependent enzyme PAD4, this post-translational modification of histones is thought to drive chromatin decondensation [459]. Several studies have demonstrated that following receptor ligation, neutrophils from older adults exhibit impaired calcium mobilisation [418;565], which has been attributed to an age-related reduction in the generation of the calcium mobilising second

messenger $IP_3$ [424]. Given the importance of calcium in triggering and sustaining PAD4 activity, it is reasonable to think that a defect in its mobilisation would adversely affect PAD4 function, a consequence of which would be a reduction in histone citrullination and chromatin decondensation. To test this proposal, initial studies could examine by Western blotting the degree of histone H3 and H4 citrullination in primed neutrophils from young and old subjects following IL-8 and LPS stimulation.

*(iii) Age-associated alterations in the composition of the circulating neutrophil pool*

Evidence is emerging that suggests neutrophils are a heterogeneous population of cells. For instance, in mice, three neutrophil subsets with unique functional and phenotypic characteristics have been described [394], whilst in humans, neutrophil heterogeneity based on differential surface expression of CD16 has been reported [566]. Interestingly, *in vitro* studies have shown that not all neutrophils generate NETs upon stimulation [443], with one group suggesting that as little as 20% are capable of performing this function [567]. Could it be that NET production is a feature of a distinct neutrophil subset? Currently, no study has attempted to answer this question, but if we assume that it is, then maybe the age-related impairment in NET formation was the result of an age-associated reduction in the frequency of neutrophils capable of generating NETs.

Results of two recent studies have challenged the long-standing notion that NETs exhibit direct microbicidal activity. Unlike previous groups who cultured micro-organisms with resting neutrophils or neutrophils that had been pre-treated with NET-inducing stimuli, and assumed that the reduced number of micro-organisms they recovered from the latter culture was the result of NET-based killing, both Menegazzi et al [447] and Parker and colleagues [445] released micro-organisms that during culture had become entangled within NETs before assessing their viability. Using this approach, neither group found evidence of NET-

mediated killing. Based on these observations, it has been suggested that rather than eliminating pathogens directly, NETs serve to merely contain pathogens at the infection foci [447;448]. In support of this claim, two independent groups have shown that *in vivo* depletion of NETs via exogenous DNase treatment significantly enhances bacterial dissemination [478;479]. Thus, if mirrored *in vivo*, the age-related decline in NET formation could prevent elderly individuals from effectively confining pathogens to the initial site of infection.

In recent years, evidence has emerged from epidemiological studies that suggests the use of statins, a family of HMG-CoA reductase inhibitors widely prescribed for the treatment of hyperlipidemia, may be beneficial for the treatment and prevention of bacterial infections. In the first study of its kind, Liappis and colleagues investigated retrospectively the association between statin therapy and mortality arising from bacteraemia in hospitalised older adults, and found increased survival rates amongst those individuals who had received statin treatment [568]. That statins reduce infection-associated mortality has since been reported by several other groups [569-571], whilst evidence can also be found to suggest that statin use reduces the risk of contracting community-acquired pneumonia [572]. From these data it has been proposed that alongside their lipid-lowering properties, statins exert immunomodulatory effects. Interestingly, in a recent study, Chow et al reported that *in vitro* statin treatment could directly influence the bactericidal capacity of neutrophils by enhancing their generation of NETs [550]. Based on this observation it was investigated whether pre-treating neutrophils from old donors with simvastatin could increase NET production upon subsequent exposure to IL-8. In contrast to the result of Chow et al [550], no difference in NET formation between vehicle and simvastatin-treated neutrophils treated with IL-8 was found. Inter-study differences in experimental design are likely to be responsible for these contrasting observations, the most significant being the dose of simvastatin used. Whilst in this study

neutrophils were treated with 1 nM simvastatin, which is akin to the concentrations present in the circulation of patients undergoing standard statin therapy (20-40 mg per day; Dr. Elizabeth Sapey, personal communication), Chow et al [550] pre-treated cells with 10 μM simvastatin, which represents a supraphysiological concentration. Other differences in experimental set-up include the length of simvastatin treatment (40 minutes Vs. 1 hour), the source of neutrophils (old Vs. young adults) and the stimuli used to trigger NET formation (IL-8 Vs. PMA). Another caveat is that due to time constraints, data presented in this thesis was collected from a small cohort of only seven donors, meaning it is possible that the study was under powered.

Although this study found statin treatment had no significant effect upon NET production, a small but significant increase in ROS production was seen in simvastatin-treated neutrophils in response to IL-8 stimulation. As mentioned above, ROS generation by neutrophils is dependent upon activation of the multicomponent enzyme system NADPH oxidase, which is mediated primarily through phosphorylation of its cytosolic and membrane components [554]. One kinase that is known to be involved in NADPH oxidase activation is PKC, whose phosphorylation of the trans-membrane protein cytochrome $b_{558}$ has been shown to be important for promoting the assembly of NADPH oxidase at the neutrophil plasma membrane [573]. Interestingly, statins have been reported to significantly increase PKC activity [574]. Thus, a possible mechanism that could explain how pre-treatment with simvastatin enhanced IL-8-induced ROS production is that prior to IL-8 challenge, simvastatin-treated neutrophils had already begun to assemble NADPH oxidase at the plasma membrane.

# Chapter 5

# General Discussion

# 5.0 General Discussion

## 5.1 Mechanism(s) underlying the age-associated reduction in NKCC

When this project was conceived, it had been known for many years that physiological ageing was accompanied by a marked reduction in NK lytic activity at the single cell level [42;365;371]. However, no group had identified the underlying cause(s), although from the results of a small number of studies it was known that the defect lay downstream of NK-target cell interaction [365;372;374]. In this thesis, it has been shown that upon target cell contact, NK cells from older adults release less perforin into the IS. This defect resulted in reduced perforin binding to the target cell surface, an event that was found to correlate strongly with target cell lysis. Thus, it is proposed that an age-related impairment in perforin secretion into the IS mediates the decline in NKCC that accompanies physiological ageing.

From the findings presented in this report, the age-related decline in perforin secretion can be attributed to defective polarisation of lytic granules to the NK-target cell interface, and the data suggest that this results in part from delayed activation of the MAPK, ERK1/2, which plays a critical role in the mobilisation and polarisation of secretory lysosomes [263]. However, the fact that no age-associated difference was seen in the number of granules that had fused with the NK cell plasma membrane despite an impairment in granule polarisation in NK cells from older adults, suggests that another mechanism may contribute to the decline in perforin secretion. As discussed earlier, an intriguing possibility is that ageing is also associated with a reduction in the activity of ASMase, as this enzyme, which catalyses the hydrolysis of sphingomyelin to ceramide, has been shown to play a crucial role in the secretion of cytolytic effector molecules into the IS [541]. Interestingly, impaired activation of both ERK1/2 and ASMase could occur as a result of an age-related defect in a single signalling molecule: PKC.

PKC is a large family of serine-threonine kinases consisting of 11 isoenzymes that are classified into three subfamilies: classical PKCs, which include PKC α, βI, βII and γ, novel PKCs, which are represented by PKC δ, ε, η and θ, and the atypical PKCs to which PKC ζ and ι belong [575;576]. These subfamilies are distinguished from one another by the co-factors that regulate their activity. For example, whilst classical PKCs require calcium and diacylglycerol (DAG) to function, novel PKCs are regulated entirely by DAG [575;576]. In contrast, atypical PKC isoenzymes possess no calcium or DAG binding domains, and instead are thought to be regulated through protein-protein interactions and phosphorylation [575]. Several studies have demonstrated that upon binding to K562 target cells, NK cells generate significant amounts of DAG and the calcium mobilising second messenger $IP_3$[577-580], suggesting a role for PKC signalling in natural cytotoxicity. Indeed, the addition of broad-acting PKC inhibitors to NK-K562 co-cultures significantly reduces target cell lysis [581]. Importantly, in the context of the findings presented in this thesis, it seems that PKC is part of the signalling cascade that triggers the secretion of cytotoxic molecules by NK cells, since PKC inhibition has been shown to dramatically reduce PMA-induced granule exocytosis [582]. NK cells express the PKC isoenzymes, PKC α, β, δ, ε, θ and ζ [583-585]. With respect to the work presented here, PKCδ is particularly interesting given that it has been shown to not only modulate ERK1/2 activity [586] but to also bind, phosphorylate and activate ASMase [587;588]. Thus, the delayed kinetics in ERK1/2 phosphorylation that were observed in NK cells from older adults following stimulation with plate-bound anti-NKG2D and NKp30 antibodies, and the decrease in ASMase activity that is proposed may occur with age could both be explained by an age-related reduction in PKCδ activity. Importantly, studies have already been performed that have shown signalling events leading to PKC activation are impaired in NK cells from older adults. In an elegant study, Mariani and colleagues demonstrated a marked age-related decrease in the ability of NK cells to generate

$IP_3$ following K562 stimulation, which was borne from a delay in phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis [373]. As DAG is also a product of PIP2 breakdown, one would assume that the levels of this second messenger are also lower in K562-stimulated NK cells from older adults. As a novel PKC, PKCδ requires binding of DAG to its regulatory domain in order to become activated [575;576]. Thus, it is reasonable to suggest that the activity of PKCδ may be lower in NK cells from older subjects following K562 stimulation. Given that PKCδ lies upstream of ERK1/2 [586], then a reduction in its activity could contribute to the age-related impairment we observed in the delivery of lytic granules to the NK-target cell interface since ERK signalling is essential for lytic granule mobilisation and polarisation [263]. Furthermore, as ASMase is activated by PKCδ–mediated phosphorylation [587;588], reduced PKCδ activity would lead to a decline in ASMase activity.

When NK cells were stimulated with PMA and ionomycin, which mimic the signals provided by DAG and $IP_3$ respectively, no age-related difference in either perforin release or in the kinetics of ERK1/2 phosphorylation were found. These findings are similar to those of Ghoneum and co-workers who reported PMA treatment could correct the defective cytolytic activity of NK cells from aged mice [589]. As both PMA and ionomycin bypass cell-surface receptors to activate PKC, these two sets of data support the previously described work of Mariani et al [373] by suggesting that signalling events that precede PKC activation are impaired with age. As reported for other cells types [65;425], age-related alterations in the lipid composition of the NK cell plasma membrane may be responsible for these proximal signalling defects. Present within the plasma membrane are specialised microdomains called lipid rafts. Enriched for sphingolipids and cholesterol, lipid rafts fuse together to form large signalling platforms that assist in signal transduction by serving as a site at which signalling complexes interact. Interestingly, ceramide, which is generated predominantly by ASMase-

mediated hydrolysis of sphingomyelin, drives the fusion of lipid rafts into large platforms. Since ASMase is a PKCδ activated enzyme [587;588], the decline in PKCδ activity that may accompany physiological ageing would lead to impaired activation of ASMase, a consequence of which would be reduced production of ceramide at the plasma membrane. This would affect the efficiency of lipid raft fusion, leading to impaired signal transduction upon ligation of activatory receptors.

Bringing together the data discussed in the above two paragraphs, it is proposed that impaired activation of PKCδ following target cell binding could be responsible for the age-related decline in NKCC. The result of delayed PIP2 hydrolysis following ligation of activatory receptors, reduced PKCδ activity could explain both the delayed kinetics of ERK1/2 phosphorylation that were observed in NK cells from older adults following stimulation with plate bound antibodies, as well as the age-associated impairment we noted in ERK1/2-dependent polarisation of lytic granules to the IS, as c-Raf, an upstream activator of ERK1/2 is a direct target of PKCδ [586]. Furthermore, a reduction in PKCδ activity could explain our rather intriguing finding that despite comparable granule fusion and perforin expression, NK cells from older subjects released less perforin into the IS when compared to their younger controls.

To provide support for this theory it will be necessary to show that PKCδ activity is reduced in stimulated NK cells from older donors. Whilst the PMA and ionomycin data suggest that signalling pathways that trigger PKC activation upon target cell binding are impaired with age, more direct evidence is needed. As translocation of PKCδ from the cytosol to the plasma membrane is a hallmark of its activation, comparing the location of this kinase in stimulated NK cells from young and old subjects would be a useful starting point.

When the expression of the early activation marker CD69 on the surface of young and old NK cells following K562 stimulation was compared, no age-related difference was observed. Interestingly, Borrego and co-workers have shown that PKC signalling is heavily involved in K562 cell-induced expression of CD69 [590]. Thus, if impaired activation of PKC underlies the age-related decline in NKCC, then why was surface CD69 expression not reduced in NK cells from older adults? As mentioned earlier, NK cells express classical, novel and atypical PKC isoenzymes [583-585], which differ in the co-factors that regulate their activity [575;576]. From the work of Mariani et al, which showed an age-related delay in PIP2 hydrolysis [373], one would assume that the activity of both classical and novel PKCs is reduced in K562 stimulated NK cells from older adults since the PIP2 products IP3 and/or DAG are required for their activation [575;576]. However, atypical PKC isoenzymes possess no calcium or DAG binding domains, and as such their activity may be comparable in young and old NK cells. Which PKC isoenzyme is responsible for driving CD69 expression on the surface of K562-stimulated NK cells is unknown since Borrego et al used a broad acting PKC inhibitor in their experiments [590]. If we assume that PKCζ, the atypical PKC expressed by NK cells, is responsible then this may explain why no age-related decline in CD69 expression was seen, though this is pure speculation at this point.

A feature of physiological ageing is the accumulation of senescent cells. These cells, which have been detected in such tissues as skin [591], bone [592] and endothelium [593] reside in a state of irreversible cell cycle arrest, yet remain metabolically active, secreting an array of growth factors, pro-inflammatory cytokines (e.g. IL-6 and IL-8) and proteases (e.g. matrix metalloproteinases). In light of these functional properties, the sites at which they reside and the fact that they accumulate with age, senescent cells have been suggested to contribute to the development of a number of age-related pathologies by compromising tissue homeostasis

and function [594]. Recently, Baker and colleagues [595] tested this proposal by using an innovative transgenic strategy that enabled them to specifically eliminate senescent cells from tissues in which age-associated pathologies develop. In a group of young mice, life-long removal of senescent cells was shown to delay the onset of such age-related conditions as sarcopenia and cataracts [595]. More striking however was the fact that the elimination of senescent cells in older mice, who were already displaying ageing traits, attenuated the subsequent age-associated loss of muscle mass and fat tissue that was observed in littermate controls [595]. Thus, it seems that senescent cells can be assigned a causative role in the development of a number of age-associated pathologies. Interestingly, Sagiv and co-workers have recently suggested that via perforin-mediated cytotoxicity, NK cells eliminate senescent cells *in vivo* [596]. In a murine model of liver fibrosis, a setting in which the removal of senescent cells is important to limit damage and facilitate repair [597], the group found an increased frequency of senescent cells in the livers of perforin deficient mice when compared to their wild type counterparts, which was accompanied by greater tissue scarring and extracellular matrix deposition [596]. Whilst the use of perforin deficient mice is not the best model in which to assign a direct role for NK cells in senescent cell clearance, given that both iNKT cells and $CD8^+$ T cells also use this form of cytotoxicity, Sagiv et al did show *in vitro* that through the granule exocytosis pathway, primary human NK cells elicit potent cytotoxicity towards senescent fibroblasts [596]. Given that this thesis has reported a marked age-related decline in this form of NK cell-mediated defence, could it be that this impairment contributes to the increased frequency of senescent cells found in aged tissue? A simple study that could be undertaken to begin testing this hypothesis would be to perform the *in vitro* cytotoxicity assays described by Sagiv et al [596] using NK cells from young and old donors and comparing the efficiency by which senescent cells are lysed. Should an age-related impairment be found, it would be of interest to move to their *in vivo* model of liver fibrosis

and compare the rate of senescent cell clearance between cohorts of young RAG1$^{-/-}$ mice into which NK cells from young or aged mice had been adoptively transferred.

In a recent study, Bigley and co-workers showed that infection with CMV has a marked impact on the composition of the circulating NK cell pool [364]. When compared to their seronegative counterparts, subjects infected with CMV were found to have a lower frequency of KLRG1$^{+}$/CD57$^{-}$ NK cells and a higher proportion of KLRG1$^{-}$/CD57$^{+}$ NK cells [364]. These observations mirrored previously described age-related changes in NK cell phenotype [42;352;354], yet the study was performed entirely on samples from young donors, leading the group to propose that the changes in NK cell phenotype that had previously been assigned to the ageing process may have in fact been driven by CMV [364]. With this in mind, it was investigated whether donor CMV status influenced the novel finding of this thesis: the age-related decline in perforin release upon K562 cell contact. Whilst statistical analysis revealed age to be the main determinant for reduced perforin release [$(F1,15) = 8.03$, $p = 0.013$, $\eta^2 = 0.349$], a trend for CMV positive individuals to release less perforin upon K562 stimulation was found [$(F1,15) = 4.21$, $p = 0.058$, $\eta^2 = 0.219$] (Figure 5.1). This observation, albeit not statistically significant, is in line with previous reports that have shown interaction with CMV reduces the cytotoxic capacity of human NK cells [598;599]. However, unlike these studies, where NK cells were cultured *in vitro* with HCMV-infected cells prior to the analysis of NKCC, the findings of this thesis were obtained using NK cells whose cytotoxicity was examined immediately following isolation, suggesting their lytic nature had been altered *in vivo*.

An emerging concept within the NK cell field is that in addition to mediating direct lysis of transformed cells, NK cells play an important immunoregulatory role in times of viral infection. Murine-based studies have shown that via perforin-dependent cytotoxicity, NK

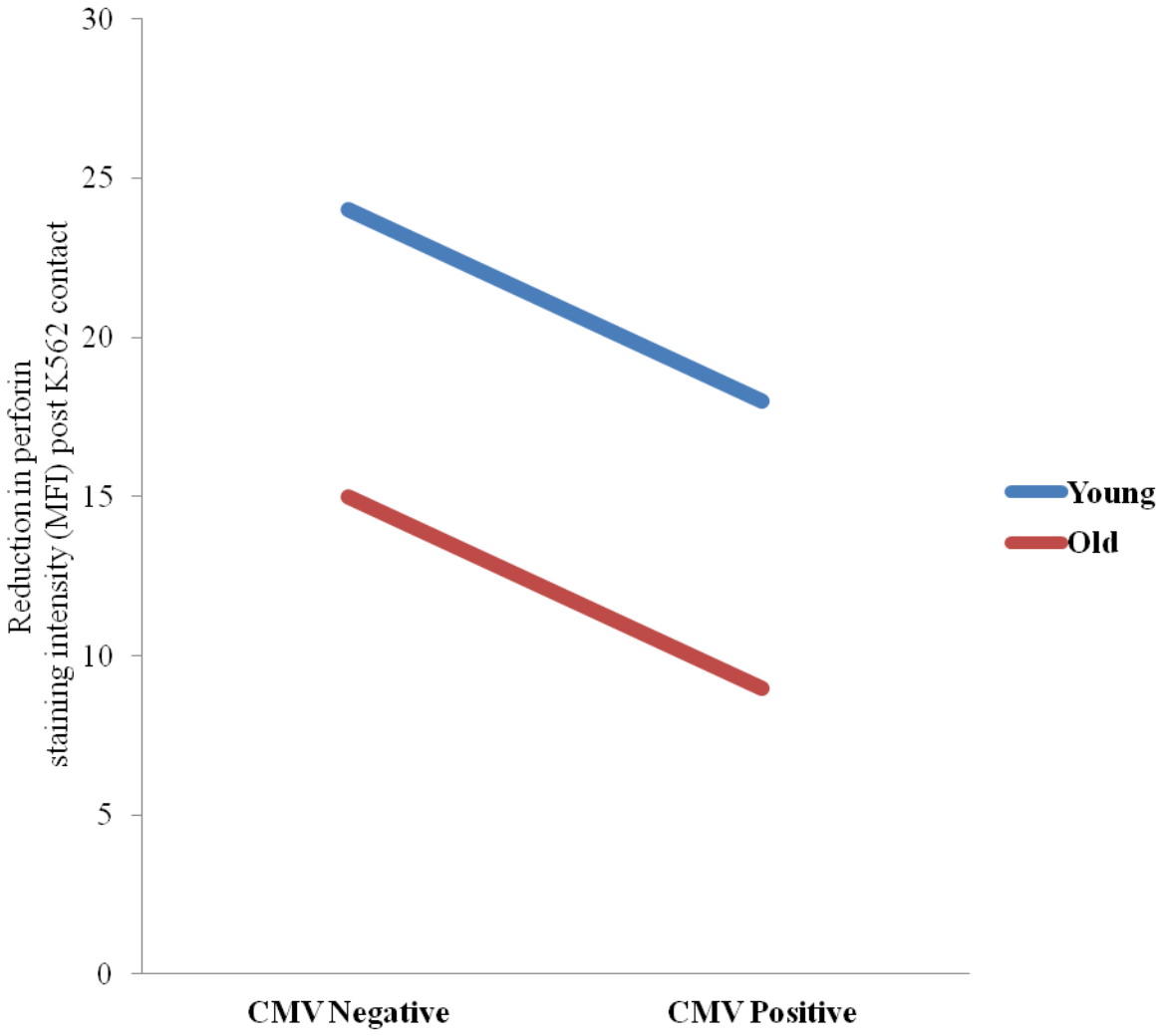



**Figure 5.1 Effect of age and CMV status on perforin release by NK cells following K562 stimulation.**

cells negatively regulate virus-specific $CD8^+$ T cell responses either through directly eliminating these cells [600] or by removing immune cells that assist in $CD8^+$ T cell responses such as DCs and $CD4^+$ T cells [601;602]. Although not a view shared by all groups [600], it is thought that by limiting T cell immunity, NK cells promote host survival by preventing excessive immune-mediated pathology [601]. As a persistent virus that undergoes continual low-level replication, CMV requires life-long immunological control. Therefore, could it be that in CMV positive individuals, NK cells are having to constantly regulate on-going CMV-specific T cell responses, a consequence of which could be their functional exhaustion? Commonly observed in virus-specific $CD8^+$ T cells in settings of chronic viral infection, functional exhaustion is characterised by a loss of cell function. Interestingly, functionally exhausted $CD8^+$ T cells exhibit reduced perforin-mediated cytotoxicity *ex vivo* [603]. Thus, maybe the trend observed here for reduced perforin secretion by NK cells from CMV seropositive donors was due to an increased frequency of functionally exhausted NK cells within the peripheral pool of these subjects. In T cells, functional exhaustion is associated with increased expression of the inhibitory receptors programmed death 1 and T-cell immunoglobulin domain and mucin domain 3 [603;604]. Comparing the expression levels of these receptors on NK cells isolated from CMV positive and negative donors would be a quick and easy approach by which to begin testing whether CMV infection is associated with functional exhaustion in NK cells.

Another mechanism that is worth considering with respect to how CMV may influence NKCC is based around the concept of NK cell licensing. This phenomenon dictates that to become functionally competent, NK cells must express on their surface inhibitory receptors that actively engage self-MHC class I molecules. Indeed, numerous studies have shown NK cells isolated from MHC class I deficient hosts are hyporesponsive to stimulatory signals

[288-291], a trait shared by NK cells that lack inhibitory receptors specific for self-MHC class I molecules [288;292]. For many years, it was thought that NK cells were licensed during their development in the BM, and that when released into the periphery their functional capacity was fixed. However, the findings of two recent studies suggest that changes in the peripheral MHC environment can alter the responsiveness of mature circulating NK cells [296;297]. Of particular interest is the work of Joncker and colleagues, which demonstrated that, when transferred into MHC class I deficient mice, mature responsive NK cells from wild type mice became hyporesponsive, suggesting that NK cells must continually sample self-MHC class I molecules in order to retain their reactivity [297]. As a persistent virus, CMV possesses several immune evasion strategies, one of which is to down-regulate expression of MHC class I molecules from the surface of infected cells [605;606]. Thus, it is reasonable to consider the possibility that due to CMV-mediated alterations in the MHC class I environment, a greater proportion of circulating NK cells may be hyporesponsive in CMV positive donors, which may explain our observation for a trend towards reduced perforin secretion by NK cells from CMV positive individuals upon target cell stimulation. This proposal may also extend to other persistent viruses such as EBV and VZV, which like CMV, reduce cell surface expression of MHC class I molecules [607;608]. It must be said however that whilst environmentally-induced alterations in NK cell responsiveness can be detected *in vitro* [296;297], its significance *in vivo* is unclear given the recent work of Orr et al, which revealed that in times of infection, "unlicensed" NK cells play a more important role in host protection than their licensed counterparts [298].

### 6.2 Ageing and NET formation

NETs were discovered in 2004 by Brinkmann and colleagues [435], who noted that when challenged with pro-inflammatory cytokines or bacteria, neutrophils released into the

extracellular environment chromatin fibres decorated with granule-derived peptides and enzymes [435]. Since this seminal paper, our understanding of how NETs are formed, their constituents and the stimuli that trigger their generation has grown considerably [465]. However, as a relatively novel field, many aspects of NET biology require more in depth analysis. For instance, there is a need to: (i) further define the mechanisms that govern NET formation, (ii) determine the fate of microbes that become entangled within NETs and (iii) gain a greater understanding of the contribution NETs make to host defence *in vivo* [448].

When first described, NETs were thought to confer protection through pathogen entrapment and direct killing. However, based on the recent observations of Parker et al [445] and Menegazzi and colleagues [447], who both found that pathogens entangled within NETs remained viable, current opinion within the field is that NETs serve only to contain pathogens to the initial site of infection. Thus, could the age-related decrease we observed in NET formation contribute to the more invasive infections experienced by older adults? Results of a study that came to our attention whilst this discussion was being written suggest that this is a possibility. After observing *in vitro* that in response to S. aureus stimulation, neutrophils from aged mice generated significantly fewer NETs than those isolated from younger littermates, Tseng et al [609] examined its biological relevance in a murine model of skin infection. Staining of infected skin tissue obtained from young mice revealed the presence of NETs at the edge of the infection foci, with very few bacteria beyond the initial site of inoculation [609]. In contrast, staining of skin tissue obtained from aged mice, who by day three post-infection exhibited a significantly increased systemic bacterial burden, revealed a disorganised and much smaller meshwork of extracellular DNA, which had been penetrated by many bacteria [609]. Based on these findings, the group proposed that whilst unlikely to

be solely responsible, an age-associated impairment in NET generation contributes to the increased incidence of invasive bacterial diseases reported by older adults [609]. Our observation, albeit *in vitro*, that NET formation by neutrophils from elderly humans is also reduced provides some support to this claim.

When concluding their findings, Tseng and colleagues suggested that gaining a greater understanding of the mechanisms that underlie the age-related decrease they observed in NET formation could lead to the development of therapeutic strategies to enhance NET production in aged hosts [609]. Whilst appealing, attempting to enhance NET formation *in vivo* is a strategy fraught with danger. For instance, as well as promoting thrombosis [610] and directly inducing epithelial and endothelial cell death [446], NETs have been linked to the pathology of several inflammatory disorders [485;611] and proposed to contribute to the development of certain autoimmune conditions [480;487;488].This latter association between NETs and autoimmunity is particularly pertinent in the context of trying to enhance NET formation in older adults, given that ageing has been shown to be accompanied by an increase in the circulating levels of auto antibodies against the two major constituents of NETs: DNA and histones [612-614].

Although a characteristic feature of the innate immune response, NET generation has recently been reported to influence the activity of adaptive immune cells. In a series of *in vitro* assays, Tillack and colleagues [451] demonstrated that through a contact-dependent mechanism, NETs directly prime $CD4^+$ T cells, thereby reducing their activation threshold. Indeed, when compared to T cells that had been cultured with resting neutrophils, the functional response elicited by NET-primed T cells was significantly greater not only in times of antigenic specific challenge but also following sub-optimal stimulation [451]. Thus, whilst not directly microbicidal, NETs may promote pathogen clearance by increasing the activity of bystander

immune cells. Given the age-related reduction in NET generation presented in this thesis and by Tseng et al [609], then NET-mediated T cell priming may not be as efficient in older adults, further exacerbating the well-documented dysregulation in T cell function that accompanies physiological ageing [57;58;61;62].

Despite the fact that numerous studies have examined the effect of human ageing on neutrophil function, only one group has performed a longitudinal-based study to determine the effect that age-associated changes in neutrophil behaviour have on human health [411]. Thus, in an effort to establish whether a direct link exists between neutrophil immunesenescence and the increased incidence of infection reported by older adults, retrospective and prospective studies should be at the forefront of future research. In the context of this thesis, in which it was demonstrated for the first time that physiological ageing in humans is accompanied by a marked reduction in NET formation, a longitudinal study that enrols both young and older adults would be of particular interest as its results would provide an insight into whether the increased susceptibility older adults exhibit towards bacterial and fungal infection [544-547], two pathogens that are potent inducers of NET formation, is attributable in any way to the age-related decline in NET generation we observed *in vitro*.

# CHAPTER 6

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## Appendix I – Primary papers and review articles published during the completion of this PhD

1. **Hazeldine J**, Hampson P and Lord JM. Reduced release and binding of perforin at the immunological synapse underlies the age-related decline in natural killer cell cytotoxicity. Aging Cell, 2012. October; 11(5) 751-759.

2. Wang K*, Hampson P*, **Hazeldine J**, Krystof V, Strnad M, Pechan P and Lord JM. Cyclin-dependent kinase 9 activity regulates neutrophil spontaneous apoptosis. PLoS One. 2012;7(1):e30128. Epub 2012 Jan 19.

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3. Hampson P*, **Hazeldine J*** and Lord JM. Neutrophil apoptosis and its induction as a potential treatment for chronic inflammatory disease. Curr.Opin.Heam. (In press)

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4. **Hazeldine J**, Arlt W and Lord JM. Dehydroepiandrosterone as a regulator of immune cell function. J.Steroid.Biochem.Mol.Biol. 2010 May 31;120(2-3):127-36.