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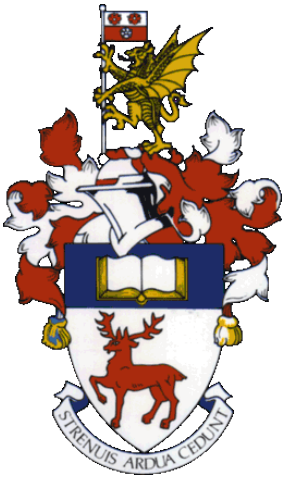

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Volume 1 of 1

Maturation and function of natural killer cells during aging

by

Serena Martelli

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

UNIVERSITY OF SOUTHAMPTON

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MATURATION AND FUNCTION OF NATURAL KILLER CELLS DURING AGING

By Serena Martelli

Current population aging is without parallel in human history and it brings significant socio-economic and political challenges with it. The immune system is profoundly affected during aging, a process termed immunosenescence. A hallmark of aging is the increased susceptibility to infections and cancer. Since Natural Killer (NK) cells play a critical role in immune-surveillance against virally-infected and transformed cells, a clearer understanding of the key players in NK cell maturation might help to better design innovative therapeutic strategies relevant to aging and other conditions of persistent immune activation, such as chronic infections and cancer.

The aim of this thesis is to detail the age-associated alterations in human and murine NK cell subset repartition, maturation, phenotype, transcriptional regulation and effector functions. We compared the phenotype and function of human NK cell subtypes using three models of persistent immune activation: aging, cytomegalovirus (CMV) infection and hepato-cellular carcinoma (HCC). This study established that CD57, NKG2C and TIM-3 were hallmarks of NK cell immunosenescence and that acquisition of these markers correlated with CMV and inflammation status of the donors. Mature NK cells gained poly-functionality but lost cytotoxicity potential in older donors and their functionality was, at least partially, regulated by the TIM-3/Ceacam-1 pathway. Also, work demonstrated that HCC progression was associated with tumor infiltration of exhausted and cytotoxic-deficient NK cells expressing CD57, TIM-3 and Ceacam-1. Clinical stages of HCC could be segregated according to co-expression of Ceacam-1 and TIM-3.

Moreover, we sought to expand the knowledge on how aging impacts NK cell differentiation and function in murine models, such as C57BL/6 wild-type mice and *Timp-3* KO mice. We demonstrated that, in aged C57BL/6 wild-type mice and aged *Timp-3* KO mice, NK cells are reduced in frequency and numbers and exhibit an altered phenotype in the blood and spleen. Investigating the expression of a variety of cell surface markers associated with the maturation process, we showed that aging is characterized by an accumulation of immature NK cells coupled with a reduction in the late differentiated subset. This phenotypic immaturity reflected a relevant functional immaturity. Our results showed that cytokine secretion, cytotoxicity and gene expression of NK cells are modulated by the aging process along a maturation pathway defined by CD11b and CD27 and, in

some cases, LY49H and KLRG1. In particular, NK and T cells from older *Timp-3* KO mice experienced the same qualitative age-related changes as the lymphocytes from the wild-type counterparts; however, the kinetic of these changes was accelerated in old *Timp-3* KO animals, resulting in earlier NK and T cell immunosenescence. These data offer new insights into TIMP-3 biological role in adaptive and innate immunity, especially its importance during the aging process.

This project deepened our understanding of human and murine NK cell differentiation and functionality during aging, leading to novel insights into age-related dysfunctions in NK cell responses and innate immunosenescence.

Our findings could support the identification of new immunological targets for checkpoint blockade therapies in order to rescue early innate defense upon aging, chronic infections and cancer.

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DECLARATION OF AUTHORSHIP

I, Serena Martelli, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Maturation and function of natural killer cells during aging

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:
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   Martelli S., Pender S.L.F., Larbi A. “Compartmentalization of immunosenescence: a deeper look at the mucosa”. Biogerontology 2016, 17:159-176

Signed: .....

Date: .....

Acknowledgements

"It is imperfection - not perfection - that is the end result of the program written into that formidably complex engine that is the human brain, and of the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development."
Dr. Rita Levi-Montalcini - Neurobiologist, Nobel prize winner, Italian senator, Champion of women in science and beyond. From "In Praise of Imperfection: My Life and Work", 1987

Undertaking this Ph.D has been a truly life-changing experience for me and it would not have been the same without the guidance that I received from many people. A Ph.D is more than a hollow piece of paper hanged on the wall; it is definitely not a normal job (also judging from my bank account). It is a life lesson. It taught me how to be intellectually curious and recover from failure, how to develop and accept my character, how to value and depend on my friends and family to help me get through and keep my motivation, how to be extremely patient and celebrate small wins. Now, I feel communicative, self-aware, resilient and, most of all, way more open to exploration and encounter.

For all these reasons, I wish to express my appreciation to my supervisors Dr. Sylvia Pender and Dr. Anis Larbi for the invaluable opportunity they offered me. Dr Sylvia L.F. Pender is Associate Professor Reader in Mucosal Immunology, within Medicine at the University of Southampton. As a mucosal immunologist, her research is based on how the mucosal immune and innate responses of the host can make the disease get better. She was one of the first to discover the involvement of matrix metalloproteinase in mucosal tissue inflammation in inflammatory bowel disease. Her current research shows new functions of MMPs and the related proteins which play important roles in anti-microbial infection, metabolic syndrome and aging disease such as osteoporosis. Dr. Anis Larbi is Principal Investigator and Head of the Flow Cytometry Facility at the Singapore Immunology Network (SIgN), Singapore. His group studies immunosenescence of the adaptive and innate branches of our immune system with a special focus on the impact of chronic infections on the aging process. In addition to basic immunology studies, the lab is also involved in prospective studies in Singapore to analyse biomarkers linked to successful aging.

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A good support system is important to surviving and staying sane in graduate school. I am indebted to all my friends who made my house a melting pot of different experiences, ethnicities and ideas, who shared the burden and the honour of doing a Ph.D and who were always so helpful in numerous ways. Special mention to Anna Doffini and her beetle fights, Elena Colangelo and her healthy courgette dinners, Alessandro Riccio and his crazy moments, Digvijay Singh with Pablo Sandino Morales Chavez and María Martínez Valado for being my three-continents housemates, Dana Neo for being such a sweet girl, Will Mothersole for the treasured suggestions on books and travel, Arran Gill and Stuart Berry for the lunches, Giampaolo Cetraro with Giuseppe Cannella, Dario Vignali and Luigi Simeone for being the trio pizza-spaghetti-mandolino of England, and the Salsa crews in Italy, the UK and Singapore who made many of my evenings. I am also thankful for all the diversions and hobbies, my trekking in the nature and travels in South-East Asia, my dance, my books, my painting, my music, my yoga and my cooking for making these years more enjoyable than they could have been.

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and myself and donated unconditional love and care. GRAZIE ragazzi. I warmly hope I made them proud of me. To them I dedicate this thesis.

I would like to extend my appreciation to my family-in-law, Martina and the rest of my relatives who make a noisy and generous Italian famiglia.

And finally to Davide, my smart, sweet and handsome husband. My companion of the last eleven years (and counting). We got married in the middle of our Ph.D, in a hot summer evening in the Italian countryside. He has always been patient despite my crazy peaks of stress and insecurity as I have been patient despite his. We moved to three different countries together and cherished every single instant of this unforgettable adventure. I feel that we both learned a lot about existence and strengthened our commitment and determination to each other and to live life to the fullest. Without him, I would not have had the courage to embark on this journey in the first place and would have not travelled around the world, experiencing the best moments of my life so far. GRAZIE Dado.
This thesis is dedicated to you.
P.S. To make this section totally honest, I would like to take the freedom to acknowledge my own tenacity for not throwing in the towel during the wild and wonderful journey on this sometimes bumpy road. I am sure it was worthy and will be adequately valued in the future to come. Now, time has come to take a new, unknown route across The Netherlands (or should I say bike ride?), hoping that life will be as inspirational as it has been so far.

Abbreviations

ACK: Ammonium-Chloride-Potassium

ADAM: A Disintegrin And Metalloproteinase

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity

AFAR: Federation for Aging Research

APC: AlloPhycoCyanin

BLIMP-1: B Lymphocyte-Induced Maturation Protein-1

BMI: Bone Mass Index

BP: Binding Protein

BTB: Broad-Complex, Tramtrack and Bric a brac

CCD: Charge Coupled Device

CEACAM1: carcinoembryonic antigen cell adhesion molecule 1

CLP: Common Lymphoid Progenitor

CMV: Cytomegalovirus

CPT: Cell Preparation Tube

CRP: C-Reactive Protein

CTLA-4: Cytotoxic T Lymphocyte Antigen 4

CVLT: California Verbal Learning Test

DAP : Death Associated Protein

DC: Dendritic Cells

DDR: DNA Damage Response

DEPC: DiEthyl Pyrocarbonate

DHEAS: Dehydroepiandrosterone

DMSO: DiMethyl SulfOxide

DNA: DeoxyRibonucleic Acid

DNAM-1: DNAX Accessory Molecule-1

dNTP: deoxyNucleotide TriPhosphates

DTT: DiThioThreitol

EAT2: Ewing's sarcoma-Associated Transcript 2

EBV: Epstein-Barr Virus

EDTA: EthyleneDiamineTetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

FACS: Fluorescence-Activated Cell Sorting

FADD: Fas-associated protein with death domain

FasL: Fas Ligand

FBS: Foetal Bovine Serum

FEV1: Forced Expiratory Volume 1

FoxO: Forkhead box O

FSC: Forward Scatter

Gal-9: Galectin 9

GAPDH: GlycerAldehyde 3-Phosphate DeHydrogenase

gDNA: Genomic DNA

GDP: Gross Domestic Product

GH: Growth Hormone

GHR: Growth Hormone Receptor

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

HA: hemagglutinins

HbA1c: Glycated Haemoglobin A1c

HBV: Hepatitis B Virus

HCC: Hepato-Cellular Carcinoma

HCMV: Human CytoMegaloVirus

HCV: Hepatitis C Virus

HIV: Human Immunodeficiency Virus

HLA: Human Leukocyte Antigen

HLA-DR: Human Leukocyte Antigen - antigen D Related

HMGB1: High-Mobility Group Box 1 protein

HN: Hemagglutinin Neuraminidases

HRP: HorseRadish Peroxidase

HSC: Hematopoietic Stem Cell

HSV: Herpes Simplex Virus

HTLV1: Human T Lymphotropic Virus 1

IACUC: Institutional Animal Care and Use Committee

ICS: Intracellular Cytokine Staining

Id2: Inhibitor of DNA binding 2

IFN: Interferon

IGF-1: Insulin-like Growth Factor 1

IGFR: Insulin-like Factor Receptor

IL: InterLeukin

ILC: Innate Lymphoid Cells

iNK: immature NK cells

IRB: Institutional Review Board

IRP: Immune Risk Phenotype

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibitory Motifs

JAK: JAnus Kinases

KIR: Killer cell Immunoglobulin-like Receptor

KO: Knock Out

LAG-3: Lymphocyte Activation Gene

LAMP-1: Lysosome-Associated Membrane Protein 1

LED: Light-Emitting Diode

LIR: Leukocyte Ig-like receptor

LPS: LipoPolySaccharide

MAIT: Mucosal-Associated Invariant T cells

MCMV: Murine CytoMegaloVirus

MHC: Major Histocompatibility Complex

MIC: MHC class I Chain-related protein

MIP: Macrophage Inflammatory Proteins

MMP: Matrix MetalloProteinases

mNK: mature NK cells

MTOC: MicroTubule-Organizing Centre

mTOR: mammalian Taget of Rapamycin

mTOR: Mammalian Target Of Rapamycin

NCR: Natural Cytotoxicity Receptor

ND: Not Determined

NER: Nucleotide excision repair

NIH: National Institute of Health

NK: Natural Killer

NKC: Natural Killer Cluster

NKG: Natural Killer Group

NKP: NK cell Precursor

OECD: Organisation for Economic Co-operation and Development

PBMC: Peripheral Blood Mononuclear Cell

PBS: Phosphate Saline Buffer

PCNA: proliferating cell nuclear antigen

PD-1: Programmed cell Death 1

PE: Phycoerythrin

PKC: Protein Kinase C

PLZF: Promyelocytic Leukaemia Zinc Finger protein

PMA: Phorbol 12-myristate 13-acetate

PRDMI: PR/SET Domain 1

PS: phosphatidylserine

PSMT: Picture Sequence Memory Test

qPCR: quantitative Polymerase Chain Reaction

RANTES: Regulated on Activation, Normal T cell Expressed and Secreted

RAVLT: Rey Auditory Verbal Learning Test

RBC: Red Blood Cell

RNA: RiboNucleic Acid

RNAi: RNA Interference

RPMI: Roswell Park Memorial Institute medium

SAM: Senescence-Accelerated Mice

SAMP: Senescence-Accelerated Prone

SAMR: Senescence-Accelerated Resistent

Scr: Scrambled

SHP-1: Src Homology region 2 domain-containing Phosphatase-1

Siglec-7: Sialic acid binding Ig-like Lectins 7

siRNA: Small Interfence RNA

SIV: Simian Immunodeficiency Virus

SLAS: Singapore Longitudinal Aging Study

SRBC: Sheep Red Blood Cell

SSC: Side Scatter

STAT: Signal Transducer and Activator of Transcription

TACE: TNF-α Converting Enzyme

T-bet: T-box protein Expressed in T cells

Terc: Telomerase RNA component

Tert: Telomerase reverse transcriptase

TF: transcription factor

TGF-β: Transforming Growth Factor β

Th: T helper

TIGIT: T cell Immunoreceptor with Ig and ITIM domains

TIM-3: T cell immunoglobulin domain and Mucin domain protein 3

TIMP-3: Tissue Inhibitor of MetalloProteinases

TMB: 3,3',5,5'-TetraMethylBenzidine

TNF: Tumour Necrosis Fcator

TNF-α: Tumour Necrosis Factor α

TOF: Time Of Flight

TRAIL: TNF-Related Apoptosis-Inducing Ligand

Treg: Regulatory T cells

tSNE: t-Distributed Stochastic Neighbor Embedding

WHO: World Health Organization

Zbtb32: Zinc finger And BTB domain containing 32

ZEB2: Zinc Finger E-Box binding homeobox 2

γδT: Gamma/Delta T cells

Chapter 1: Introduction

1.1 The age of aging

"Population aging is both a challenge and an opportunity. If nothing is done, population aging poses serious economic and social challenges. But it is also a tremendous opportunity if longer and healthier lives are matched by longer working lives".
Organisation for Economic Co-operation and Development (OECD) (2006)

Population aging on the current scale has no precedents. It is a process without parallel in the history of humanity. For the first time, the elderly (65+ years old) will outnumber children aged <5 years by 2020 (Fig. 1.1) (Organization, 2011).

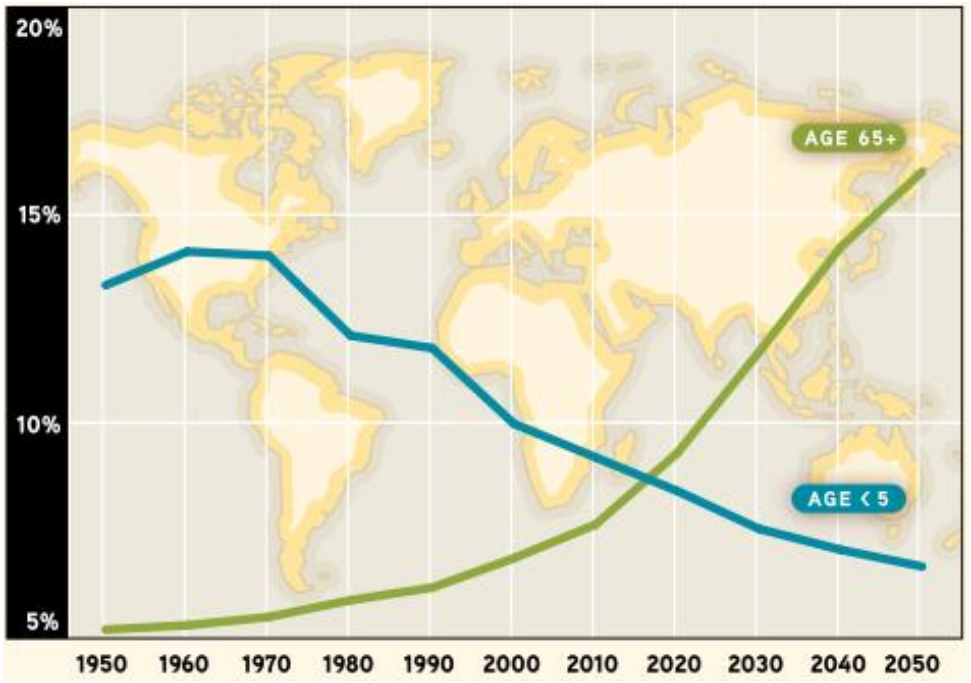


Figure 1.1 Young (<5 yrs) and old (>65yrs) as a percentage of the global population over time.
Adapted from (Organization, 2011).

Globally, the number of persons aged 60 and above is expected to more than double by 2050 and more than triple by 2100, increasing from 901 million in 2015 to 2.1 billion in 2050 and 3.2 billion in 2100. 66% of the increase between 2015 and 2050 will occur in Asia, 13% in Africa, 11% in Latin America and the Caribbean and the remaining 10% in other areas (Nations, 2015). This surge in older people is clearly illustrated by the most populous countries on earth: China and India (Fig. 1.2). Chinese elderly are projected to swell to 330 million by 2050 from 110 million today. Indians

over 65 will rise from the current 60 million to 227 million in 2050, an increase of 280 percent (Nations, 2015).

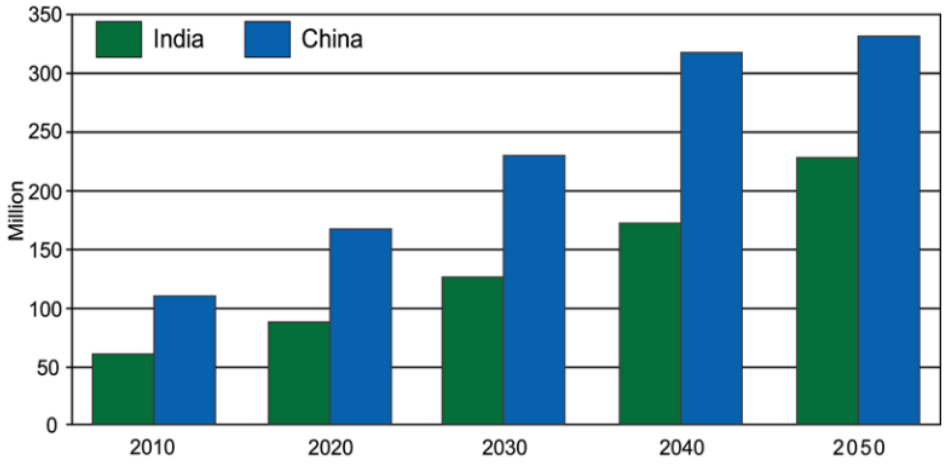


Figure 1.2 Growth of the population aged 65 and older in India and China (2010-2050).

Adapted from (Nations, 2015).

This significant increase in life expectancy stems from a drop in mortality rates and a change in the major causes of diseases and death (Nations, 2007). At the beginning of the 20th century, the main health concerns were infections such as pneumonia, tuberculosis and influenza. With the introduction of antibiotics and improvements in nutrition and hygiene, mortality rates from infectious diseases plummeted and today the first cause of death in developed countries are age-related chronic illnesses (Jones et al., 2012). Examples of aging-associated diseases are atherosclerosis and cardiovascular diseases, cancer, cataracts, osteoporosis and osteoarthriris, chronic lower respiratory diseases, type 2 diabetes and Alzheimer's disease. The incidence of all of these pathologies increases rapidly with aging (exponentially in the case of cancer) (Fig. 1.3) (Niccoli and Partridge, 2012). Of the roughly 150,000 people who die each day across the globe, about two thirds—100,000 per day—die of age-related causes (Lopez et al., 2006). In industrialized nations, the proportion is much higher, reaching about 90%. Thus, albeit indirectly, aging is by far the leading cause of death and main risk factor for the prevalent pathologies listed above. Global average life expectancy at birth jumped from less than 50 years old in 1950-1955 to almost 70 in 2010-2015 and it is projected to reach 77 by 2045-2015 and even to 83 years in 2095-2100. All major geographic areas shared the same trend in the life expectancy gain over this period, but the greatest increase was recorded in Africa (Nations, 2015).

Along with the reduction in mortality rates, decreasing birth rates have been one of the primary causes of increased average age. The fertility rate has dropped almost by half globally, from 4.9

children per woman in 1950-1955 to 2.6 in 2005-2010 and it is expected to keep on declining to reach 2.0 children per women in 2045-2050 (Nations, 2007).

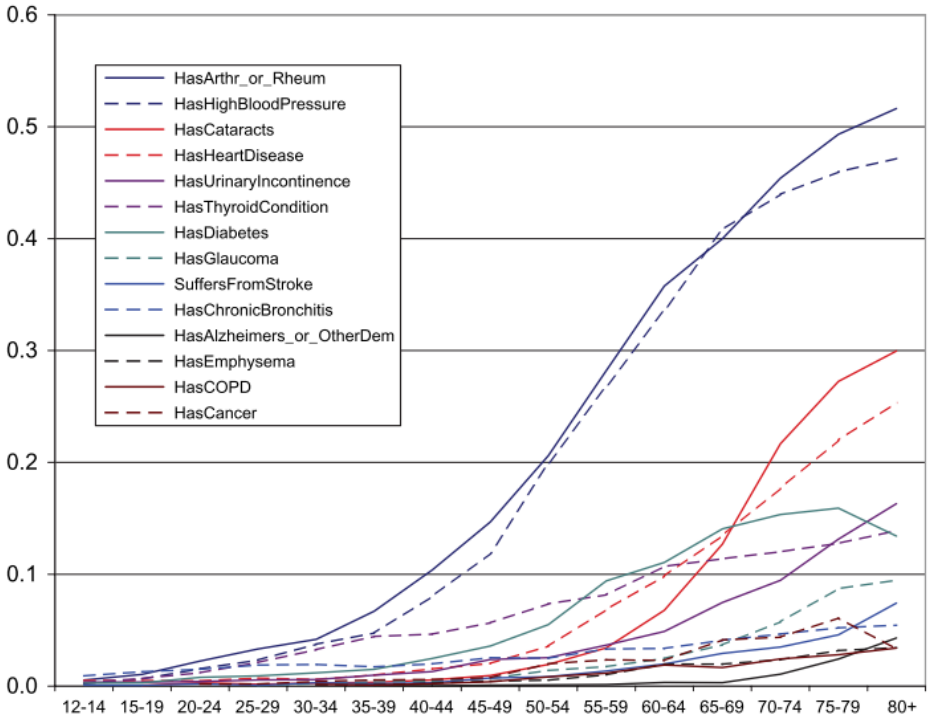


Figure 1.3 Prevalence rates for chronic conditions associated with old age, 2005.

Adapted from (Denton and Spencer, 2010)

Not only the magnitude but also the rate at which world's population aging is running is to be taken into account. Most developed countries have had decades to adjust to their changing age structures (Fig. 1.4) (Kinsella, 2009). For example, it took more than a century for the French population aged ≥65 to rise from 7% to 14%. In contrast, many less developed countries, such as South Korea, China, Thailand and Brazil, are experiencing an amazingly rapid increase in the number and percentage of older people, often within a single generation. This poses a huge socio-economic burden to governments since some developing nations may become old before becoming developed.

Global aging is a success story, a triumph for medical advancement and socio-economic development over some diseases that limited human life for millennia. On the other hand, such a rapid and massive growth in the older population brings formidable challenges with it. Population aging is an often underestimated serious concern, having important consequences and implications for all aspects of human life. In the economic and political aspects, population aging influences labour markets, economic development, pensions, insurances, health systems and welfare policies. A prominent economic concern is the decrease of the workforce relative to the number of retirees, leading to the shrinking of financial resources dedicated to public pensions. This is worsened by the

fact that over time the number of years spent in retirement inflated. In the Organisation for Economic Co-operation and Development (OECD) countries, in 2007, the average man/woman left the labour force before age 64/63 and could expect 18/22 years of retirement, with more than 26 years in France, Italy, Belgium and Austria (OECD, 2013). Supporting a big share of old people for about two decades is not sustainable in the long term prospect. This means governments must rise retirement age, increase the contribution or tax rate on younger workers and boost the productivity of elderly workers, with continuing education, workplace design and part-time employment opportunities.

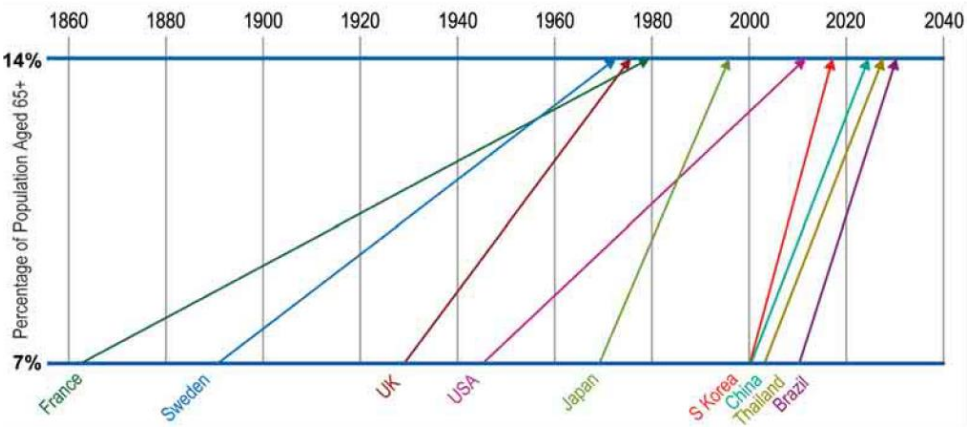


Figure 1.4 The speed of population aging. Time required or expected for percentage of population aged 65 and over to rise from 7 percent to 14 percent. Adapted from (Kinsella, 2009)

Total health care for the elderly represents on average around 9% of GDP in OECD countries and around three-quarters of this are financed through the public sector (OECD, 2013). The process of aging in OECD countries is further accelerating since the baby-boom generation is beginning to enter retirement. Since 40 to 50% of health care expenditures is directed towards the elderly and per-capita health care costs for those over 65 are three to five times higher than for those under 65, pressure on health and long-term care costs in the OECD will surely become unsustainable (Gregersen, 2014).

With regards to the social area, population aging has a significant impact on family composition and care. As people live longer and have fewer children, the emerging family structure is the so-called “beanpole family”, a vertically extended structure characterized by an increase in the number of living generations within a lineage and a decrease in the number of people within each generation (Organization, 2011). Since informal home care given by relatives is the most common long-term support for the aging society, the impact on family finance and commitment is concerning.

For all these reasons, policies should help promoting a holistic view of healthy aging, defined as the absence of overt or severe diseases and disabilities and the preservation of good physical, cognitive,

social and productive abilities (Franceschi et al., 2008). Section 1.1.1 reports an extensive summary of the recent priority interventions that sovranational entities and individual countries have been putting into place to tackle population aging challenges and, at the same time, encourage a new vision of heathy older adults as resources for their communities.

1.1.1 Policies and interventions for healthy aging

"And in the end, it's not the years in your life that count. It's the life in your years".
Unknown

World Health Organization (WHO) has put healthy aging high on its regional and global policy agenda. The fact that in the WHO European Region the median age is the highest in the world prompted the launch of the European action plan on healthy aging for 2012–2016. The main goal of this policy is to "optimise opportunities for health, participation and security in order to enhance quality of life as people age. It applies to both individuals and population groups. Active ageing allows people to realize their potential for physical, social, and mental well-being throughout the life course and to participate in society, while providing them with adequate protection, security and care when they need" (Organization, 2012). The WHO Regional Office has been working with governments to design and implement priority interventions that are logistically and politically feasible and whose results can be achieved and measured in a relatively short time. The main interventions are:

- Influenza vaccination and infectious diseases prevention. Infections in the elderly are more frequent and more severe than in younger people because of epidemiological elements, senescence of the immune system and malnutrition, as well as age-associated physiological and anatomical alterations. For instance, influenza is usually mild and self-limiting in healthy adults while it can causes life-threatening complications in the elderly, such as pneumonia, leading to costly hospitalisation and often death. During seasonal epidemics, 90% of influenza-related deaths occur in people aged 65 or older. WHO suggests that the elderly are annually vaccinated before the season begins. Influenza vaccination is the most efficient intervention currently available to reduce morbidity and mortality but the lower protection offered to older people urgently calls for new studies on immune system aging, vaccine efficacy and alternative vaccination technologies. Novel investigations must be supported by the increase of vaccine intake and implementation of more robust vaccination programme across countries.

- Prevention of falls. Fall-related injuries (mainly hip fractures) are more common among older people (especially women) and more likely to cause pain, disability, loss of independence and premature death. About 30% of people aged ≥65 and 50% of those aged over 80 fall each year. The financial costs for hospital admissions and rehabilitation are substantial and increasing worldwide. A combination of exercise and nutritional programmes, home safety assessments, physical therapy and balance re-training can significantly reduce number and severity of falls.

- Promotion of physical activity. The age-related muscle loss (sarcopenia) in the age range 70-79 affects more than 40% of men and 50% of women in the European region. Physical activity is one of the strongest predictors of healthy aging. Regular moderate activity in older age prevents diseases and injuries, improves mental and cognitive health and promotes social well-being (McPhee et al., 2016).

The strategy plan discussed above has just been reaffirmed and expanded in January 2016 by the WHO for the time frame 2016-2020 (Organization, 2015).The five strategic objectives added in this policy are:

- Improving research, measurement and monitoring on healthy aging. Multi-country and multidisciplinary studies are compelling in order to widen the basic and applied knowledge on aging in the fields of biology, medicine, politics, economy and sociology. Of crucial relevance is developing and reaching consensus on metrics, measurement strategies, instruments, tests and biomarkers for key concepts related to healthy aging including intrinsic capacity, functional ability, well-being, environments, genetic inheritance, social position, vulnerability and resilience, multimorbidity and the need for care and other social services.

- Fostering healthy aging in every country. Recognition by governments and public of the value that healthy aging represents in terms of health and economic returns and other social benefits is at the base of any healthy aging promotion policy. This includes ensuring that all countries take into account healthy aging policies in their regulatory frameworks and budget lines. There is the need, through efficient communication campaigns, to build and embed in the thinking of all generations a new understanding of aging in order to combat ageism, defined as the age-based stereotypes and discrimination that influence behaviours of institutions, individuals and even research.

- Creating age-friendly environments. An age-friendly environment includes non-physical factors (social, economic and health system) and physical factors (buildings, transportation, housing, information, streets and parks) to allow the elderly to be mobile, build/maintain relationships and contribute to families and communities.

- Aligning health systems to the needs of the older populations. Healthcare is often designed to treat acute manifestations and distinct diseases independently. With age, medical needs become more chronic and complex and health systems should address these multidimensional demands in an integrated and holistic way. This objective requires ensuring a sustainable and appropriately trained health workforce with gerontological and geriatric skills.

- Developing systems for providing long-term care. Long-term care systems at home, in communities or within institutions enable care-dependent older people to live with dignity. Importantly, since most care (in terms of hours) is given at home by non-professional care givers (mainly women/wives), supporting long-term care means freeing care givers to pursue other social and economic roles. Additionally, this intervention can reduce inappropriate use of acute health services and help families avoid overwhelming expenditures.

The translation of the directions given above into actions and results in every country will require commitment by stakeholders, including not only WHO and its state members but also health workers and their professional associations, private sector partners, academia and research institutes and media.

1.2 Biology of aging

"Aging is arguably the most familiar yet least-well understood aspect of human biology".
Murgatroyd, Wu, Bockmuhl, and Spengler (2009)

The biology of aging is a rapidly expanding but still immature and puzzling field. Even though the aging phenomenon has fascinated medical researchers, philosophers, anthropologists, and the general public all over history, our knowledge of what aging is, why and how it occurs is limited. This is mainly because aging do not act as a single disease with specific causes, signs, symptoms, biomarkers, tests and endpoints. Even defining aging is not so straightforward. And we are not talking about mere semantic since some gerontologists would identify aging with the age-related conditions, others would say it is a risk factor for age-related diseases. Other basic questions that do not find any consensus are: is aging a lifelong, initially asymptomatic process or starting at the end of the reproductive life of humans? Is aging physiologic or pathologic?
All the (sometimes personal and subjective) views reported above led to the proliferation of different theories of aging, none of which outclasses the others. The brief review of literature below lists the most accepted hypotheses that are not mutually exclusive.

1.2.1 Theories on aging

Evolutionary theories (also referred to programmed theories) of aging hypothesize that aging is not a random process but driven by Darwinian evolution. Some scientists support that this is accidental (evolution did not worry much about negative consequences of aging since they happen post-reproduction) and others that aging is selected by evolution (as nearly immortal organisms would destroy their environment leading to extinction). According to the theory of the accumulation of mutations at old age proposed by Medawar in 1952 (Holliday, 2006), deleterious mutations expressed at young age are severely selected against in order to avoid passing them to the next generation. On the other hand, the same mutations, confined to old people, will experience no selection, because their bearers have already transmitted their genes to the offspring. Over successive generations, deleterious mutations will passively accumulate in old age, causing an increase in mortality rates late in life. In 1957, Williams suggested that some genes are pleiotropic, in the sense that they have favourable effects on fitness at young age and deleterious ones at old age (antagonistic pleiotropy theory) (Blagosklonny, 2010). These genes are actively favoured by selection since they have any beneficial effects early in life. An example of pleiotropic gene is the one encoding for target of rapamycin (TOR), a conserved kinase which regulates cell growth and metabolism in response to environmental clues. The TOR pathway drives both mass growth and aging and its inactivation increases lifespan, while decreasing fitness early in life (Blagosklonny, 2010).

Conversely, damage-based or stochastic theories propose that aging results from a continuous process of damage accumulation originating in by-products of normal cellular processes and/or inefficient repair systems. Two main damage-based hypotheses are the cross-linking theory and free radicals theory. The cross-linking theory of aging, proposed by Bjorksten in 1942 (Bjorksten, 1968), postulated an accumulation of cross-linked proteins that damage cells and tissues. The free radicals theory, which was first introduced by Gerschman in 1954 (Gerschman et al., 1954), proposed that superoxide and other free radicals damage the macromolecular components of the cell, especially nucleic acids.

The development of many unsatisfactory aging theories proves that our comprehension of the mystery of aging has still a long way to go. If we are far from the complete understanding of why we age, at least it is feasible to do accomplish something more practical: how to measure aging. In the next section, I report a general overview regarding the hot topic of aging biomarkers, highlighting their role in assessing the health state of elderly.

1.2.2 Biomarkers of aging

The desperate need for a biomedically useful definition of aging is prompting a series of studies with the common aim to define a naturally occurring molecule/s, gene/s or characteristic/s by which the aging process can be identified and measured. Chronological age, the simplest criterion to evaluate for a geriatrician, often is not a useful measure to describe the functional capacity and the biological age of an individual. Because of the intrinsic multi-casual and multi-system nature of the aging process, scientific criteria for biological aging have been investigated (Fig. 1.5) but no candidate biomarker has so far proven to evaluate adequately the underlying process.

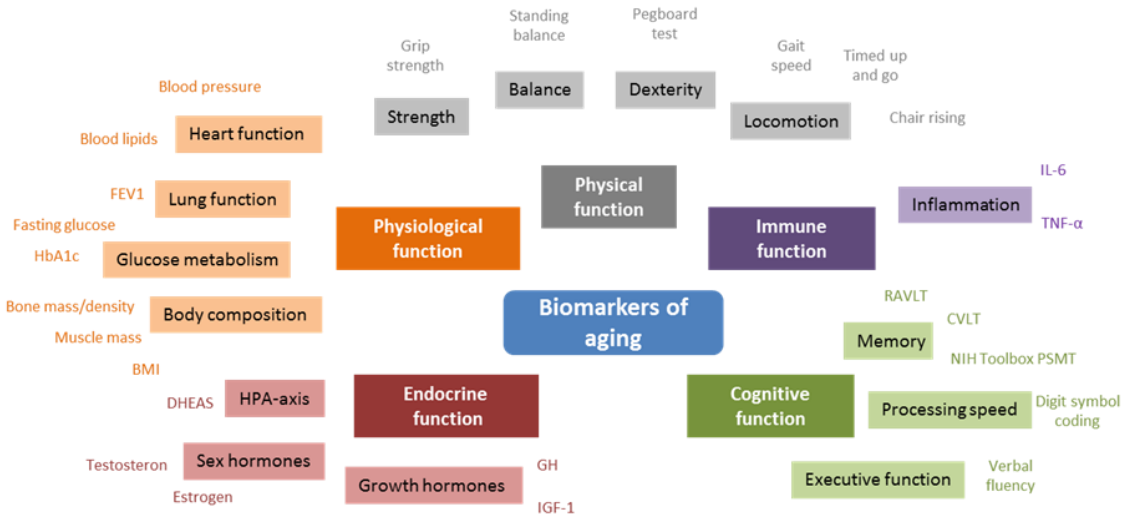


Figure 1.5 Proposed biomarkers of aging. RAVLT, Rey auditory verbal learning test; CVLT, California verbal learning test; PSMT, picture sequence memory test; FEV1, forced expiratory volume 1; HbA1c, glycated haemoglobin A1c; BMI, bone mass index; DHEAS, dehydroepiandrosterone; GH, growth hormone; IGF-1, insulin-like growth factor 1; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α. Adapted from (Lara et al., 2015)

The American Federation for Aging Research (AFAR) has proposed the following criteria for a biomarker of ageing (Burkle et al., 2015):

- It must predict the rate of ageing independently of chronological age.
- It must monitor a basic process that underlies the aging phenomenon, not the effects of disease.
- It must be tested easily without harming the subject (e.g. a blood test or an imaging technique).

- It must work in humans and in animal models, such as mice, since preliminary testing is always done in non-human subjects (even though this criterium may raise objections since some parameters such as telomere shortening show importance for the ageing process may differ between mammalian species).

Lack of accurate biomarkers of aging creates enormous difficulties when comparing studies and it is one of the research topics that must be addressed with most urgency. Another issue hindering the research on aging is the ethical and logistical limitation in the use of humans as experimental subjects. An extensive analysis of the animal models of aging with their advantages and pitfalls is discussed below.

1.2.3 Murine models of aging

Using humans as subjects in aging mechanistic research is complicated because of many ethical issues, the long natural life span, genetic diversity, environmental influences and various other limiting factors. Therefore, many animal models have been developed to study the fundamental biology of aging. Intrinsic and extrinsic influences on the aging process such as genetic background, diet, environment and health status can be strictly controlled in many models.

Much has been learned from the study of aging in worms and flies, but it is important to test the knowledge derived from these lower organisms in a mammalian species. Mice are the best choice in this type of research for several reasons. First of all, mice are similar to humans in much of their genetics, physiology, cellular functions and, to a lesser degree, in their anatomy. Mice share 99% of their genes with humans (Boguski, 2002). They also show similarities in disease pathogenesis. Secondly, there are some technical and economic justifications for the use of murine models. They are low-cost test subjects, easy to manipulate genetically and have shorter lifespan allowing aging research during the relatively short period, for instance, a PhD project. Indeed, longitudinal studies can be conducted on mice and all tissues can be analysed at all stages of the aging process.

However, the main problem in any research involving animal model is whether these similarities in cellular processes and disease pathogenesis are sufficiently robust that experimental findings from mouse models can be extrapolated to study aging in human systems. Despite the advantages of mouse models of aging, there are some important age-related differences between mice and humans. Maximum lifespan potential is 4 years in mice and 120 years in humans (Demetrius, 2006). Another discrepancy involves the dynamics of telomeres, ribonucleoprotein structures that cap the chromosomes extremities to overcome the inevitable DNA shortening during mitotic cell division. Telomere attrition is the molecular mechanism responsible for the “Hayflick limit” and replicative

senescence that will be discussed in details in section 1.3.1.1 (Hayflick, 1965). Thus, telomeres shorten with each cell division, but telomerase, a reverse transcriptase, elongate telomeres in very specific cells, such as embryonic and adult stem cells. Although telomere sequence is identical in mice and humans and telomeres play the same role in both species, mice have 5 to 10 times longer telomeres and higher telomerase activity in many organs (Calado and Dumitriu, 2013). Finally, aged mice do not show all the typical age-related diseases seen in humans, e.g., cardiovascular disease and Alzheimer's disease (Vanhooren and Libert, 2013).

Some principles should be kept in mind when using mice in aging research. First, animals should be healthy, free of pathogens and disease, without any signs of tumours or lesions to ensure that aging and not disease is being studied. We should take great care to distinguish between the aging process per se and aging-like phenotypes resulting from pathological changes. It is also important to use mice that have already reached maturity to avoid observation of maturation rather than aging effects.

Several mouse systems have been established to investigate immunosenescence and the specific pathways altered by normal or pathological aging. In the next paragraph, I discuss several murine models that provide significant data on the changes in immune system with advancing age especially those characterized by a shortened lifespan.

1.2.3.1 Mouse models with a normal lifespan

Many inbred strains have been used in the aging research although most studies rely on one mouse strain only (C57BL/6). Lifespan is strain-specific. The Jackson Aging Center performed an in-depth lifespan study of 31 genetically diverse inbred mouse strains housed in specific pathogen-free conditions (Yuan et al., 2009). Median life spans range from 251 to 964 days. The shortest was that of AKR/J and the longest was in female WSB/EiJ and male C57BL/6J. The results from this broad study confirmed that genetics plays an important role in determining longevity and that sex did not significantly affect lifespan for most strains.

While maximum lifespan has been the fundamental metric of aging research, longevity alone is not completely informative since it does not allow for assessment of health. Pathology in aged mice also is strain-dependent. For instance, the most common death causes in studies on aging C57BL/6 mice are lymphoma and hematopoietic neoplasms in general (Pettan-Brewer and Treuting, 2011).

The best murine model for studying human aging is the mouse with a normal lifespan because, like humans, the normal aging mice develop cancer, cataracts, muscle weakness, immune abnormalities, cognitive impairment, impaired fertility, joint problems, central obesity, skin atrophy and other aspects of normal mammalian aging (Miller, 2004). However, models with altered

lifespan might be useful depending on the study aim and on technical and/or economic feasibility issues.

1.2.3.2 Mouse models with an extended lifespan

The mouse is ideal for the investigation of compounds or interventions that could delay aging, followed by the eventual development of drugs to slow aging and retard disease progression in humans.

As discussed below, the main models with prolonged lifespan (calorie-restricted mice, rapamycin-treated mice and dwarf mice) rely on the same basic principle: food, energy and/or growth factor restriction allows a significant lifespan extension in mice and more work is needed to translate these finding to the clinics.

1.2.3.2.1 Caloric restriction

Caloric restriction (limiting food intake without causing nutritional deficiencies) has been shown to increase both the mean and the maximum life span in different rat and mouse strains and in many other species, including yeast, invertebrates, fish, hamsters and dogs, in both genders (Masoro, 2005). Caloric restriction delays the onset and/or slows down the progression of most age-associated diseases, including neoplastic, degenerative and auto-immune diseases. Several mechanisms have been suggested such as lower oxidative stress and growth hormone (GH)/insulin-like growth factor-1 (IGF-1) levels (Masoro, 1996).

Additionally, caloric restriction is the most extensively studied strategy to delay or prevent the alterations of immune functions that lead to immunosenescence, including reduction in naïve T cells and in T cell proliferation upon mitogen stimulus, decline in anti-viral response as well as increase in inflammatory cytokines production (Pahlavani, 2000). To date, there are no definitive data that caloric restriction prolongs lifespan in humans (because of the long life span) but data on health benefits (metabolic syndrome, type-2 diabetes, inflammation, hypertension and cardiovascular disease) are convincing (Roth and Polotsky, 2012).

1.2.3.2.2 Compounds extending lifespan

In mammals, seven sirtuin genes have been identified (SIRT1-7). SIRT1 regulates glucose and insulin production, fat metabolism and cell survival (Dong, 2012). Resveratrol, a molecule produced by a variety of plants in response to stress, emerged as the most potent enhancer of SIRT1 activity *in vitro* and it has been shown to extend lifespan in different lower organisms and mice (Baur et al., 2006). Of note, reduced risk for coronary heart disease and a possible extension of lifespan was

found in human populations consuming wine with a higher amount of resveratrol (Chachay et al., 2011). The NIH Aging Interventions Testing Program showed that also aspirin and rapamycin increased lifespan in mice (Strong et al., 2008). Interestingly, use of aspirin in humans was associated with a lower risk of cancer incidence and mortality (Bardia et al., 2007). The antifungal mammalian target of rapamycin (mTOR), a protein kinase, helps to integrate cellular activities in response to nutrients, stress and extracellular signals, including hormones and growth factors. It was shown that rapamycin, an inhibitor mTOR signalling, fed both at young and old age, extends lifespan in genetically heterogeneous mice (Harrison et al., 2009). Treatments like rapamycin, that slow the aging process when begun late in life, might delay or reduce the severity of age-related pathologies. This would suggest the use of rapamycin in early-stage age-related diseases, such as Alzheimer's and in families predisposed to other age-related diseases (Sharp and Strong, 2010). For human application, an obvious potential problem is the possible immunosuppressive effects of rapamycin. Currently, the anti-cancer effect of the mTOR inhibitor rapalog CCI-779 is being tested and no immunosuppressive effects were detected in patients with advanced cancer in a phase I clinical trial (Sharp and Strong, 2010). Additionally, the mTOR inhibitor RAD001 has been shown to ameliorate immunosenescence in elderly volunteers, as assessed by their response to influenza vaccination. RAD001 also reduced the percentage of CD4 and CD8 T lymphocytes expressing the T cell exhaustion marker PD-1 (Mannick et al., 2014). In summary, the clinical use of TOR pathway inhibitor as antiaging intervention still suffers from many caveats but is promising.

1.2.3.2.3 Genetic mutations extending lifespan

Single-gene mutations that prolong lifespan are another tool for the study of the molecular basis of age-related cellular alterations and diseases. Several mutations that cause dwarfism have been reported to increase lifespan in mice. The most valuable models are the Ames dwarf mouse and the Snell dwarf mouse, which have mutations in the transcription factors *Prop1* and *Pit1* genes respectively. Mutations of *Prop1* or *Pit1* lead to defective differentiation of pituitary cells resulting in decreased production of growth hormone (GH), prolactin and thyroid-stimulating hormone and consequent severely retarded growth (Li et al., 1990). Furthermore, the dwarf mice show a slower metabolism with reduced circulating levels of serum insulin, insulin-like growth factor (IGF-1) and glucose (Bartke et al., 1998). Interestingly, lower IGF-1 also characterizes the aforementioned caloric restriction model and some of the physiological properties of dwarf mice are similar to those of dietary restricted mice. Onset of fatal neoplastic disease is delayed in Ames dwarf mice compared to normal controls (Ikeno et al., 2003). Snell dwarf mice are resistant to chemically induced cancers (Alderman et al., 2009). Snell and Ames dwarf mice do not experience various age-related changes

such as decline in locomotor activity and learning/memory skills, osteoarthritis and collagen cross-linking (Kinney et al., 2001). Studies of immune function in these mice are much less extensive than those performed on the caloric restriction strategy. In Snell dwarf mice, some immunosenescence processes are delayed or reversed, including increase in splenic memory T cells and decrease in IL-2 production (Flurkey et al., 2001).

Humans with dwarfism due to a *Prop-1* gene mutation can live a long life but there is no demonstrated association with longevity (Krzisnik et al., 2010). The same pathophysiological findings have been observed in the growth hormone receptor/binding protein (GHR/BP) knockout (KO) mice. 99 Ecuadorian individuals with the Laron syndrome due to GHR deficiency were monitored for 22 years and did not develop type 2 diabetes and were almost free from cancer (Guevara-Aguirre et al., 2011).

Heterozygous KO mouse for IGF-1R (IGF-1R+/−) is another model with mutations in the growth hormone pathway that shows a longer lifespan and is characterized by a greater resistance to oxidative stress (Holzenberger et al., 2003). Italian centenarians carry a polymorphic variant of the IGF-1R resulting in lower IGF-1 plasma levels (Bonafe et al., 2003). Ashkenazi Jewish centenarians show an overrepresentation of heterozygous mutations in the IGF-1R gene reducing activity of the IGF-1R (Bonafe et al., 2003).

All the mouse models and related human populations that have been discussed above confirm the important roles of GH/IGF-1 axis and glucose metabolism in aging and suggest a deeper investigation of the hormonal/metabolic regulation of longevity.

1.2.3.3 Mouse models with a shortened lifespan

Since it is expensive and time-consuming to follow mice during their entire life, studying animals with a reduced life span and signs of accelerated aging is of great advantage. However, these studies should be treated with extreme care because the causes and mechanism of accelerated aging in such models are not fully understood yet. These mice do show some typical features of aging and inflammation but also some others that are not seen in normal aged mice, suggesting that other pathways can be responsible of their phenotype.

1.2.3.3.1 SAM mice

The Senescence-Accelerated Mice (SAM) are a group of inbred mouse strains generated as a result of an accidental outcrossing of AKR/J mice with another unknown albino mouse strain. Litters showing an accelerated senescence phenotype and short lifespan were selected to become the progenitors of senescence-prone strains (SAMPs), while litters with normal aging were selected as

progenitors of senescence-resistant strains (SAMRs). The median survival time of SAMP mice is 9.7 months, 40% shorter than that of the SAMR strains (16.3 months) (Takeda et al., 1994). To date, the SAM series includes nine SAMP strains and three SAMR strains. SAMP strains share a combination of gene mutations responsible for the common senescence-prone phenotype. In addition, each strain is thought to carry its own gene mutation(s) causing age-related pathological phenotypes that are unique to each strain (Shimada and Hasegawa-Ishii, 2011). Table 1.1 reports the pathophysiological phenotype in the different SAM strains.

SAMP mice demonstrate age-associated decline in various functions including immunity. In SAMP1 mice, involution of the thymus and decrease of CD4 T cells and CD4/CD8 ratio in the peripheral blood occur earlier than in SAMR1 mice (Toichi et al., 1994). *In vitro* studies on splenocytes from 2 month-old SAMP1 have revealed that the antibody-forming capacity to T-independent antigens, such as DNP-Ficoll, and the activity of NK cells undergo an early onset of regression with a sharp decline from the level of age-matched control SAMR1 mice. SAMP1 mice also exhibit profound defects in the antibody responses to T-dependent antigens, such as sheep red blood cells (SRBC) and ovalbumin (OVA) (Hosokawa et al., 1987). Nishimura et al. observed that splenic CD4 T cells from young SAMP1 mice showed abnormal IL-2 production and proliferation upon stimulation with concanavalin A (con A) (Nishimura et al., 2002). Studies of intranasal inoculation with influenza A (Dong et al., 2000) virus as well as with respiratory syncytial virus (Liu and Kimura, 2007) have indicated that SAMP1 mice are more susceptible to viral infection and exhibit a higher rate of mortality and prolonged virus growth in the lungs, due to diminished cellular immunity by local virus-specific CTLs and NK cells. SAMP8 mice share similar immune system deficiencies with SAMP1 mice. At 2 months of age, the SAMP8 spleen cells showed markedly decreased NK cell activity, anti-SRBC antibody responses, cell proliferation, and IL-2-producing activity in response to con A (Abe et al., 1994). Most of the age-dependent geriatric disorders seen in humans are included in the phenotypes of SAMP mice, such as senile amyloidosis, osteoporosis, impaired hearing and retinal atrophy, supporting the SAM model as a valid and useful tool for aging research. However, genetic changes and mechanisms of premature aging must be further investigated. In particular, there are very few recent updates on deficient immune functions and systematic as well as extensive studies regarding immunosenescence in different tissues across SAMP strains are compelling.

Table 1.1 Pathophysiological phenotype in the different SAM strains

STRAIN	AGING	PATHOLOGY
SAMR1 **SAMR4** **SAMR5**	Normal	Non thymic lymphoma Histiocytic sarcoma Ovarian cyst
SAMP1	Accelerated	Impaired immune response Senile amyloidosis Impaired hearing Retinal atrophy Contracted kidney Lung hyperinflation Ileitis
SAMP2	Accelerated	Impaired immune response Senile and secondary amyloidosis
SAMP3	Accelerated	Degenerative arthrosis
SAMP6	Accelerated	Senile osteoporosis Secondary amyloidosis
SAMP7		Senile amyloidosis Thymoma
SAMP8	Accelerated	Impaired immune response Age-related emotional disorders, behavioural disorders, deficit in learning and memory, altered circadian rhythm Age-related brain alteration, amyloidosis Age-related declines in retinal function Accelerated aging of the reproductive organs
SAMP9	Accelerated	Cataract
SAMP10	Accelerated	Brain atrophy Impaired learning and memory Depression
SAMP11	Accelerated	Thickened thoracic aorta Senile amyloidosis Contacted kidney

1.2.3.3.2 Klotho mice

The Klotho gene accelerates aging and shortens lifespan when disrupted and extends it when overexpressed (Kurosu et al., 2005). This molecule inhibits intracellular insulin and IGF-1 signalling, which is a major mechanism involved in other aging models presented in this thesis.

Klotho-deficient mice exhibit a syndrome resembling human premature aging characterized by infertility, decreased spontaneous activity, premature thymic involution, ectopic calcification, skin atrophy, arteriosclerosis, osteoporosis and pulmonary emphysema. They also have dramatically accelerated age-related decline in B cell lymphopoiesis (Kuro-o et al., 1997). The InCHIANTI study, a longitudinal population-based study of aging in Italy, measured the plasma Klotho levels in 804 adults aged above 65 years (Turturro et al., 1999). Participants with low plasma Klotho levels had a higher risk of death.

1.2.3.3.3 DNA-repair mutant mice

The findings that efficiency of the DNA repair machinery declines with age and that human progeroid syndromes involve defects in DNA maintenance suggest that progressive genome instability is an important aspect of aging (Campisi, 2005). Telomerase, the enzyme that prevents telomere shortening during cell division, is composed of two basic units, telomerase reverse transcriptase (Tert) and telomerase RNA component (Terc). Mice deficient for Terc are initially normal, but after 5-6 generations demonstrate a premature aging phenotype (higher cancer incidence, alopecia, skin lesions, lower fecundity) and shorter lifespan. These mice also show bone marrow failure, decreased antibody response, altered germinal center formation upon T-dependent antigens and reduced T and B cell proliferation *in vitro* (Lee et al., 1998). These data confirm that shortening of the telomeres can affect immune response efficacy.

In humans, mutations in telomerase components are responsible for the onset of dyskeratosis congenita, that is a multisystem premature aging syndrome characterized by muco-cutaneous features, bone marrow failure, early greying, dental loss, osteoporosis, and malignancy (Dokal, 2001). Nucleotide excision repair (NER) removes helix-distorting DNA lesions, such as UV-induced pyrimidine dimers and also repairs oxidative damage. Defects in different NER factors exist in some patients and in certain mutant mice, resulting in phenotypes that resemble premature aging (Mitchell et al., 2003).

1.2.3.3.4 *Timp-3* knock out mouse

Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an inhibitor of matrix metalloproteinases (MMPs) along with other three members of the same family (TIMP-1, TIMP-2and TIMP-4). The *Timp-3* KO mouse was generated in 2001 and shows spontaneous air space enlargement in the lungs, dilated cardiomyopathy, interstitial nephritis and fibrosis, spontaneous osteoarthritis, collagen degradation and abnormal bone growth, impaired cognitive function (Brew and Nagase, 2010).

Unlike most of the mitogen-induced genes, that are constitutively expressed during the normal cell cycle, TIMP-3 expression is tightly regulated in a cell cycle-dependent fashion, showing a clear peak of expression around mid-G1 (Wick et al., 1994). Thus, TIMP-3 has a role in processes that are associated with the G1 phase of the cell cycle such as the control of cell cycle progression, terminal differentiation, and cellular senescence. It has been demonstrated that TIMP-3 is a mediator of hematopoietic stem cell regulation, recruiting cells into active cell cycle and expanding the multipotent progenitor pool (Nakajima et al., 2010). Wick and colleagues (Wick et al., 1994) showed that TIMP-3 expression is up-regulated during terminal differentiation of the myeloid cell line HL-60 and down-regulated during replicative senescence. Two papers have suggested a relationship between TIMP-3 and aging. Kamei and Hollyfield (Kamei and Hollyfield, 1999) showed an increase in TIMP-3 protein levels in Bruch's membrane in patients with age-related macular degeneration and during normal aging. Macgregor and colleagues (Macgregor et al., 2009) characterized the distribution of TIMP-3 in human lungs, kidneys, retinas and vascular tissues. They found that TIMP-3 protein increases with age in lung, kidney and retina and that TIMP-3 protein accumulation is a normal age-dependent phenomenon.

TIMP-3 is a pleiotropic molecule that shows MMPs-independent functions. It has been demonstrated that TIMP-3 inhibits several ADAM (a disintegrin and metalloproteinase) metalloproteinases, also called "sheddases", that influence cell behaviour by proteolytically shedding the ectodomain of cell surface molecules such as growth factors, cytokines and cell adhesion molecules (Khokha et al., 2013). Other TIMPs can block the same enzymes but with a lower efficiency. An exclusive ability of TIMP-3 is to inhibit the shedding activity of ADAM17 also known as tumour necrosis factor (TNF)-α converting enzyme (TACE), which sheds the ectodomain of membrane anchored TNF-α, raising the levels of the soluble form of TNF-α (Black et al., 1997). Mohammed and colleagues (Mohammed et al., 2004) showed that *Timp-3* KO mice develop chronic inflamed livers due to an increase in local TNF-α. Smookler and collegues (Smookler et al., 2006) observed that *Timp-3* KO mice are more susceptible to lipopolysaccharide (LPS)-induced mortality than wild-type mice, suggesting that the loss of TIMP-3 and the consequent raise in TNF-α levels leads to pathological inflammation due to an unregulated innate immune response. Moreover, TACE is able to catalyse the cleavage of other molecules involved in inflammation such as IL-6R (Althoff et al., 2000), IL-1RII (Reddy et al., 2000), IL-15Ra (Budagian et al., 2011), TNF-αR (Wang et al., 2003), V-CAM (Garton et al., 2003), I-CAM (Tsakadze et al., 2006), JAM-1 (Koenen et al., 2009a) and L-selectin (Peschon et al., 1998).

Increased concentration of TNF-α is found not only in acute inflammation but also in chronic inflammatory conditions. Human aging is accompanied by persistent, low-grade inflammation

(Vasto et al., 2007), known as 'inflammaging', that has been associated with age-related diseases as detailed later in the text (Franceschi et al., 2000). Given the importance of TNF-α in inflammation and the aging processes and the role of TIMP-3 as TACE inhibitor, the *Timp-3* KO mouse might be seen as an animal model in order to study not only inflammatory diseases but also aging, age-related pathologies and especially the relationship between aging and inflammation.

1.3 Aging of the immune system

"Immunosenescence is currently a prognostic factor for human longevity, and thus a more sophisticated appreciation of immune dysfunction will contribute to increased quality of life of the elderly population". Larbi, Franceschi, Pawelec (2008)

While at the social, political and economic levels healthcare institutions and governments have clearly defined the concept of healthy and active aging, at the biological level there is not much consensus on the specific signature that may characterise this process. We know that the immune system is profoundly affected. Deterioration of immune protection plays a pivotal role in increasing susceptibility of elderly persons to infectious diseases, vaccine failure and possibly autoimmunity and cancer compared with the young (Ershler, 1993; Goronzy and Weyand, 2012; Pawelec, 1999; Targonski et al., 2007). Therefore, a deeper understanding of the erosion of the immune system with age is necessary.

This phenomenon was first termed immunosenescence by Roy Walford in 1969 (RL, 1969). In the evolutionary view, immunosenescence is not a random process, but is subject to the laws of evolution. The trend of thymic ontogenesis and involution supports this hypothesis (Shanley et al., 2009). As aforementioned, nowadays humans live on average an enormous amount of time longer than that predicted by evolutionary forces. For this reason, the elderly have to deal with an unforeseen lifelong antigenic load. In fact, the quality of aging depends heavily on the individual's resilience to stressors. The immunological history (antigenic exposure, latency and persistence) is an important determinant of later health (Franceschi and Bonafe, 2003). Many factors determine quality and quantity of the antigenic exposure such as historical period, geography (developed or underdeveloped countries), social-economic status and education.

In the next sections, I will be discussing the age-related alterations that occur in the different subsets of immune cells. The aging process affects both innate and adaptive immunity as summarized in Tab. 1.2 and detailed thereafter.

Table 1.2 Main age-related changes in the immune system

Type of immunity	Cell type	Age-related changes
Innate immunity	Neutrophils	↓ Oxidative burst ↓ Phagocytic activity ↓ Chemotaxis
	Macrophages	↓ Oxidative burst ↓ Phagocytic activity ↓ MHC II complexes Conflicting results on pro-inflammatory cytokines production
	NK cells	↓ $CD56^{bright}CD16^{neg}$ subset ↑ $CD56^{dim}CD16^{pos}$ subset ↓ Responsiveness to cytokines ↓ Cytotoxicity
	Dendritic cells	↓ Antigen presenting capacity ↑ Cytokines production ↓ Homing to lymph nodes
Adaptive immunity	T cells	↑ Number of memory/effector T cells ↓ Number of naïve T cells ↓ Diversity of T cell repertoire Specific phenotypic signature
	B cells	↑ Number of memory B cells ↓ Number of naïve B cells

1.3.1 Adaptive immunosenescence

The adaptive immune system acts later during an infection, using pathogen-specific responses and highly depends on innate cell presentation of antigens. It is composed of two main cell populations: B cells which play a role in the humoral immune response (antibody production), and T cells involved in cell-mediated immune responses (helper function for CD4 and cytotoxic function for CD8 T cells). Both B and T cells can be at three stages of differentiation. Naïve lymphocytes have matured, left the bone marrow/thymus, have entered the lymphatic system but have not met their cognate antigen yet. Effector cells have been activated by their cognate antigens and are actively involved in immune responses. Memory lymphocytes are very long-lived antigen-experienced lymphocytes (Alberts B., 2007). Naïve cells ensure a specific response to any potential new foreign antigen and memory cells guarantee a more rapid and magnified response to subsequent encounters of a previously experienced antigen (Weng, 2006).

It is worth to note that adaptive immunity is more susceptible to the deleterious effects of aging than innate immunity.

1.3.1.1 T cells

The main populations of T lymphocytes are T helper (Th) cells (CD4), cytotoxic T cells (CD8), regulatory T cells (Treg), gamma/delta T cells (γδT), intraepithelial lymphocytes (IEL) and mucosal-associated invariant T cells (MAIT). T helper lymphocytes influence the functions of other immune cells by releasing a variety of cytokines. They help activate B cells to secrete antibodies and promote killing activity of macrophages and cytotoxic T cells. Cytotoxic T lymphocytes use perforins and granzymes to induce apoptosis in cancer cells, infected cells (particularly by viruses) or cells damaged for other reasons. Regulatory T cells are a subpopulation of T cells which modulate the immune system and maintain tolerance to self-antigens to avoid autoimmune reactions (Alberts B., 2007). The γδ and MAIT cells have been identified more recently and the focus of many scientists is to discover the antigen they respond to and their biological role while very few studies identified a link to immunosenescence (Walker et al., 2014).

When discussing T cells and immunosenescence one always tend to include the involution of the thymus as a possible player. Actually, the modification of thymic environment start from the first years of life and declining thymopoiesis is almost complete by the age of 40–50 years in humans (Busse and Mathur, 2010). Most vertebrates show thymic involution but an evolutionary explanation has yet to be proven. Shanley et al. proposed that, once a T cell repertoire has been built, the host benefits from a downregulation of thymopoiesis and rechanneling of energy to other systems, especially reproduction (Shanley et al., 2009).

Repeated antigenic stimulation throughout life may compromise T cells through two processes. First, persistent antigen challenge can lead to exhaustion and loss of crucial functional activities. Second, they may undergo the so-called replicative senescence, losing permanently their ability to proliferate as a result of telomere erosion and/or unrepaired DNA damage. There is still considerable confusion about the relationship between senescence and exhaustion when referring to highly differentiated T cells. Senescent and exhausted T cells share some common defects and emerge as a way of preventing excessive and damaging T cell responses to persistent infections. However, we currently know that these two mechanisms are independently regulated and define cell populations that are phenotypically and functionally different.

Exhaustion is characterized by the hierarchical and progressive loss of T cell functions due to high chronic antigenic load (Akbar and Henson, 2011). Exhausted T cells express the isoform CD45RO and show a low level of chemokine receptor CCR7 and co-stimulatory molecules CD27/CD28, whereas they upregulate various inhibitory receptor on their surface, including CD28 family

member programmed cell death 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin domain and mucin domain protein 3 (TIM-3) and lymphocyte activation gene 3 (LAG-3), CD244/2B4, CD160, T cell immunoreceptor with Ig and ITIM domains (TIGIT) and others (Blackburn et al., 2009; Wherry, 2011). IL-2 production and proliferative ability are the first functions to be lost, while TNF-α production fails later; cytotoxic activity is also inhibited in exhausted human T cells. At a severe stage of exhaustion, interferon-γ (IFN-γ) production is compromised and, ultimately, exhausted T cells are deleted (Wherry et al., 2003). Exhaustion develops when there is a high antigenic load as during cancer (Speiser et al., 2014) and chronic infections with human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and human T lymphotropic virus 1 (HTLV1) (Wherry, 2011). The discovery that blockade of the PD-1 and TIM-3 pathway could partially reverse exhaustion and reduce viral or tumor load was a significant breakthrough (Barber et al., 2006; Hirano et al., 2005; Sakuishi et al., 2010).

The phenomenon of replicative senescence was first described by Hayflick and colleagues in 1965 in human lung fibroblasts cultures (Hayflick, 1965). Thereafter, many researchers investigated this process in other somatic cell types such as epithelial cells, hepatocytes, endothelial cells and keratinocytes. More recently this phenomenon has been examined in the context of the immune system. Cultured senescent T cells share similar functional changes with other senescent somatic cells. They express cell cycle inhibitors including cyclin dependent kinase (Cdk) inhibitors p21CIP1/WAF1 (p21) and p16INK4a (p16) (Campisi and di Fagagna, 2007; Liu et al., 2009) and acquire a pro-inflammatory secretion pattern called senescence-associated secretory phenotype (SASP) (Coppe et al., 2010). Resistance to apoptosis triggered by a variety of stimuli, such as anti-Fas antibody, IL-2 withdrawal, mild heat shock, galectin-1 and staurosporine, is elevated (Spaulding et al., 1999). However, these cells have been selected for their ability to survive *in vitro* and may not be representative of freshly isolated highly differentiated T cells. The irreversible cycling arrest can be caused by the erosion of telomeres, the repeating hexameric sequences of nucleotides at the ends of linear chromosomes, occurring at each replication cycle. Indeed, senescent T cells appear to have shorter telomeres (Hodes et al., 2002; Plunkett et al., 2007). If telomerase, the enzyme that elongates telomeres, declines in activity, the chromosome is recognized as a double strand initiating a process commonly referred to as the DNA damage response (DDR). Senescence can also be triggered by telomere-independent DNA damages, including reactive oxygen species or ionizing radiation, chromatin perturbation and activation of p53 and stress pathways in response to growth factor deprivation. If the damaged DNA is repaired, the DDR ceases and the cell resumes

cell cycling. However, in case the DNA damage is not repaired, proliferation arrest becomes irreversible.

Currently little information about replicative senescence mechanisms in T cells is available and investigations are hindered by significant differences in the regulation of this process in rodents and humans. For instance mice have considerably longer telomeres than humans but have a much shorter lifespan (Akbar et al., 2000). Even though senescent T cells can no longer proliferate, they retain some, albeit dysregulated, effector functions in response to antigenic stimulation *in vitro*. As CD8 T cells are driven to senescence in cell culture, they maintain potential cytotoxic ability (through granzyme B and perforin) (Rufer et al., 2003) and produce more proinflammatory cytokines, mainly IL-6 and TNFα (Effros et al., 2005; Parish et al., 2009), than their more early-passage counterparts. Interestingly, these same inflammatory cytokines are strongly associated with frailty in the elderly (Hubbard et al., 2009). Unlike IL-6 and TNFα, there is no definitive consensus on the capacity to produce the anti-viral cytokine IFN-γ. Most studies reported a decreased IFN-γ production (Effros et al., 2005; Ouyang et al., 2000).

A critical phenotypic and genetic change in T cell senescence is the loss of gene and surface expression of CD28 marker, an essential co-stimulatory receptor that activates T cell pathways such as the Akt and NF-κB pathway and modulates crucial functions such as lipid raft formation, IL-2 gene transcription, apoptosis, stabilization of cytokine mRNA, glucose metabolism and cell adhesion (Vallejo, 2005). Indeed, one of the most striking age-related changes observed in the T cell compartment is an increase in the number of $CD28^{neg}$ cells (Weng et al., 2009). At birth, all human T cells express the co-stimulatory molecule CD28. However, in the elderly 10–15% of CD4 and 50–60% of CD8 T cells lack CD28 expression. $CD28^{neg}$ CD8 T cells display limited antigen-induced proliferation, reduced diversity of T cell receptors, shortened telomeres and increased resistance to apoptosis (Vallejo, 2005).However, the expansion of senescent cells seen in old individuals and the related impaired immune function is likely a consequence of constant exposure to pathogens, some of which persist throughout life, more than a consequence of the aging process itself (Brunner et al., 2011). Along with CD28, other phenotypical characteristics have been attributed to senescent T cells, mainly the lack of CD45RO isoform, of costimulatory molecules CD27 and of chemokine receptor CCR7 accompanied by reacquisition of CD45RA isoform and up-regulation of KLRG1 and CD57 (Larbi and Fulop, 2014).

In CD4 T cells, there is a defect in IL-2 production and thus decreased CD154 (CD40 ligand) expression (Haynes et al., 1999); hence, the capacity of CD4 T cells to interact with CD40 on the B cell surface and help B cell proliferation and antibody production is reduced.

Overall, the T cell pool is highly modified during aging; however many of these changes are physiological as T cells are very responsive to pathogens and will express the history of their activity on the surface or intracellularly.

1.3.1.2 B cells

The number of B cell precursors in the bone marrow undergoes some changes over time. While the number of pro-B cells remains the same in young and old animals, there is a profound decrease in the number of pre-B cells in aged animals. Paradoxically, this decrease is not accompanied by a similar decline of mature B cells in the periphery (Stephan et al., 1996). A possible interpretation of these results is that aged bone marrow is able to compensate for the reduction in the number of pre-B cells by allowing a greater proportion of pre-B cells to mature into newly formed B cells. This would mean that the mechanisms that tightly regulate the production of new B cells in young mice are relaxed in old mice. Therefore, important quality control checkpoints may be compromised in exchange for quantity of output, potentially leading to the production of autoreactive and functionally defective B cells (Johnson et al., 2002).

Similarly to the T cell pool, the B cell pool shows a reduction in naïve lymphocytes ($CD27^{neg}$) and a clonal expansion of memory B cells ($CD27^{pos}$) (Colonna-Romano et al., 2003). Furthermore, memory B lymphocytes are less susceptible to apoptosis in the elderly (Chong et al., 2005). The accumulation of memory B cells may limit the diversity of the repertoire and influence the outcome of vaccinations in elderly individuals. Annual influenza vaccination has a lower efficacy in the elderly and although serum immunoglobulin levels are stable during aging, the antibodies generated in old age are of lower affinity and avidity (Johnson and Cambier, 2004). A tight interplay between B cells and other immune cells is crucial for antibody production. Indeed, changes in humoral response with age derive not only from age-related defects in maturation and function of B cells but also from dysregulated interactions with other cell types of the immune system. As mentioned above, aged CD4 T cells produce less IL-2 and thus express less CD154 (CD40 ligand), which is crucial in the interaction of B and T cells. A gap in the understanding of B cell biology in aging is the lack of information on possible alterations of the cell-to-cell interactions involving the B cells.

1.3.2 Innate immunosenescence

Innate immunity represents the first non-specific line of host defence that is already present at birth. It is an evolutionary conserved process and exists in all species of plants and animals. It does

not depend on a host's prior exposure to the specific pathogen and provides the basis for an adequate adaptive response to pathogens. Innate immune response consists of physical and chemical barriers, sets of proteins and cells such as neutrophils, monocytes-macrophages, natural killer cells and dendritic cells (Alberts B., 2007).

While there are strong evidences for changes in the adaptive immunity, the consequences of aging on the innate branch of the immune system have been less well investigated until recent years. It is noteworthy that, during aging, adaptive immunity declines whereas some innate responses are even activated, inducing a characteristic pro-inflammatory profile (Salminen et al., 2008). The potential up-regulation of the innate immune response in the elderly may come from a lack of negative feedback from T cells and decreased Treg activity with age (Tsaknaridis et al., 2003). The elevated plasma concentrations of IL-6, IL-1β, C-reactive protein (CRP) and TNF-α have been described in the elderly and postulated as predictive markers of age-associate pathology and mortality (O'Mahony et al., 1998). These factors are thought to contribute to a lifelong stimulation of the immune system, resulting in the subclinical chronic inflammatory status seen in the elderly and defined as inflamm-aging (Franceschi et al., 2000). The consequent inflammatory response, along with tissue damage and production of reactive oxygen species, also elicits the release of additional cytokines (Cannizzo et al., 2011). This leads to a self-perpetuating vicious cycle that sustains the pro-inflammatory status. Inflamm-aging supports the development and progression of age-related diseases with an inflammatory basis, such as dementia (Bruunsgaard et al., 1999), atherosclerosis (Bruunsgaard et al., 2000), sarcopenia and frailty syndrome (Krabbe et al., 2004), cachexia (Ruscin et al., 2005), osteoporosis (Ginaldi et al., 2005), diabetes (Argiles et al., 1994) and obesity (Norman et al., 1995).

According to the antagonistic pleiotropy theory of aging, the beneficial effects of inflammation devoted to the neutralization of harmful agents early in life and in adulthood may become detrimental late in life in a period not foreseen by evolution. The inflamm-aging may be the price to pay in older age for an active immune system at younger age (Franceschi et al., 2000).

Cells producing pro/anti-inflammatory molecules are not limited to the immune system as most of cells in the human body such as endothelial cells, adipocytes, fibroblasts and others are capable. For this reason inflammaging should not be seen as an immunity-restricted phenomenon.

1.3.2.1 Neutrophils

Neutrophils are short-lived phagocytes that act as the first responders during acute inflammation. They represent more than 50% of the white blood cells in the circulation. They ingest and kill

bacteria and fungi through production of oxygen and nitrogen oxidative species and generation of proteolytic enzymes and microbicidal peptides. In some circumstances, neutrophils are also able to kill pathogen extracellularly, extruding DNA coated with antimicrobial agents (neutrophil extracellular traps). Once neutrophils have cleared the invaders, they undergo programmed cell death (apoptosis) (Alberts B., 2007).

The number of neutrophils is well preserved with age and there is no loss of ability to generate a robust neutrophilia in response to infection (Chatta et al., 1993). However, some functions, such as chemotaxis, phagocytosis, superoxide anion production and apoptosis are reduced (Kovacs et al., 2009; Shaw et al., 2010). Interestingly, phagocytosis of unopsonized bacteria occurs at the same level in young and old subjects (Emanuelli et al., 1986). This suggests that receptors for innate recognition of bacterial components (e.g. the LPS receptor CD14) are not affected by aging. In contrast, the expression of the Fcγ receptor CD16 and Fc-mediated superoxide production is significantly reduced in neutrophils from elderly donors (Butcher et al., 2001). Since both Fc receptor-mediated superoxide production and phagocytosis are reduced, it is possible that Fc signalling pathways are altered in old people. Fulop et al. found that without stimulation the susceptibility of neutrophils to apoptosis is slightly increased with aging (Fulop et al., 1997). They also observed that agents, such as granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2 and LPS, which prevent apoptosis of neutrophils in young subjects, are unable to inhibit the programmed cell death of neutrophils obtained from elderly subjects (Fulop et al., 1997).

1.3.2.2 Dendritic cells (DCs) and Monocytes

Dendritic cells act as messengers between the innate and the adaptive immune branches. They represent only a minute fraction of immune cells (<3%) in the circulation. Their primary role is up-taking, processing antigens and presenting the resulting peptides to T cells. In addition, dendritic cells release a variety of cytokines that have pro-inflammatory action (IL-1, IL-6) or affect adaptive immunity (IL-12) (Alberts B., 2007). Aged dendritic cells were shown to lose ~50% efficiency in up-taking antigens through various mechanisms such as macropinocytosis and endocytosis than their young counterparts (Agrawal et al., 2007). Thus they may not be able to stimulate T cells effectively. Furthermore, dendritic cells were found to have increases in LPS- and single-stranded RNA-induced TNF-α and IL-6 production (Agrawal et al., 2007), contributing to inflamm-aging. Finally, dendritic cells trafficking to drain lymph nodes is affected by aging, as a result of impaired expression of the lymph node homing marker CCR7 (Grolleau-Julius et al., 2008). Unfortunately, many of these aging studies utilise DCs derived from *in vitro* differentiation of monocytes despite clear evidence showed

the significant functional difference between *ex-vivo* dendritic cells and monocyte-derived dendritic cells (Wimmers et al., 2014).

The number of monocytes in peripheral blood does not change substantially with age, although there is a decreased number of macrophage precursors and bone marrow macrophages (Plowden et al., 2004). The non-classical $CD14^{pos}$ $CD16^{neg}$ monocytes significantly increased with age, but displayed reduced HLA-DR and CX3CR1 surface expression in the elderly (Seidler et al., 2010). It seems that the pool of monocytes maintain with aging mainly due to change in monocyte subsets which are mostly the non-classical $CD14^{pos}$ $CD16^{pos}$, $CD14^{pos}$ $CD16^{neg}$ and $CD16^{pos}$ $CD14^{low}$. Other studies have reported similar results (Nyugen et al., 2010) in which numbers of $CD14^{high}$ $CD16^{pos}$ and $CD14^{low}$ $CD16^{pos}$ monocytes were significantly increased in aging. The production of cytokines such as IL-6 and TNF-α by the subsets is altered in aging. The associated ERK-MAPK signalling seems faulty in the monocyte subsets in aging. One exercise training intervention study showed that ghrelin and adiponectin increased levels were negatively correlated with the percent change in $CD14^{pos}$ $CD16^{pos}$ monocytes in the exercise group. This suggests that lifestyle and intervention are important modulators of the inflammatory capacity of monocytes. Finally, in flu-vaccine challenged individuals it was recently reported that the sustained IL-10 response by monocyte subsets in elderly was associated with the antibody response. The induction of the $CD14^{pos}$ $CD16^{pos}$ inflammatory monocytes after vaccination was paralleled by the production of TNF-α and IL-6 in the classical $CD14^{pos}$ $CD16^{neg}$ monocytes. Levels of IL-10 were significantly higher in monocytes from elderly individuals during the course of vaccination (Mohanty et al., 2014).

While neutrophils and macrophages are able to phagocytose extracellular pathogens, NK cells remove intracellular microbes, especially viruses. In addition to this, they also possess tumouricidal activities. The ability to perform such a large spectrum of functions renders NK cells of major importance during immune responses. Alteration of this equilibrium in old age may increase susceptibility of the elderly to infections and malignancy. In section 1.4, I give detailed description of the state-of-the-art of NK biology and the changes that accompany the aging phenomenon.

1.4 NK cell biology and immunogerontology

"…besides the much publicised increase in viral infection rates, several features of the ageing process may be attributable in part to the decline in NK cell function that accompanies human ageing. If true, then developing strategies to prevent, delay or reverse NK cell immunesenescence may be one way by which to improve the health of older adults".

Hazeldine and Lord (2013)

The traditional cell surface phenotype defining human NK cells in the lymphocyte gate on the flow cytometric analyser lacks CD3 (thereby excluding T cells) and shows expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) (Caligiuri, 2008). Three NK cell subpopulations can be identified in humans: $CD56^{dim}CD16^{pos}$ are terminally differentiated cells with cytotoxic functions; $CD56^{bright}$ $CD16^{neg}$ cells are immature cytokine-secreting cells; $CD56^{neg}CD16^{pos}$ are cells with poor proliferative and cytotoxic capacity, described in HIV, hepatitis B and C patients (Cooper et al., 2001). In the murine model, all NK cells express CD161, a C-type lectin receptor recognized by the NK1.1 antibody in C57BL/6 and C57BL/10 strains (Huntington et al., 2007b).

Historically, NK cells have been considered short-lived innate lymphocytes that can rapidly respond to pathogen-derived or stress-induced molecules in an antigen-independent manner and then undergo cell death (Cerwenka and Lanier, 2001). More recently, they have been classified as cytotoxic, IFN-γ-producing members of the relatively new group of innate lymphoid cells (ILCs) (Artis and Spits, 2015). Once thought to be important only as killer cells, after four decades since their first observation, our comprehension of NK cells has expanded to include regulatory activity (Fu et al., 2014), antigen-specific memory-like responses (O'Sullivan et al., 2015), tissue repair (Zenewicz and Flavell, 2011), cross-talk with other immune cells (Malhotra and Shanker, 2011), clearance of senescent cells (Sagiv et al., 2013), resolution of inflammation (Waggoner et al., 2012) and so on. These data clearly highlight that NK cells comprise a more heterogeneous, sophisticated and enigmatic cell population than originally suggested. Furthermore, accumulating insights into NK cell clinical applications (especially cancer immunotherapy and vaccination) (Berrien-Elliott et al., 2015; Rydyznski and Waggoner, 2015) make their study a current hot topic in the immunology field.

1.4.1 NK cell functions

1.4.1.1 Cytotoxic ability

NK cells can directly kill abnormal cells via two contact-dependent mechanisms: granule exocytosis and death receptor ligation (Smyth et al., 2005). Granule exocytosis is mainly executed by $CD56^{dim}$ NK cells and characterised by the secretion of cytotoxic molecules, namely perforin, granzymes and granulysin. To ensure that NK cells do not kill indiscriminately, this process is tightly regulated and coordinated (Topham and Hewitt, 2009). Upon binding to the target membrane, an immunological synapse forms at the point of contact with the target cell and there is a rearrangement of the actin

cytoskeleton. Then the microtubule-organizing centre (MTOC) of the NK cell and the secretory lysosomes are polarized towards the lytic synapse. The secretory lysosomes dock with the plasma membrane at the lytic synapse, before finally fusing with it and releasing their cytotoxic contents. CD107a, also known as lysosome-associated membrane protein 1 (LAMP-1) is located on the luminal side of lysosomes. After activation, CD107a is transferred to the cell membrane of activated NK cells and for this reason it is commonly used as a marker of degranulation. Once released, perforin polymerises on the target cell surface, causing the creation of pores and the co-endocytosis of perforin and granzymes (Thiery et al., 2011). Inside the target cell, perforin triggers endosomal lysis, leading to the release of granzymes into the cytoplasm (Thiery et al., 2011). Eleven granzymes (A, B, C, D, E, F, G, H, K, M, and N) have been described. Ten of these (A–K, M and N) are expressed in mice, and five in humans (A, B, H, K and M) (Smyth et al., 2005). Granzyme B is the most studied. It is an aspartase able to activate several members of the caspase family, including caspase 3 (Goping et al., 2003). Caspase 3 induces apoptosis by activating the endonuclease caspase-activated DNase (CAD) or degrading proteins involved in DNA repair (Taylor et al., 2008). Granzyme B can also activate caspases 3 and 7 indirectly through mitochondrial permeabilisation, leading to the release of the pro-apoptotic protein cytochrome c into the cytosol (Alimonti et al., 2001). Consequently, cytochrome c binds to ATP, apoptosis-activating factor 1 (Apaf-1) and pro-caspase 9, forming a complex named apoptosome. This results in the activation of caspase 9 that mediates apoptosis by cleaving and activating caspases 3 and 7. (Bao and Shi, 2007).

The second pathway involves death receptor engagement. Upon cytokine stimulation (Sato et al., 2001) or binding of activating receptors (Chua et al., 2004), NK cells express Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) on their surface, which ligate their cognate molecules Fas and TRAIL on the target cell. This interaction results in the formation within the target cell of a signalling complex composed by the adaptor molecule Fas-associated protein with death domain (FADD) and pro-caspases 8 and 10 (Bao and Shi, 2007). Recruitment of several caspase precursors into this complex drives their activation through an 'induced-proximity' mechanism, which enables the enzymes to process each other proteolytically. The death signal propagates into the cell either by processing of effector caspases (caspases-3 and -7) or alternatively by cleaving of Bid into tBID, which induces mitochondrial permeabilisation and the release of cytochrome c (Lavrik et al., 2005).

1.4.1.2 Immunoregulation of other cells

Beside their ability to kill aberrant cells, NK cells show also significant immunoregulatory functions. This job is mainly responsibility of the regulatory $CD56^{bright}$ subpopulation of NK cells NK cells

activated by cytokine stimulation (Mariani et al., 2002) or target cell challenge (De Maria et al., 2011) secrete multiple cytokines and chemokines. In mice, it has been demonstrated that NK cell–derived cytokines are critical in early responses to intracellular parasites such as Listeria, Toxoplasma and Leishmania and in resistance to cytomegalovirus infection (Orange et al., 1995; Wherry et al., 1991). The most prominent cytokines produced by NK cells are TNF-α and IFN-γ. NK cells have been reported to secrete several other molecules, namely immunoregulatory cytokines such as IL-5, IL-10, IL-13, IL-2, the chemokines macrophage inflammatory proteins (MIP) MIP-1α, MIP-1β, IL-8 and RANTES (CCL5) and GM-CSF (Fauriat et al., 2010). Through these factors, NK cells are able to affect adaptive immune responses by promoting DCs maturation and directing T cell differentiation. Indeed, NK cells boost the antigen presenting ability of macrophages and drive the maturation of DCs (Vitale et al., 2005). Furthermore, it has been demonstrated that, after migrating to draining lymph nodes, NK cells help drive Th1 cell polarisation by inhibiting Th2 cell differentiation while promoting Th1 cell development, through secretion of IFN-γ (Martin-Fontecha et al., 2004).

1.4.1.3 Regulation of NK cell activity

Quality and quantity of NK cell responses are heavily impacted by the surrounding cytokine microenvironment and the interactions with other cells. Activation and survival are positively regulated by cytokines such as IL-2, IL-15, IL-12, IL-18 and type I IFNs and dampened by molecules with immunosuppressive activities such as transforming growth factor β (TGF-β).

IL-2 is produced by activated T cells and DCs and signals through activation of the Janus kinases (JAK) Jak1/Jak3 and the signal transducers and activators of transcription (STAT) STAT3/STAT5 pathway; the results on NK cells are promotion of survival through the induction of the anti-apoptotic protein Bcl-2 and increased IFN-γ secretion, cytotoxicity and proliferation (Yu et al., 2000).

IL-15 is produced by activated DCs and macrophages and, as IL-2, signals in NK cells through activation of Jak1/Jak3 and STAT1/STAT3/STAT5 regulating NK cell activation, proliferation, survival (Huntington, 2014).

IL-12, a cytokine that is secreted by monocytes, macrophages, and DCs in response to microbial insults, stimulates NK cells to secrete IFN-γ and augments the proliferation and cytolytic activity. IL-12 signals by promoting the activation of Jak2 and STAT3/STAT4, which triggers cytokine secretion and cytotoxicity by NK cells (Ferlazzo et al., 2004).

IL-18 is secreted by DCs and macrophages and signals through activation of Tyk2 and STAT4. This cytokine is involved in the stimulation of the migratory potential of NK cells, therefore, contributing

to the cooperation between NK cells and DCs for the activation and polarization of the adaptive immune response (Mailliard et al., 2005).

Type I IFNs (IFN-α/β) are strong stimuli for NK cells, which are produced during viral infections mainly by plasmacytoid DCs. IFN-α signals through Jak1 and different STATs. Activation of STAT1 and STAT2 regulate IFN-α-mediated cytotoxicity, whereas activation of STAT4 promotes IFN-γ secretion (Nguyen et al., 2002).

All stimuli coming from other cells, either soluble factors and/or direct receptor engagement in *trans*, are integrated on the NK surface by a complex network of inhibitory and activating receptors. While T and B cells possess a single antigen receptor that dominates their activation, NK cells instead rely on a vast array of germline-encoded receptors that do not undergo somatic recombination (Paust et al., 2010). No single receptor dominates on others; on the contrary, synergistic signals from multiple receptors are integrated to activate or block natural cytotoxicity and cytokine production (Long et al., 2013). NK cells activity regulation is thus remarkably unique in terms of recognition strategies and genomic diversity. Complexity and variety of this receptor system is illustrated in the next sections and in Tab. 1.3.

Table 1.3 NK cell activating and inhibitory receptors in human and mouse

Receptor	Ligand	Function	Host
KIR			
KIR2DL1	HLA-C^{Lys80}	Inhibitory	Human
KIR2DL2	HLA-C^{Asn80}	Inhibitory	Human
KIR2DL3	HLA-C^{Asn80}	Inhibitory	Human
KIR2DL4	HLA-G	Inhibitory	Human
KIR2DL5a	N.D.	Inhibitory	Human
KIR2DL5b	N.D.	Inhibitory	Human
KIR3DL1	HLA-bw4	Inhibitory	Human
KIR3DL2	HLA-A3 and HLA-A11	Inhibitory	Human
KIR3DL3	N.D.	Inhibitory	Human
KIR2DS1	HLA-C^{Lys80}	Activating	Human
KIR2DS2	N.D.	Activating	Human
KIR2DS3	N.D.	Activating	Human
KIR2DS4	N.D.	Activating	Human
KIR2DS5	N.D.	Activating	Human
KIR3DS1	N.D.	Activating	Human
LIR			
LIR1 (CD85J)	HLA-A, B, C, E, F and G, UL18	Inhibitory	Human
LIR2	HLA-A, B, C, E, F and G	Inhibitory	Human
LIR3	N.D.	Inhibitory	Human
LIR5	N.D.	Inhibitory	Human
LIR6	HLA-B	Activating	Human
LIR7	N.D.	Activating	Human
LIR8	N.D.	Inhibitory	Human
Ly49			
Ly49A	H-$2D^b$, D^d and D^k	Inhibitory	Mouse
Ly49B	N.D.	Inhibitory	Mouse
Ly49C	H-$2D^b$, K^d, D^d and D^k	Inhibitory	Mouse
Ly49D	H-$2D^d$	Activating	Mouse
Ly49E	N.D.	Inhibitory	Mouse
Ly49F	H-$2D^d$	Inhibitory	Mouse
Ly49G	H-$2D^b$ and L^d	Inhibitory	Mouse
Ly49H	M157	Activating	Mouse
Ly49I	H-$2K^d$	Inhibitory	Mouse
Ly49J	N.D.	Inhibitory	Mouse
Ly49Q	N.D.	Inhibitory	Mouse
NKG2			
NKG2A	HLA-E (human); H2-Qa1 (mouse)	Inhibitory	Human/mouse
NKG2B	HLA-E (human); H2-Qa1 (mouse)	Inhibitory	Human/mouse
NKG2C	HLA-E (human); H2-Qa1 (mouse)	Activating	Human/mouse
NKG2D	MIC A and B, UL16 (human); Rae-1α-ε, H60a-c, Mult-1 (mouse)	Activating	Human/mouse
NKG2E	HLA-E (human); H2-Qa1 (mouse)	Activating	Human/mouse
NKG2F	N.D.	Activating	Human/mouse
NKG2H	HLA-E (human); H2-Qa1 (mouse)	Activating	Human/mouse
NCR			
NKp30	HCMV pp65, viral HA, Heparin or heparan sulfate	Activating/ Inhibitory	Human/mouse
NKp44	Proteoglycans, viral HA and HN, PCNA, Heparin or heparan sulfate	Activating/ Inhibitory	Human
NKp46	Viral HA and HN, Heparin or heparan sulfate	Activating	Human/mouse
Others			
CD16	IgG	Activating	Human/mouse
DNAM-1 (CD226)	CD112, CD155	Activating	Human/mouse
TIM-3	Gal-9, PS, HMGB1, CEACAM-1	Inhibitory	Human/mouse
CEACAM-1	CEACAM-1 and 5	Inhibitory	Human/mouse
CD96 (Tactile)	CD155	Inhibitory	Human/mouse
TIGIT	CD112, CD155	Inhibitory	Human/mouse
2B4 (CD244)	CD48	Activating/ Inhibitory	Human/mouse
KLRG1	Cadherins	Inhibitory	Human/mouse
Siglec-7	Sialic acid	N.D.	N.D.

Abbreviations: HA, hemagglutinins; HN, hemagglutinin neuraminidases; PCNA, proliferating cell nuclear antigen; PS, phosphatidylserine; N.D., not determined

1.4.1.3.1 Inhibitory receptors

NK cells are able to recognize virus-infected, stressed and malignant cells by sensing self-molecules levels on the surface of target cells. Invariably all healthy nucleated cells of mammalian organ systems possess transmembrane immunological molecules namely MHC (Major Histocompatibility Complex) class I molecules. Their counterparts in humans are HLAs (Human Leukocyte Antigens). Any situation lowering or altering MHC class I expression (cancerous transformation, viral infection etc.) leads to "missing-self" cell lysis (Biassoni, 2009).

Two major families of MHC class I inhibitory receptors evolved convergently in different species but they display common features such as organization in multi-gene groups, expression patterns, MHC-I specificity, signalling and effects on NK-cell functions. For these reasons, insights in defining inhibitory receptor functions in small animal models could have direct relevance in human studies. Killer cell immunoglobulin-like receptors (KIRs) developed in primates while rodents and others evolved type II integral membrane Ly49 molecules, belonging to the C-type lectin superfamily (Yokoyama et al., 2004).

A common feature of inhibitory receptors is the presence in their cytoplasmic region of conserved immunoreceptor tyrosine-based inhibitory motifs (ITIM). After the inhibitory receptor binds its ligand, the ITIM becomes phosphorylated in a specific tyrosine, which will recruit src homology 2 domain-containing tyrosine phosphatases (SHP)-1. This signalling cascade terminates with the inhibition of NK cytolytic activity (Long, 2008).

KIR and Ly49 loci are extremely polymorphic, both in terms of gene numbers and alleles present, creating a huge heterogeneity. The 15 KIR genes map on human chromosome 19 and recognize HLA-A, HLA-B and HLA-C alleles. KIR receptors are named based on the number of their extracellular Ig-like domains (2D or 3D) and by the length of their cytoplasmic tail (long (L), short (S), or pseudogene (P). The number following the L, S or P differentiates KIR receptors with the same number of extracellular domains and length of cytoplasmic tail. KIRs recognize groups of HLA class I molecules according to the amino acids at the C-terminal portion of the HLA class I α1 helix (Boyington and Sun, 2002). In particular, the recognition of inhibitory KIR2D depends to a large extent on the nature of the MHC class I amino acid present at position 80: for instance, KIR2DL1 recognises the group of HLA-C molecules with a Lys80 residue (HLA-C2 specificity), whereas the KIR2DL2 and KIR2DL3 allelic forms recognize the group of HLA-C with an Asn80 residue (HLA-C1 specificity). Regarding inhibitory KIR3D, KIR3DL1 interacts with Bw4-containing HLA-B alleles and KIR3DL2 binds HLA-A3 and HLA-A11 allotypes (Parham, 2005). KIR3DS1 and KIR2DS1/2/3/4/5 lack

ITIM motifs and instead carry a lysine residue in their tail that anchors DAP12, and adapter molecule that transduces activation signals (Lanier, 1998).

At least 23 Ly49 members exist (from Ly49A to W) on the mouse chromosome 6, in a region termed the NK gene complex (NKC). Most of the Ly49 NK cell receptors are described as inhibitory, based on functional data or on the presence of the ITIM sequence in their intracytoplasmic tails. On the contrary, Ly49D, H, L, M, P, R, U and W are believed to be activating.

Other inhibitory receptors are the leucocyte Ig-like receptors (LIR) (especially CD85j, also known as LILRB1, ILT-2 or LIR-1) (Lanier, 2005) in humans and CD94/NKG2A that is present in both primates and rodents (Borrego et al., 1998). CD85j has first been detected in searching for the counterpart of UL18, a cytomegalovirus encoded HLA class I homolog that is expressed on infected cells (Cosman et al., 1997). CD94-NKG2A heterodimers recognize the non-classical MHC class I molecule HLA-E in humans and Qa1 in the mouse (Natarajan et al., 2002).

1.4.1.3.2 Activating receptors

The ligands for NK cell activating receptors are non-self molecules and self-proteins that become up-regulated on abnormal cells, such as stress-inducible MHC class I chain-related (MIC) molecules MICA and MICB. The most studied activating receptor on NK cells is CD16 (FcγRIIIa), a glycoprotein of the Ig superfamily with low affinity for IgG. Upon CD16 binding, NK cells release cytokines and mediate antibody-dependent cell-mediated cytotoxicity (ADCC). During ADCC, the Fab portion of the IgG antibody binds to epitopes on the target cell causing the NK cell to bind to the Fc portion of IgG through CD16 and leading to degranulation and target cell death (Seidel et al., 2013).

The NKG2 family comprises four closely related molecules: NKG2A (and its splice variant NKG2B), NKG2C, NKG2E (and its splice variant NKG2H), and NKG2F. With the exception of the homodimer-forming NKG2D and the orphan receptor NKG2F, NKG2 family members heterodimerize with CD94, a C-type lectin-like molecule that allows for interaction of the complex with HLA-E (Orbelyan et al., 2014). As discussed above, NKG2A is inhibitory, whereas other members are activating receptors. As NKG2A, NKG2C binds HLA-E while NKG2D recognizes MICA and MICB as well as the HCMV glycoprotein UL16-binding proteins (ULBPs) in humans and Rae-1 in mice (Arase et al., 2002).

The natural cytotoxicity receptors (NCRs) consist of two constitutively expressed molecules (NKp46 and NKp30) and one inducible (NKp44). These receptors signal through coupling with the immunoreceptor tyrosine-based activation motif (ITAM)-containing CD3ζ and/or FcεRIγ adaptor proteins and are involved in the recognition of various tumour cells. Functions of NCRs is mainly activating but they have been shown to inhibit NK cells upon binding of specific ligands.

1.4.1.3.3 Newly described receptors

Several other recently described receptors are crucial regulators of NK cell functions. Among them, a family of adhesion proteins has emerged. CD226 (also known as DNAM1) is the most studied member of this group and is able to control NK cell cytotoxicity. It binds to the nectins CD112 and CD155 that are regulated by cellular stress and involved in cancer and infection (Chan et al., 2014). Two inhibitory receptors (CD96, also known as Tactile, and TIGIT) interact with CD226 ligands counterbalancing CD226-mediated activation of NK cells (Bernhardt, 2014; Stanietsky et al., 2009). Furthermore, it has lately been shown that NK cells constitutively express high levels of type I transmembrane receptor Tim-3 (Gleason et al., 2012; Ndhlovu et al., 2012). Tim-3 was originally identified as a marker of terminally differentiated CD4 Th1 cells and subsequently associated with T cell exhaustion (as reported above in this thesis) in HIV-1, HCV and HBV infections (Golden-Mason et al., 2009; Sakhdari et al., 2012; Wu et al., 2012). To date, three ligands have been described for Tim-3, including Galectin-9 (Gal-9, a 40-kD S-type β-galactoside-binding lectin), cell-surface phosphatidylserine and the DNA-binding protein called high-mobility group box 1 (HMGB1) (Freeman et al., 2010). Gal-9 is highly expressed in immune tissues and its engagement of Tim-3 on T cells can trigger either inhibitory or activating signals, with the outcome depending on the interaction with other receptors and still unidentified ligands. The main investigated roles of Gal-9/TIM-3 pathway are reduction of proliferation and IFN-γ production as well as induction of apoptosis in CD4 Th1 cells to dampen Th1 immunity and induce peripheral tolerance (Zhu et al., 2005). A new layer of regulation of TIM-3 function in T cells has been recently discovered. TIM-3 is co-expressed and forms heterodimers in *cis* and *trans* with carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1 also known as CD66a) (Huang et al., 2015). The presence of CEACAM1 endows TIM-3 with inhibitory function and facilitates its maturation and cell surface expression. Simultaneous blockade of CEACAM1 and TIM-3 enhance anti-tumour response in murine colorectal cancer models. Thus, CEACAM1 acts as a heterophilic ligand for TIM-3 that is necessary for TIM-3 ability to mediate T cell tolerance and inhibition, uncovering a significant role in autoimmunity and anti-tumour immunity (Huang et al., 2015). Other studies have reported the expression of TIM-3 on Th17 cells (Seki et al., 2008), NK and NKT cells (Gleason et al., 2012; Liu et al., 2010; Ndhlovu et al., 2012), dendritic cells {Nagahara, 2008 #200}, mast cells (Nakae et al., 2007) and macrophages (Monney et al., 2002), suggesting a far more complex role for this molecule, beyond T cell exhaustion. Although TIM-3 is present on several immune cell types, its highest expression has been found on NK cells. Little is known about the impact of Tim-3 on NK cell responses and its interactions

with other NK cell receptors. Tim-3 has recently been proposed as a marker for mature and fully functional NK cells, with those expressing the highest levels of the receptor displaying the most potent cytotoxic activity or cytokine production (Ndhlovu et al., 2012). On NK cells, Tim-3 can act as an activating co-receptor, since exposure to Gal-9 enhances IFN-γ production by Tim-3^{pos} NK cells (Gleason et al., 2012) and it can also deliver inhibitory signals, given that the ability of NK cells to kill target cells is decreased upon Tim-3 cross-linking (Ndhlovu et al., 2012). Indeed, up-regulation of Tim-3 on NK cells has been associated with reduced anti-viral properties in chronic HBV infection and NK cell cytotoxicity was enhanced by Tim-3 blockade (Ju et al., 2010). Therefore, the regulatory effects of Tim-3 on NK cells are distinct depending on immune microenvironments and interaction with different ligands such as CEACAM1.

Novel strategies harnessing the vast array of NK receptors to target infected and malignant cells are of crucial importance, especially checkpoint blockade of inhibitory receptors and the use of agonist antibodies to stimulate activating receptors.

1.4.2 NK cell development and maturation

The sequential acquisition of the receptors and functional capabilities described above can be monitored by flow cytometry during *in vitro* NK cell differentiation. Three main stages are identified, namely NK cell precursors (NKPs), immature NK cells (iNKs), and mature NK cells (mNKs).

1.4.2.1 NK cell precursors

NK cell precursors (NKPs) are the first step toward NK cell commitment and result from a sequential loss of pluripotency. Human NK cells originate from CD34^{pos} hematopoietic stem cells (HSCs) passing through common lymphoid progenitors (CLPs). For long, the bone marrow has been considered the only site of human NK cell differentiation. However, there is evidence that NK cell development from HSCs occur in tissues other than bone marrow. In culture, CD34^{pos} cells isolated from different sites (peripheral blood, umbilical cord blood, thymus, secondary lymphoid organs, foetal and adult liver and decidua) give rise to mature NK cells (Montaldo et al., 2013). NKPs can be identified as CD34^{neg} CD117^{pos} CD244^{pos} CD161^{pos} CD56^{neg} cells that produce mainly GM-CSF, IL-5 and IL-13 (Loza et al., 2002). In the mouse model, NKPs have been identified in the foetal thymus and the bone marrow of adult animals by the acquisition of CD122, the beta chain of IL-2 and IL-15 receptor complex and by the following phenotype: Lin^{neg}CD244^{pos}CD127^{pos}NK1.1^{neg}DX5^{neg}CD27^{neg}CD11b^{neg} (Di Santo, 2006).

Transcription factors of the Id family support generation of NKPs from the CLPs. Id2 can inhibit E2A, a transcription factor associated with B and T cell development (Engel and Murre, 2001). Thus the relative balance of Id2 and E2A determines lineage commitment at this stage.

1.4.2.2 Immature NK cells

The acquisition of CD56 marks the passage to iNKs that are described as $CD117^{pos}$ $CD244^{pos}CD161^{pos}NKp44^{pos}CD56^{bright}CD16^{neg}$ cells. These cells are cytokine-secreting cells that constitute about 10% of the peripheral NK cells and are mostly present in secondary lymphoid organs (Montaldo et al., 2012). They comprise the commonly termed immunoregulatory subset. Indeed, upon DCs-derived signals such as IL-2, IL-12, IL-15, IL-18, they can readily proliferate and produce high levels of cytokines like IFN-γ, TNF-α, GM-CSF, IL-13, IL-10, IL-8 and IL-22 (Fauriat et al., 2010).

In mice, development of iNKs is accompanied by acquisition of NK1.1 (C57BL/6 and C57BL/10 mouse strains), CD94, NKp46, NKG2D, TRAIL and CD27 (Di Santo, 2006).

Development of iNKs is modulated by the interplay between the transcription factors NF-IL3 (E4BP4) and T-bet (Bernardini et al., 2014).

1.4.2.3 Mature NK cells

As they mature, iNKs upregulate other receptors such as CD16, NKp46, KIRs, NKG2D and CD226 (DNAM1) while CD56 expression dims. Different experimental evidences support the idea of progression from iNKs ($CD56^{bright}CD16^{neg}$) to mNKs ($CD56^{dim}CD16^{pos}$) that compose approximately 90% of circulating NK cells (Chan et al., 2007). This process is accompanied by a decrease in CD94/NKG2A, CCR7, CD117, CD27 and CD62L expression and a lower proliferative potential (Moretta, 2010). Hyporesponsiveness to cytokines leads to a weaker secretion of IFN-γ; however, acquisition of CD16 makes $CD56^{dim}CD16^{pos}$ NK cells the main IFN-γ-producing population upon target cells recognition (Luetke-Eversloh et al., 2014).Additionally, $CD56^{dim}CD16^{pos}$ cells show higher expression of KIRs accompanied by a stronger cytolytic activity than $CD56^{bright}$ $CD16^{neg}$ cells (Jacobs et al., 2001). Maturation from $CD56^{brigth}CD16^{neg}$ to $CD56^{dim}CD16^{pos}$ NK cells also modulate adhesion molecules (CD11a/CD18 also called LFA-1) and chemokine receptors (including CCR7, CXCR1, CX3CR1, and ChemR23), changing their homing target from secondary lymphoid organs to inflamed tissues (Parolini et al., 2007).

In mice, mNKs upregulate CD11b (Mac-1), CD43, KLRG1, DX5 (CD49b), Ly49, LY6C while losing CD127, TRAIL and CD27 expression (Di Santo, 2006). After exiting the bone marrow, NK cells continue to mature and gain further functional competence as they undergo homeostatic proliferation in peripheral organs. Several distinct phases of mNK cells differentiation occur in periphery and have been described in the literature mainly using co-expression of CD11b and CD27. According to these markers, murine mNK cells are divided in two populations with different effector function potency and proliferation ability: mature 1 ($CD27^{pos}CD11b^{pos}$) and mature 2 or terminal ($CD27^{neg}CD11b^{pos}$) (Chiossone et al., 2009; Hayakawa and Smyth, 2006; Huntington et al., 2007b).
Generation of mNKs and their functional differentiation are genetically regulated mainly by Zbtb32 (Beaulieu et al., 2014), Blimp-1, Eomes and T-bet (Daussy et al., 2014; Townsend et al., 2004). Function of T-bet needs coordination with Zeb2 expression in order to complete differentiation of NK cells in mice (van Helden et al., 2015). Furthermore, Deng et al uncovered a pathway by which Foxo transcription factors play an inhibitory role in terminal NK cell development (Deng et al., 2015).

1.4.2.4 Terminally differentiated $CD57^{pos}$ NK cells

Björkstrom (Bjorkstrom et al., 2010) and Lopez-Verges (Lopez-Verges et al., 2010) independently showed that human NK cells continue to differentiate beyond the transition from $CD56^{bright}CD16^{neg}$ to $CD56^{dim}CD16^{pos}$ cells. They investigated the expression of the T cell senescence marker CD57 during NK cells maturation, suggesting its importance in the identification of highly mature human NK cells.
CD57 was originally attributed specifically to cells with natural killer activity. However, it is commonly used to identify terminally differentiated senescent T cells. Heterogeneity of CD57 expression is observed in NK cell subsets. Immature $CD56^{bright}CD16^{neg}$ regulatory NK cells do not express CD57, even during chronic infections. The main innate populations expressing CD57 are $CD56^{dim}CD16^{pos}$ cytotoxic NK cells and $CD56^{neg}CD16^{pos}$ inflammatory NK cells (Bjorkstrom et al., 2010; Lopez-Verges et al., 2010). The acquisition of CD57 thus follows the natural differentiation of NK cells (Nielsen et al., 2013).
Similarly to T cells, it could be thought that CD57 is a marker of terminal cell differentiation for NK cells too. However, its expression on NK cells is not associated with senescence of the innate immune system and its role is still not definitive. Indeed, anti-retroviral therapy in chronic HIV-infected patients was able to restore phenotype and functions of NK cells (Brunetta et al., 2010).
Since CD57 is a marker of NK maturation, its expression is coupled with loss of antigens associated with $CD56^{bright}$ NK cells such as NKp46, Nkp30, NKG2D and CD62L and with the up-regulation of

CD16, KIR and CD85j. The strong association of CD57 with adhesion molecules and the acquisition of homing molecules restricted to inflamed peripheral tissues (CXCR1, CX3CR1) suggest a local role of CD57 NK cells during inflammation (Carrega and Ferlazzo, 2012).

Differentiation of $CD57^{neg}CD56^{dim}$ to $CD57^{pos}CD56^{dim}$ NK cells is coupled with a loss of proliferative abilities in response to inflammatory cytokines *in vitro*. The decrease of IL-2Rβ, IL-12Rβ and IL-18Rα may explain this proliferation defect (Bjorkstrom et al., 2010; Lopez-Verges et al., 2010). However, assessment of proliferation marker Ki-67 in cytotoxic NK cells did not reveal any differences between $CD57^{neg}CD56^{dim}CD16^{pos}$ and $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells (Lopez-Verges et al., 2011). This suggests that mature NK cells were cycling recently *in vivo* in response to specific stimulation such as viral antigens or indirectly in response to pro-inflammatory cytokines.

Elevated expression of CD16 by $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells renders them hyper-responsive to CD16 ligation that mimics ADCC. Cytotoxicity potential including degranulation (CD107a) and expression of granzyme B and perforin is thus increased in mature NK cells. Finally $CD57^{neg}CD56^{dim}CD16^{pos}$ NK cell secreted more IFN-γ than $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells only in response to inflammatory cytokines but not after cross-linking with CD16 (Lopez-Verges et al., 2011).

Functional role of $CD57^{pos}$ NK cells during human physiology and pathology has been reviewed recently by our group (Kared et al., 2016). The observations that CD57 expression is associated with T cells replicative senescence, absent or low on foetal and newborn NK cells (Abo et al., 1984) and increases with age on NK cells (Le Garff-Tavernier et al., 2010) highlight the significant role of CD57 during aging in the NK cell population. Indeed, at birth all $CD56^{dim}$ NK cells in blood lack CD57 expression, then 25-60% of $CD57^{pos}CD56^{dim}$ NK cells are present among European adults between the age of 18 and 60 years old and this percentage increases in the elderly (>80 years old) (Le Garff-Tavernier et al., 2010).

$CD57^{pos}$ NK cells may accumulate over time as a consequence of cumulative exposure to infections. Notably, while among Caucasians NK differentiation is highly age-dependent, $CD56^{dim}$ $CD57^{pos}$ NK cells rapidly accumulate in Gambian children reaching adult levels by 5 years of age due to the different infection burden (Goodier et al., 2014). These data stress the difference between chronological and immunological age. In cleaner environment, the more an individual age, the more she/he has to deal with a greater antigenic load. Thus the aging process and one's immunological history (antigenic exposure, latency and persistence) are intimately interrelated. After the acute phase of infection, members of the herpesvirus family, such as CMV, Epstein-Barr virus (EBV) and herpes simplex virus (HSV) establish a simptomatically silent latent phase for the lifetime of the host. However, immune suppression due to old age, diseases or drugs can cause reactivation and

clinical manifestation (Sinclair and Sissons, 2006). T cells and NK cells are crucial responders in the immune response against infection, especially against herpesviruses (Mossman and Ashkar, 2005). In particular, CMV prevalence increases with age and chronic infection in old individuals is associated with accumulations of late-differentiated $CD8^{pos}$ T cells, characteristic of CD8 T cell immunosenescence, and with the so-called 'Immune Risk Phenotype' (IRP), predictive of early mortality in the elderly (Pawelec et al., 2012). This suggests that CMV is a major driving force of T cell immunosenescence. It has more recently been shown that CMV markedly shape the NK cell population repertoire as well, leading to a clear accumulation of CMV-specific highly mature NK cells expressing the surface markers CD57 and NKG2C, as further described later in the text (Heath et al., 2016; Lopez-Verges et al., 2011). This subset is the same that expands during aging and is considered a hallmark of NK immunosenescence. However, this skewing is reported to be strongly associated to CMV infection rather than to aging as no significant differences are observed between young and old CMV seropositive donors (Campos et al., 2014). Indeed, only in CMV-seronegative donors (with the lowest frequencies of $NKG2C^{pos}CD57^{pos}$ NK cells) there was a significant correlation between $NKG2C^{pos}CD57^{pos}$ NK expansion and age (Heath et al., 2016). It is still not clear if the virus by itself or the associated inflammation drive the differentiation of NK cells. *In vivo*, a better prognosis has been associated with increased frequency of mature NK cells in CMV-infected patients receiving solid organ transplantation. A slower disease progression is also observed in chronic HIV patients presenting elevated frequency of poly-functional NK cells expressing CD57 (Ahmad et al., 2014).

These results show the complexity of dissecting the role of aging and infections in driving NK cell maturation and draw attention to the need of including a detailed infectious status in all gerontologic studies. Indeed, even though HCMV infection is a major confounder of the association between age and NK cell properties, many published studies do not report HCMV status.

The first functional characterization of NK cells was based on their potential to kill malignant cells presenting a reduction of MHC class I expression and increase of danger signals-associated molecules (such as stress related molecules) (Vivier et al., 2012). Several studies sought to understand if accumulation of mature NK cells late in life may affect their role in the tumour immuno-surveillance. It has been observed a positive cancer prognosis with higher peripheral frequency of NK cells expressing CD57 during leukemia, lymphoma or carcinoma cases (Balch et al., 1983; Nielsen et al., 2013). Moreover, it was recently observed a local expansion of $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells in tumour-infiltrated lymph nodes of melanoma patients with higher survival (Ali et al., 2014).

Finally, depletion of $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells is observed during autoimmune disorders such as atopic dermatitis (Matsumura, 1990) or psoriasis (Batista et al., 2013). In contrast, a localized expansion of $CD57^{neg}$ $CD56^{dim}$ $CD16^{pos}$ NK cells occurs in the inflamed target tissues such as skin during psoriasis (Ottaviani et al., 2006), synovial fluid of joints in rheumatoid arthritis patients (Dalbeth and Callan, 2002) or in pancreas during type 1 diabetes (Dotta et al., 2007). Mature $CD57^{pos}$ NK cells seem to have a regulatory role on auto-reactive cells during autoimmune diseases. They may act as sentinels to detect self-modified molecules and to prevent alteration of tissue.

The protective role of $CD57^{pos}$ NK cells during viral infection and tumour progression may confer to these cells clinical applications. New adoptive immunotherapy could be based on the conversion/expansion of mature NK cells before administration to patients. Potential CMV components and soluble factors to drive *in vitro* differentiation of cytototoxic NK cells may be promising. Vaccine strategies favouring innate immunity and specifically boosting expansion of mature cytotoxic NK cells could also be developed.

1.4.3 NK cell memory

The ability of the immune system to respond rapidly and provide enhanced protection against a previously encountered pathogen is defined as immunological memory. This phenomenon has been extensively studied in T cells and proceeds in three phases: cells vastly proliferate upon encounter with the cognate antigen (expansion phase), then the vast majority of them undergo apoptosis (contraction phase) to form a small, but stable, pool of memory T cells (memory phase). Patrolling memory cells persist in the host's tissues until the secondary antigen exposure, when they show enhanced effector functions.

Since innate cells lack the ability to undergo somatic rearrangement of their receptor genes, it was supposed that these cells, including NK cells, are not able to mediate antigen specificity and therefore cannot develop classical immunologic memory (O'Sullivan et al., 2015). However, recent work has revealed that NK cells share more features with T and B lymphocytes than previously suspected, including immunological memory (Min-Oo et al., 2013; Paust and von Andrian, 2011). The memory-like properties of NK cells exist in different settings, including antigen-specific recall response to haptens and virus-like particles (Paust et al., 2010), cytokine-induced memory (Cooper et al., 2009) and enhanced secondary response to cytomegalovirus (MCMV) (Sun et al., 2009). Researchers used the well-characterized NK cell response to MCMV to determine whether immune memory could exist in virus-specific NK cells. In C57BL/6 mice, NK cells bearing the activating receptor Ly49H specifically recognize infected cells expressing the MCMV-encoded protein m157

(Arase et al., 2002). These MCMV-specific NK cells undergo a clonal-like expansion and mediate a protective response (Brown et al., 2001; Lee et al., 2009). This proliferation is antigen-specific because infection of mice with a mutant MCMV lacking m157 could not drive Ly49H^{pos} proliferation (Sun et al., 2009). Using an adoptive transfer system, small numbers of Ly49H^{pos} NK cells proliferate 100–1000 fold in lymphoid and non-lymphoid tissues in the recipient following MCMV infection, resulting in a long-lived pool of memory NK cells. This self-renewing population of NK cells was able to undergo secondary and even tertiary expansion following several rounds of adoptive transfer and virus infection. The memory NK cells recovered from previously infected mice several months later exhibited more robust effector functions *ex vivo* and were far more effective at protection against viral challenge compared to an equal number of resting NK cells from naïve mice, demonstrating a qualitatively different secondary response in NK cells that had previously encountered viral antigen (Sun et al., 2011).

Recent works began to shade light onto the mechanisms governing the formation and expansion of memory NK cells. For instance, it has shown a role for inflammatory cytokines IL-12, IL-15, IL-18 and IL-33 and STAT4 (Firth et al., 2013; Sun et al., 2012). The Immunological Genome Project (www.ImmGen.org) described the kinetic of NK cell gene expression of naive LY49H^{pos} NK cells before MCMV infection and of effectors (day 1.5), late effectors (day 7) and memory cells (day 27) after MCMV infection (Bezman et al., 2012). The transcription factor zinc finger and BTB domain containing 32 (Zbtb32) was one of the most upregulated genes on memory NK cells. Using ZBTB32-deficient mice, it was demonstrated that ZBTB32 is essential for the proliferation and the protective capacity of the virus-specific NK cells and act by blocking the anti-proliferative factor BLIMP1 (also known as PRDM1) (Beaulieu et al., 2014). The microRNA miRNA155 is also involved in the expansion of Ly49H^{pos} NK cells and subsequent memory formation (Zawislak et al., 2013). Moreover, the co-stimulatory molecule DNAM1 (also known as CD226) is required for optimal differentiation of MCMV-specific memory NK cells (Nabekura et al., 2014). Accordingly, the DNAM1 ligand CD155 is upregulated on infected monocytes and DCs after MCMV infection *in vivo*. Following the elimination of the virus, the BIM and autophagy pathways regulate the contraction of the expanded populations of NK cells, giving rise to a stable population of MCMV-specific memory NK cells (Min-Oo et al., 2014). MCMV-specific memory NK cells cannot offer cross-protection against heterologous pathogens as they become specialized and antigen-restricted for the control of MCMV upon rechallenge. Indeed, a study revealed that MCMV-primed memory NK cells have weaker response to influenza and Listeria infections (Min-Oo et al., 2014).

Along with studies on MCMV infection, several reports on animal models proposed that NK cells contribute in recall responses to other viral infections, such as HSV-2 or vaccinia virus infection

(Abdul-Careem et al., 2012; Gillard et al., 2011), influenza (van Helden et al., 2012) and simian immunodeficiency virus (SIV) (Bostik et al., 2009; Reeves et al., 2015).

In humans, NK cells also are heavily involved in the response against HCMV. This is particularly evident in patients with primary NK cell deficiencies or NK cell functional abnormalities (Biron et al., 1989; Etzioni et al., 2005; Orange, 2006) who present uncontrolled and often fatal infections due to HCMV. Humans do not have functional Ly49 genes and so far it has not been demonstrated that proteins of the KIR family, which are the human analogous to Ly49 receptors in mice, can directly recognize antigens on HCMV-infected cells or HCMV components. Furthermore, CMV is a species-specific virus that has coevolved and adapted within its own specific mammalian host (Sun and Lanier, 2009). Currently, there is no evidence of a counterpart of the Ly49H-MCMV m157 interaction described in humans. However, the existence of human memory NK cells has been proposed after the observation that a specific NK cell subset expressing the NKG2C receptor expanded and remained stable during acute HCMV infection following solid organ transplantation (Lopez-Verges et al., 2011), during episodes of HCMV reactivation after hematopoietic cell transplantation (Foley et al., 2012) or after umbilical cord blood transplantation (Della Chiesa et al., 2012). NKG2C^{pos} NK cells show a specific signature characterized by a highly mature phenotype (CD57^{pos}NKG2A^{neg}KIR^{pos}Siglec7^{neg}CD85j^{pos}) (Della Chiesa et al., 2012; Wu et al., 2013). The proliferation of CD94-NKG2C^{pos} NK cells was also observed in *in vitro* co-coltures of human NK cells with HCMV-infected fibroblasts and was hindered by blocking CD94-specific mAb (Guma et al., 2006a). These data support the involvement of a specific receptor-viral ligand interaction in driving the expansion of this population. Similarities have been shown between the murine and the human models. Acute HCMV infection causes a huge proliferation of CD94-NKG2C^{pos} NK cells, comparable to the 100-fold expansion observed for mouse Ly49H^{pos} NK cells after MCMV infection (Kuijpers et al., 2008). NKG2C^{pos}CD57^{pos} NK cells can constitute up to 70% of the total NK cell population in some individuals (Della Chiesa et al., 2012; Foley et al., 2012). Moreover, both Ly49H in mice and CD94-NKG2C in humans associate with the adapter protein DAP12 suggesting a shared signalling pathway (Smith et al., 1998). Taken together, the evidences reported above led researchers to propose that CD94-NKG2C on human NK cells represents the functional counterpart of Ly49H receptor in mice. However, additional work is required to identify the viral component that binds the CD94-NKG2C complex. A report suggested that CD94-NKG2C interacts weakly with the HCMV UL18 glycoprotein (Kaiser et al., 2008).

NKG2C^{pos} NK cells expand in patients with viral infections other than HCMV, such as HCV, HBV, chikungunya virus, hantavirus or HIV (Beziat et al., 2012; Bjorkstrom et al., 2011a; Brunetta et al., 2010; Guma et al., 2006b; Petitdemange et al., 2011) but this occurs only in HCMV sero-positive

individuals. EBV and HSV-2 infection do not trigger a skewing of the NK cell repertoire (Bjorkstrom et al., 2011b; Hendricks et al., 2014). These studies suggest that the expansion of NKG2C^{pos} cells in humans is specific to HCMV infection rather than a generalized response to acute herpesvirus infections.

As discussed above, NK memory cells can be generated in a different experimental settings but it remains unclear whether they are relevant in pathologic contexts. Memory NK cells are surely a very attracting game changer in the vaccination setting and in the management of infectious diseases, either acting alone of supporting adaptive responses (Rydyznski and Waggoner, 2015). For instance, where T cell memory is impaired as in HIV patients, memory NK cells might partially offer protection against disease. Similarly, after haematopoietic cell transplantation, recall responses of NK cells may contribute to host defence against opportunistic viruses, in particular HCMV reactivation. On the other hand, activated NK cells could improve existing T cell-based vaccinations by rapidly providing inflammatory cytokines and chemokines. Reciprocally, T cells can support NK cell memory responses by secreting IL-2, which further drives memory NK cell proliferation. However, the possibility that memory NK cells could eliminate activated T cells should be taken into account (Waggoner et al., 2012).

Discovering the adaptive features of NK cells during viral infection prompted great enthusiasm in exploiting NK cell memory for clinical application in cancer. The biggest challenge is to optimise NK cell activation strategies before infusion and to achieve proliferation and stability of NK cells with potent anti-tumour activity in the patients after transfer. In this regard, induction of memory NK cells with IL-12, IL-15 and IL-18 before transfer led to increased survival of the recipient mice (Ni et al., 2012). Based on the same concept, a Phase I clinical trial using cytokine-induced memory NK cells for treatment of patients with acute myeloid leukemia is currently in progress at Washington University School of Medicine, St Louis, Missouri, USA (NCT01898793) and is estimated to be completed by March 2017.

Figure 6 summarizes the topics discussed above, representing the kinetic of acquisition/loss of surface markers, the functional changes and the transcriptional regulation that accompany the maturation of human NK cells.

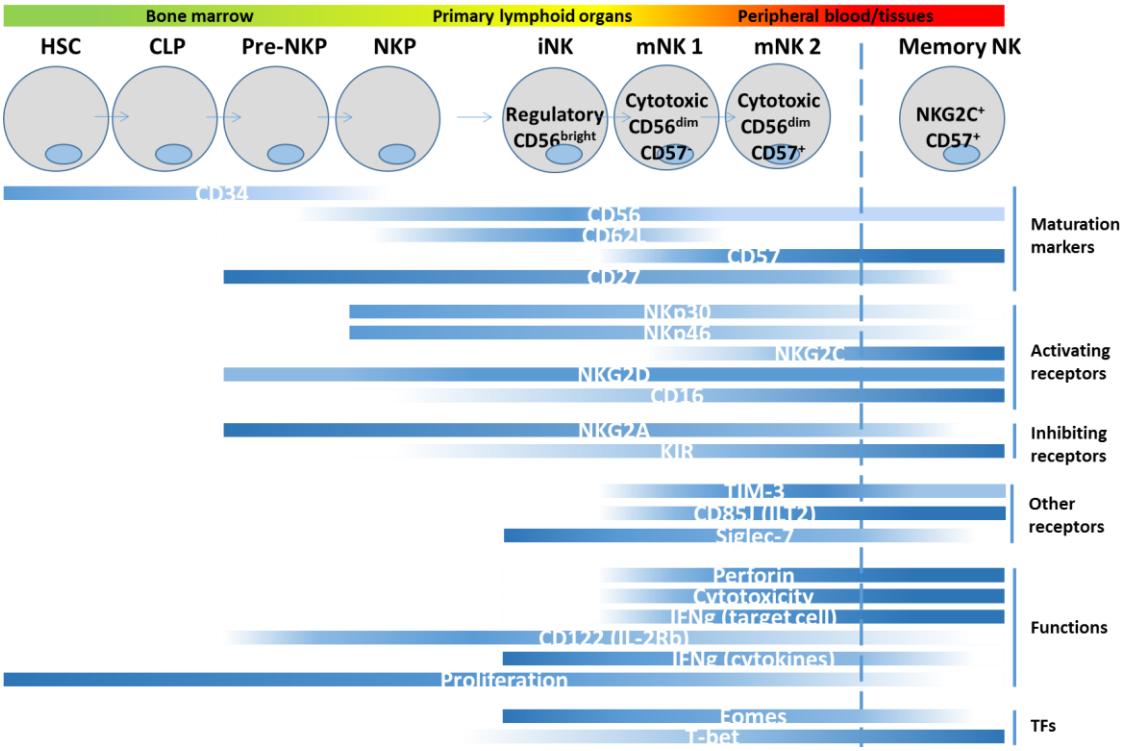


Figure 1.6 Kinetic of acquisition/loss of surface markers, functional changes and transcriptional regulation during human NK cell differentiation

1.5 NK immunosenescence

As already mentioned, NK cells constitute our bodies' frontline defence system, guarding against transformed and virally infected cells. Thus any age-associated alteration of their highly regulated functionality could seriously impact immunological responses in older age. NK immunosenescence have been documented both in mice and humans but mechanistic investigations remain scarce.

1.5.1 NK immunosenescence in mice

With aging, a decreased number and percentage of total NK cells and of $CD27^{neg}CD11b^{pos}$ mature NK cells in most tissues of aged mice has been reported (Beli et al., 2014; Fang et al., 2010). This defect resulted in the loss of resistance to lethal mousepox (Fang et al., 2010). NK cells from aged mice present several maturation defects such lower expression of maturational markers on the surface including KLRG1 (Beli et al., 2014). Nair *et al.* recently (Nair et al., 2015) confirmed and further expanded previous data showing reduced proliferation *in vivo,* dysregulated expression of Eomes and altered expression of collagen-binding integrins. They also demonstrated that this array of defects in NK maturation is the result of deficient maturational signals provided by bone marrow

stromal cells. The hypothesis that the host environment (rather than an intrinsic NK cell defect) is responsible for aging-related functional NK cell deficiency is corroborated by a second study. They found that, after being transferred to aged mice, bone marrow cells from young mice gave rise to NK cells with maturation defects. Conversely, bone marrow cells from aged mice led to $CD27^{neg}$ NK cells normally in young hosts. NK cell defect (decreased proliferation and lack of KLRG1 upregulation) was completely reversed by injecting soluble IL-15/IL-15Rα complexes, suggesting that IL-15 receptor agonists may be useful tools in treating aging-related functional NK cell deficiency (Chiu et al., 2013). Also Nair *et al.* (Nair et al., 2015) investigated the IL-15 signalling as a cause of the dysfunction. They found that treatment with complexes of the cytokine IL-15 and IL-15Rα induced massive expansion of NK cells in aged animals, but most of these NK cells remained immature and were unable to restore resistance to mousepox.

1.5.2 NK immunosenescence in humans

Human aging is correlated with alterations in the composition, phenotype and functions of NK cell, a phenomenon referred to as NK cell immunesenescence (Gayoso et al., 2011; Solana et al., 1999; Solana and Mariani, 2000). In the elderly, changes in properties of NK cell subsets have been reported but there were considerable differences in study outcome. Discrepancies are likely due to the selection criteria of the population analysed such as ethnicity, age range, nutritional and health status. Some authors met the strict SENIEUR criteria of very healthy elderly and centenarians (Fulop et al., 2012; Ligthart et al., 1984), while others studied unselected populations, analysing the relation between NK cell alterations and diseases or mortality. Very healthy elderly and centenarians have shown to have preserved NK cell functionality (Ligthart et al., 1989; Sansoni et al., 1993). Some studies support the hypothesis that preserved NK cytotoxicity is a putative biomarker of healthy aging and longevity, whereas low NK cytotoxicity associates with increased morbidity and mortality as a result of infections, atherosclerosis and poor response to influenza vaccination (Ogata et al., 2001; Solana and Mariani, 2000).

Lack of standardisation led to discordant data. The percentage and absolute numbers of NK cells have been reported to be maintained, increased or decreased in the elderly (Borrego et al., 1999; Gayoso et al., 2011; Hazeldine and Lord, 2013; Solana et al., 2012b). NK cell subsets undergo redistribution with a significant age-related increase in the $CD56^{dim}/CD56^{bright}$ ratio documented with specific accumulation of terminally differentiated $CD57^{pos}CD56^{dim}CD16^{pos}$ cells (Hayhoe et al., 2010; Solana et al., 2006). As reported above, expansion of $CD57^{pos}CD56^{dim}CD16^{pos}$ NK cells is a result of aging and CMV infection, with the latter as a prominent driving force. Decrease of $CD56^{bright}$

cells may be due to a lower output of new NK cells from the bone marrow as a consequence of the age-associated changes in the number and function of hematopoietic stem cells (Wang et al., 2011) and a reduction in new NK cell production and proliferation in the bone marrow (Zhang et al., 2007). The minor population of $CD56^{neg}CD16^{pos}$ cells expands in elderly donors (Campos et al., 2014).

The impact of age on the NK cell phenotypic profile is still controversial. Expression of activation receptors NKp30 and NKp46 have been reported to decline (Almeida-Oliveira et al., 2011; Hazeldine et al., 2012) or remain unchanged (Le Garff-Tavernier et al., 2010). Reduction in NKp30 and NKp46 expression leads to impaired crosstalk between NK cells and other immune cells such as DCs and neutrophils (Boyd and Orihuela, 2011; Solana et al., 2012b). Lack of DCs recognition means that NK cells from older people are less efficient in driving DCs maturation and consequently T cell polarization; abnormal crosstalk with neutrophils results in reduced NK-mediated neutrophil apoptosis and thus slower resolution of inflammation. Other activation molecules such as NKG2D and CD16 were consistently found to be stable with age (Hayhoe et al., 2010; Le Garff-Tavernier et al., 2010; Lutz et al., 2005). Regarding inhibitory receptors, percentage of KIRs-expressing NK cells increases or do not change (Almeida-Oliveira et al., 2011; Lutz et al., 2005). Furthermore, age-associated reductions in KLRG-1, CD94 and NKG2A have been documented (Lutz et al., 2005, 2011; Hayhoe et al., 2010).

How and at what extent aging affects NK cell functions is also a matter of debate. Since $CD56^{bright}$ NK cells are professional cytokines and chemokines producers upon cytokines challenge, their decreased proportion can be responsible of the defective production of soluble factors (especially IFN-γ) by NK cells stimulated with IL-2 or IL-12 observed in old individuals (Mariani et al., 2002). Alteration of functionality in the cytotoxic $CD56^{dim}CD16^{pos}$ cells is disputed with most of the reports showing an age-related loss of killing activity (Facchini et al., 1987; Hazeldine et al., 2012; Miyaji et al., 1997) and few others documenting increase (Krishnaraj and Blandford, 1987; Kutza and Murasko, 1994; Onsrud, 1981) or stability (Almeida-Oliveira et al., 2011; Nagel et al., 1981). The aforementioned increase in the proportion of $CD56^{dim}$ NK cells with aging might act as a mechanism to compensate the decrease in cytotoxicity at the single cell level (Mocchegiani and Malavolta, 2004). Discordant results may come from a variable experimental strategy since each group has performed a different cytotoxicity assay with various co-culture times and using PBMCs, peripheral blood lymphocytes or purified NK cells as effectors. However, all mentioned studies used the MHC class I deficient K562 cell line as target cells.

Little is known about the mechanisms that lead to the discussed abnormal cytotoxicity. NK cells from old individuals are able to recognise and bind to transformed cells with similar efficiency than those from younger subjects, suggesting that the age-related impairment in killing ability results

from a post-binding defect (Facchini et al., 1987; Ligthart et al., 1989; Mariani et al., 1998). This is likely due to reduced release of perforin and the age-related changes in the expression of NK cell receptors reported above (Almeida-Oliveira et al., 2011; Hazeldine et al., 2012). Since NK cells can eliminate senescent cells accumulating in skin, bone and endothelium of older adults through the granule exocytosis pathway (Sagiv et al., 2013), a lower perforin secretion ability could hinder the removal of cell cycle-arrested cells. Accumulation of cells that do not proliferate but secrete large amounts of pro-inflammatory factors compromises tissue homeostasis, contributing to age-related diseases such as sarcopenia, cardiovascular pathologies and cataracts (Baker et al., 2011).

A recent work illustrated gender influences NK cell activity in elderly humans. Women had a higher ratio $CD56^{bright}/CD56^{dim}$ NK cells, indicating a gender-related difference in NK cell maturation in the elderly. Mature NK cells from females responded more vigorously to K562 leukemia cells and secreted more IFN-γ upon NKp46 crosslinking. Finally, NK cells isolated from old women were more likely to produce MIP-1β in response to a variety of stimuli (Al-Attar et al., 2016).

Taken together, these data stress that, while most immunogerontologic studies focused on adaptive immunity, abnormalities in the NK population have severe and various effects on health of older adults. At the same time, it is clear that there is still much controversy in the field and standardised inclusion criteria and experimental design are needed. In particular, I highlighted the importance of including detailed infectious status and gender. Moreover, deeper studies on how aging mechanistically modulates the expression and function of NK cell receptors are required to better understand NK immunosenescence. Finally, no work so far explores the relation between NK cell immunosenescence and NK cell capacity to recall previous antigen encounters.

Chapter 2: Aim and objectives

The aim of the work described in this thesis is to characterise the signature of human and murine NK cells during the aging process and detail the age-associated alterations in subset repartition, maturation, phenotype, transcriptional regulation and effector functions. Ultimately, our research is intended to expand our knowledge of innate immunosenescence, especially NK cell maturation and functionality during aging, leading to novel insights into age-related dysfunctions in NK cell responses.

The study objectives include:

- To analyse the role of CD57, NKG2C and the TIM-3/Ceacam-1 pathway in the maturation, phenotype and function of human NK cells upon aging and other forms of persistent immune activation, such as HCMV infection and hepato-cellular carcinoma (HCC);
- To investigate the age-related abnormalities impacting differentiation, phenotype and function of murine NK cells isolated from blood and spleen of the C57BL/6 wild-type mouse model;
- To determine whether lack of the *Timp-3* gene affects the NK cell differentiation pathway during aging, by comparing the phenotype and function of spleen-derived NK cells between the *Timp-3* knock-out mouse and the C57BL/6 wild-type mouse model.

Chapter 3: Experimental methods

3.1 Donors and sample preparation

3.1.1 Human study: Aging cohort

Elderly participants were recruited from the Singapore Longitudinal Aging Study (SLAS) cohort. SLAS is a population-based prospective cohort study of aging and health of Singaporean older adults with the aims to:

- investigate psychosocial, lifestyle, behavioural, biomedical and healthcare determinants of aging and age-related health outcomes;
- generate research information to promote healthy aging and guide clinical practices in care of the aged people in Singapore.

Table 3.1 reports the main measurements derived from this study. Donors from the SLAS cohort are home-dwelling, aged >65 years old of Chinese background donors. They live in a very similar environment (nutritional, physical activity, socioeconomic status). Many chronic conditions may coexist in this population, namely hypertension, high cholesterol and diabetes. Subjects with a history of hospitalization in the past 6 months and high CRP levels (>3 mg/l) were excluded from the analysis. The study has been approved by the National University of Singapore-Institutional Review Board 04–140 and all participants gave informed consent.

Healthy, young participants of Chinese background were recruited from the National University of Singapore (NUS) students and staff (age 23–35 years old). The study on the young control cohort has been approved by the Ethics Committee of the NUS-institutional review board (IRB) 09-256. All study participants provided informed written consent.

Table 3.1 Main measurements in SLAS study

Domains	Main measurements
Medical and Biological	History of pathologies, medications, supplements, health service use (doctor visits, hospitalization) Blood pressure, electrocardiogram, spirometry, retinal photography, fasting glucose, lipids, homocysteine, albumin, cytokines, C-reactive protein
Biobank	Immune, genetic, inflammatory and other ad hoc studies
Lifestyle	Nutritional status, smoking, alcohol, coffee and tea comsuption, mobile phone and computer use, leisure-time activities
Psychological and neurocognitive	Social network & support, work and retirement, depression Memory and cognitive abnormalities, dementia
Physical fitness	Daily living activities, handgrip, knee extension strength, balance

Blood from overnight fasting participants was drawn in Vacutainer CPT Cell Preparation tubes (BD Biosciences). The Vacutainer CPT tube with sodium citrate is an evacuated blood collection tube system containing 0.1 M sodium citrate anticoagulant and blood separation media composed of a thixotropic polyester gel and a FICOLL Hypaque solution (Sigma Aldrich). As represented in Figure 3.1, the blood separation medium takes advantage of the relatively low density of mononuclear cells to isolate them from whole blood. The separation occured during centrifugation when the gel portion of the medium moved to form a barrier separating the mononuclear cells and plasma from the denser blood components. After centrifugation at 1650 rpm for 20 minutes at room temperature, PBMCs and platelets were in a white layer just under the plasma layer. Plasma was aspirated and PBMCs were collected with a Pasteur pipette.

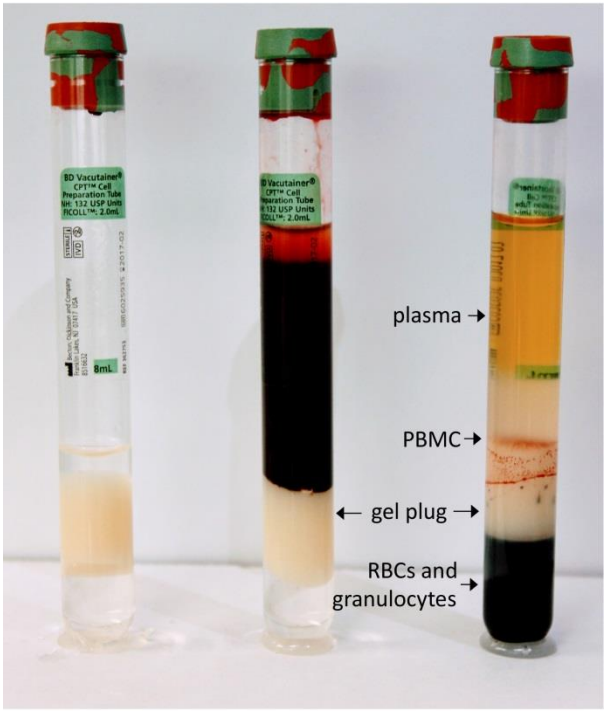


Figure 3.1 Separation of blood components with CPT tubes. Empty CPT (left), after blood draw (middle) and after centrifugation (right). Adapted from (Puleo, 2017).

Plasma was centrifuged to remove cell debris and stored in −80°C, whereas PBMCs were washed twice in phosphate saline buffer (PBS) and cryopreserved in liquid nitrogen. PBMCs were used fresh or frozen. In the latter case, freezing was performed by keeping cells in 90% foetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO). The day of experiment, cryo-vials were thawed rapidly and washed extensively with PBS containing 10% FBS. Samples offered a recovery >75%, with no specific loss of immune population. Viability was >95%, as tested by trypan blue exclusion.

3.1.2 Human study: Hepato-cellular carcinoma cohort

Immune cells were isolated from hepato-cellular carcinoma (HCC) tumours, adjacent non-tumour tissues, as well as PBMCs collected from the same patients at the National Cancer Centre, Singapore. Patient consent was obtained with institutional review board (IRB) approval (2009/524/B). Processing of blood samples was performed as above. Liver tissue specimens were obtained at the time of surgery. Single cell suspension were obtained under sterile conditions in PBS, biopsies were cut into 1-3 mm^3 pieces and digested at room temperature for 1 hour on a stirring apparatus in a solution containing DNAse I grade II (Roche, Cat# 10104159001), and collagenase IV (Sigma Aldrich, Cat# C5138) in Roswell Park Memorial Institute medium (RPMI). Enzymatically dissociated tissue was filtered through a 70 µm filter. Filtered samples were then diluted 1:2 with RPMI and layered onto FICOLL Hypaque (Sigma Aldrich) to obtain a leukocyte-enriched fraction. Cells were then washed three times in PBS to remove debris and freshly used in downstream assays.

3.1.3 Murine study

Study on C57BL/6 wild type and *Timp-3* KO mice was approved by the Institutional Animal Care and Use Committee (IACUC) in Singapore and local Biological Committees in the UK. *Timp-3* KO mice were a kind gift from Prof. Hideaki Nagase, Kennedy Institute of Rheumatology in Oxford, UK. The mice were originated at Prof. Rama Khokha's lab in Toronto. The wild type C57BL/6J mice were bred in the Biological Research Facility at University of Southampton and Singapore. Mice were killed by CO_2 euthanasia. Blood was collected through cardiac puncture, transferred into PBS + 10 mM ethylenediaminetetraacetic acid (EDTA) tubes and lysed with red blood cell (RBC) lysis buffer (eBioscience, Cat# 00-4333-57) for 15 minutes at room temperature. The remaining whole blood was clotted at room temperature for 2 hours and centrifuged (10 minutes at 3000 rpm, 4°C). The resulting serum was aliquoted and stored at -80°C. Whole spleens were collected and digested at 37°C in RPMI + 10% FBS + 0.2mg/ml collagenase IV (Sigma Aldrich, Cat# C5138)+ 20ug/ml DNase I grade II (Roche, Cat# 10104159001) for 30 minutes. Red blood cell lysis was performed on splenocytes suspension using ammonium chloride (ACK) lysis buffer for 5 minutes on ice. Cell suspension was finally washed extensively and used fresh in downstream assays or aliquoted and stored in liquid nitrogen for future use.

3.2 Cell stimulations

A cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin was used to induce activation of NK cells (50 ng/ml of PMA and 500 ng/ml of ionomycin) and T cells (20 ng/ml of PMA and 250 ng/ml of ionomycin). PMA is a potent activator of protein kinase C (PKC) *in vivo* and *in vitro*; it binds to the C1 domain of PKC, inducing membrane translocation and enzyme activation. Ionomycin is a Ca^{2+} ionophore that synergizes with PMA in enhancing the activation of PKC.

In order to obtain a more physiological stimulation, we also activated NK cells taking advantage of their natural ability to respond to the lack of MHC class I molecules and to target cell-bound antibodies during antibody-dependent cell-mediated cytotoxicity (ADCC). Detection of MHC class I molecules and ADCC as NK cell recognition mechanisms during immune responses have been already discussed in the introduction of this thesis. Based on this knowledge, we used the K562 cell line, a myeloid leukemia cell line lacking MHC class I molecules on the surface, at a 1 (target cells) : 10 (K562 cells) ratio. In parallel, activation was performed with anti-CD16 purified antibody-coated plate (1 µg/ml and 10 µg/ml) to trigger ADCC. Detailed conditions of stimulation are present in the specific experimental method sections of the Results chapters.

3.3 Flow cytometry

Flow cytometry is a widely used laser-based method that measures multiple characteristics of cells flowing in single file in a stream of fluid. Light scattering at different angles (forward scatter (FSC) and side scatter (SSC)) can distinguish differences in size and internal complexity, whereas light emitted from fluorescently labelled antibodies can identify a wide array of cell surface and cytoplasmic antigens. The overview of a flow cytometer is depicted in Figure 3.2.

In our study, flow cytometry was performed for analysing the expression of cell surface molecules (phenotype) and intracellular molecules (intracellular cytokine staining (ICS)) or sorting different cell types in a heterogeneous cell population (fluorescence-activated cell sorting (FACS)).

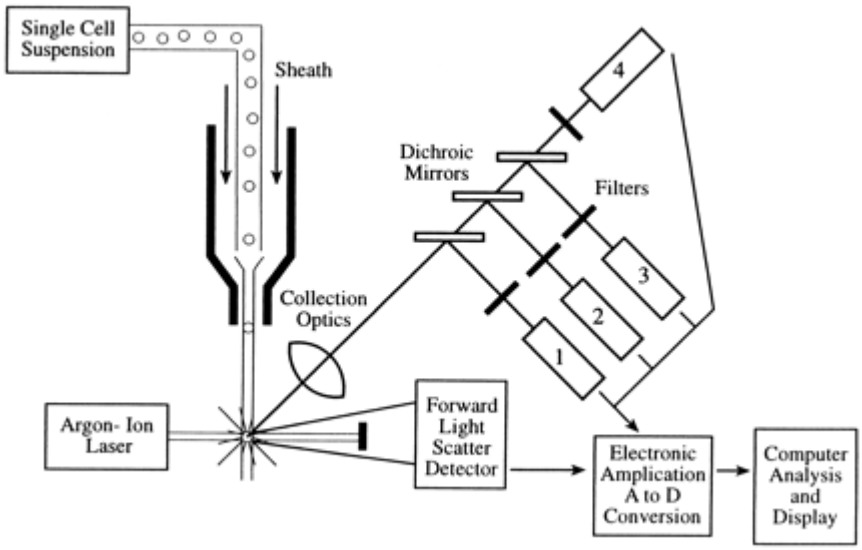


Figure 3.2 Schematic of a flow cytometer. A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Signals are collected by a forward angle light scatter detector (FSC), a side-scatter detector (SSC) and multiple fluorescence emission detectors. The signals are amplified and converted to digital form for analysis and display on a computer screen. Adapted from (Brown and Wittwer, 2000).

3.3.1 Phenotyping

Cell phenotyping was performed by flow cytometry on fresh human PBMCs and immune cells from liver tissue samples and fresh murine spleen and blood samples. For each staining, 1-2×10^6 cells were washed and stained with the fluorochrome-conjugated antibody at the titrated concentration. Specimens were incubated at 4°C for 30 minutes, washed and finally analysed on an LSR Fortessa Cell Analyzer (BD Biosciences). Lymphocytes were gated based on FSC/SSC profile and doublets/dead cell exclusion. T cells were described as CD3-expressing lymphocytes, while NK cells were identified by lack of CD3 and using CD16 and CD56 expression in human studies or NK1.1 in murine assays. The antibodies are listed in the specific experimental method sections of the Results chapters.

3.3.2 Intracellular cytokine staining

Assessment of cytokine release and cytotoxic molecule degranulation was performed by flow cytometry on human PBMCs and liver tissue samples from young and older donors. For each staining, 1×10^6 PBMC were used. Cell stimulation with PMA/ionomycin, K562 cell line or an anti-CD16 purified antibody-coated plate was performed as described above. Unstimulated and PMA/ionomycin-activated cells were used as negative and positive controls respectively. Cells were

incubated for 5 hours at 37°C and 5% CO_2 in the presence of the antibody against the degranulation marker CD107a (BD Biosciences). Protein transport inhibitors Brefeldin A (eBioscience) and Monensin (eBioscience) were added during the final 4 hours of incubation. After incubation, cells were fixed and permeabilised using 100 uL/well of Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. For intra-nuclear antigens, we used Foxp3/Transcription Factor Fixation/Permeabilisation kit (eBioscience). Samples were then washed twice with Perm/Wash buffer (BD Biosciences). Staining with titrated antibodies lasted 30 minutes at 4°C. For the list of antibodies used, refer to the specific experimental method sections of the Results chapters. Flow cytometry was performed on an LSR Fortessa Cell Analyzer (BD Biosciences).

3.3.3 Sorting

Flow cytometry was also performed in order to sort specific subpopulations of interest and assess functionality and gene expression. Cell sorting was done on human NK cells from young and elderly donors according to CD57 and NKG2C expression. Murine splenocytes were sorted to isolate CD4 T cells, CD8 T cells and NK cells. Specimens were stained on the surface using the same phenotype protocol explained above. For the list of antibodies used refer to the specific experimental method sections of the Results chapters. The sorter was FACSAria III (BD Biosciences).

3.4 Mass cytometry

Mass cytometry, or CyTOF (Fluidigm), is a variation of flow cytometry in which antibodies are labelled with heavy metal ion tags rather than fluorochromes. Readout is by time-of-flight (TOF) mass spectrometry (Figure 3.3). By replacing fluorescent labelling of probes for flow cytometry with heavy metal ion labels, the potential for higher multiplexing is greatly enhanced. Unlike the highly overlapping emission spectra of typical fluorochromes, the readout of atomic masses by mass spectrometry is very discrete and can span a wide mass window.

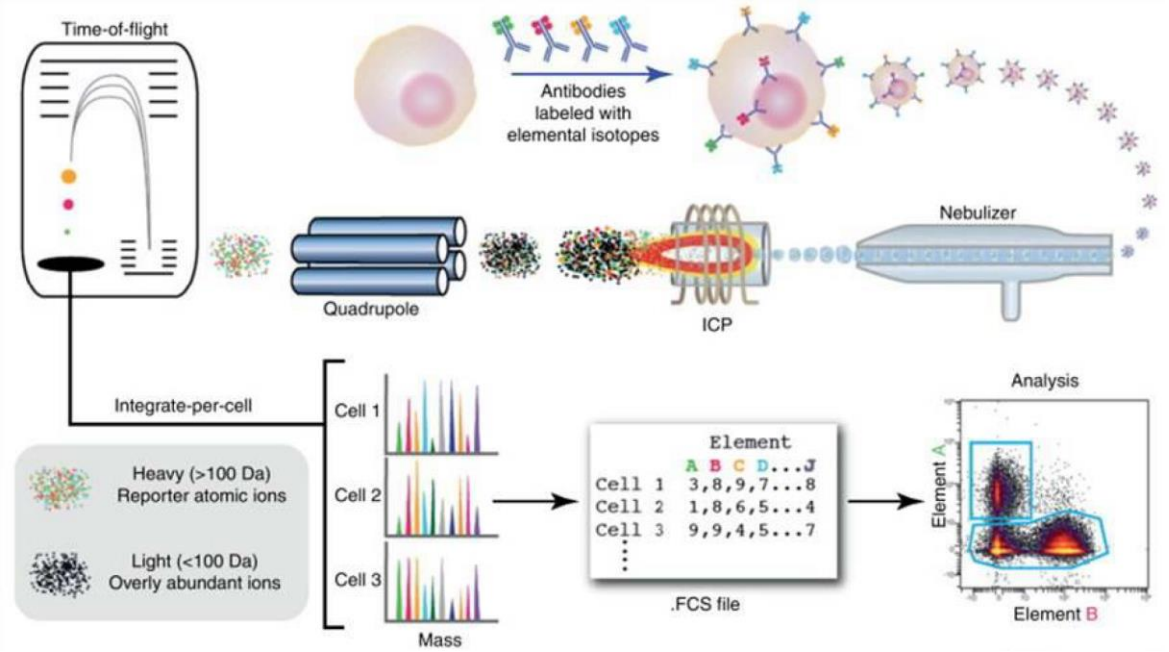


Figure 3.3 Workflow of cell analysis in a mass cytometer. Cells tagged with metal-labelled antibodies are introduced into the nebulizer that converts to a fine spray of droplets. Droplets are then carried into the plasma where they are completely atomized and ionized. The resulting ion cloud is filtered and selected for positive ions of mass range 80-200 and measured in a TOF chamber. The data is then converted to .fcs format and analysed. Adapted from (Bendall et al., 2011).

Three million PBMCs were tested for mass cytometry analysis. Prior to surface staining, cells were stained with cisplatin (viability marker) at a concentration of 5µM in PBS for 5 minutes. Cells were then stained with surface antibody cocktail in RPMI 10% FBS at 37°C for 15 minutes. After two washing steps, cells were fixed in Foxp3/Transcription Factor Fixation/Permeabilisation buffer (eBioscience) for 30 minutes at 4°C. After washing, cells were stained with intracellular antibodies for 30minutes at 4°C in permeabilisation buffer. After two washing steps, cells were barcoded. This allowed the distinct coding of all samples in a 96 well plate, which could be then combined into one tube. For barcoding, 2 mM bromoacetamidobenzyl-EDTA (Dojindo) with 0.5 mM PdCl 2 dissolved in HEPES buffer or DOTA-maleimide (Macrocyclics) with 50 mM $RhCl_3$ (Sigma) or $LnCl_3$ (Trace Sciences) dissolved in L buffer (DVS Sciences) was used. Cells were subsequently washed with permeabilisation buffer, incubated with CyFACS buffer (PBS + 2% FCS + 2 mM EDTA + 0.05% sodium azide) for 5 minutes on ice, washed once more with CyFACS and labelled at room temperature with 250 nM iridium DNA interchelator (DVS Sciences) diluted in PBS with 2% paraformaldehyde. Data were acquired on a CyTOF instrument (DVS Sciences). The antibodies used are listed in the specific experimental method sections of the Results chapters.

3.5 Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA)

Enzyme-Linked ImmunoSorbent Assays (ELISA) is method to measure the antigen concentration in an unknown sample and can be performed with a number of modifications to the basic procedure (as shown in Figure 3.4). The most sensitive and robust ELISA assay format is the sandwich assay. In the sandwich ELISA, the molecule of interest is quantified between two layers of antibodies: the capture antibody that is immobilized on the plate surface and the biotinylated detection antibody. These antibodies must bind to non-overlapping epitopes on the antigen. Streptavidin or avidin conjugated to an enzyme (usually horseradish peroxidase (HRP)) binds the biotin with high affinity and the enzyme hydrolyses its substrate (generally 3,3',5,5'-tetramethyl benzidine (TMB)), releasing a coloured product. The signal is then detected on a suitable instrumentation a spectrophotometer in the case of this study.

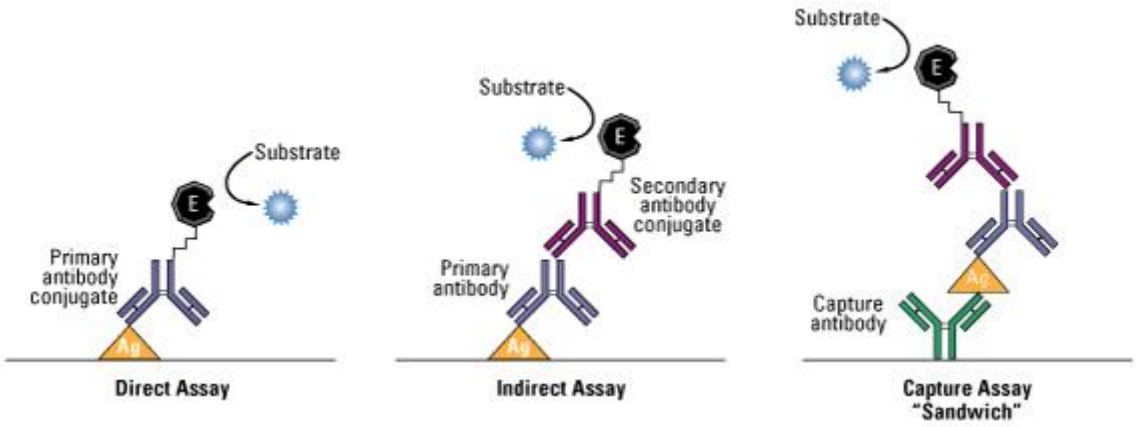


Figure 3.4 Diagram of common ELISA formats. The antigen of interest is immobilized by direct adsorption to the assay plate (left) or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection; middle) or a matched set of unlabelled primary and conjugated secondary antibodies (indirect detection; right). Ag: antigen; E: enzyme. Retrieved from "Overview of ELISA" (https://www.thermofisher.com/nl/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html).

3.5.1 HCMV IgM ELISA

Sero-positivity to HCMV was tested by a HCMV IgM ELISA kit (Genesis Diagnostics, Cat# GD85) according to the manufacturer's instructions. Briefly, plasma samples were diluted (1:100) with the

sample diluent provided. 100 uL/well of the diluted plasma specimens were incubated for 20 minutes to allow specific antibodies to HCMV to bind to the antigen-coated wells. After washing away unbound antibodies and other plasma constituents, HCMV-specific IgM were detected using 100 uL/well of rabbit anti-human IgM conjugated to HRP. After 20 minutes, unbound conjugate was removed by washing and 100 uL/well of TMB substrate was added; the plate was incubated for 10 minutes, with development of a blue colour. Addition of 100 uL/well of the stop solution turned the blue into a yellow colour. The signal was measured using an ELISA reader at 450 nm.

3.5.2 Mouse granzyme B ELISA

Frozen supernatants from sorted and stimulated NK, CD8 and CD4 T cells were thawed and tested with a Mouse Granzyme B ELISA Ready-Set-Go (eBioscience, Cat# 88-8022-22) according to the manufacturer's protocol. The ELISA plate was coated with 100 µL/well of capture antibody, washed and blocked with 200 uL/well of diluent; 100uL of standards and samples were added to the appropriate wells and the plate was incubated at room temperature for 2 hours. After incubation, 100 uL/well of detection antibody were added, plate was washed and then processed with 100 uL/well of avidin- HRP at room temperature for 30 minutes. After washing, we incubated the plate with 100 uL/well of TMB for 15 minutes at room temperature and then the process was stopped using 50 uL/well of stopping solution. Finally, plate was read at 450 nm with an ELISA reader.

3.6 Multi-analyte assays

3.6.1 LEGENDplex

LEGENDplex Human Th Cytokines kit (Biolegend) is a bead-based assay on a flow cytometer similar to the principles of the sandwich ELISA. It utilizes capture beads, biotinylated detection antibodies and streptavidin-phycoerythrin (PE) for analyte detection in order to quantify soluble analytes. Beads recognizing different analytes can be resolved by size (FSC and SSC) and internal fluorescence intensity. The internal dye can be detected by the allophycocyanin (APC) channel. Figure 3.5 represents the assay principle.

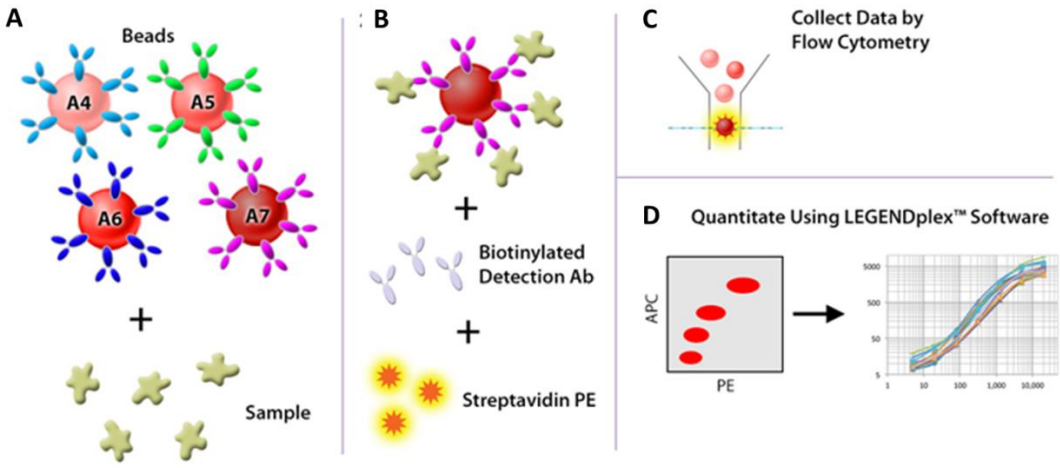


Figure 3.5 Principle of LEGENDplex assay. (A) When antibody-conjugated capture beads are mixed together and incubated with an unknown sample containing target analytes, each analyte will be bound by its specific capture bead. (B) Biotinylated detection antibodies bind to its specific analyte, thus forming capture bead-analyte-detection antibody sandwiches. Streptavidin-PE is subsequently added, which will bind to the biotinylated detection antibodies, providing fluorescent signal with intensities in proportion to the amount of bound analyte. (C) For each bead population, the PE signal fluorescence intensity is then quantified using a flow cytometer. (D) The concentration of a particular analyte is determined based on a known standard curve using the LEGENDplex data analysis software.
Retrieved from "LEGENDplex" (https://www.biolegend.com/legendplex).

The test was performed according to the manufacturer's instructions. Briefly, we added 25 uL/well of assay buffer, 25 uL/well of plasma sample and 25 uL/well of detection antibody. The plate was incubated on a shaker for 2 hours at room temperature. After incubation, the samples were treated with 25 uL/well of streptavidin-PE for 30 minutes at room temperature. Following a centrifugation at 1000 g for 5 minutes, supernatants were removed and wells were washed. Finally, we read PE signal from resuspended beads an LSR Fortessa Cell Analyzer (BD Biosciences).

3.6.2 Human CD8$^+$ T Cell Milliplex

Supernatants from sorted immune cell subsets and serum samples were tested with Milliplex MAP Human CD8$^+$ T Cell kit (Millipore, Cat# HCD8MAG-15K) or Milliplex MAP Mouse Th17 kit (Millipore, Cat# MTH17MAG-47K). MILLIPLEX MAP system is based on the Luminex xMAP technology. Luminex uses a technique to internally colour-code microspheres with multiple fluorescent dyes. Through precise concentrations of these dyes, distinctly coloured bead sets of non-magnetic or magnetic

polystyrene microspheres can be created, each of which is coated with a specific capture antibody. Figure 3.6 summarizes the assay principle.

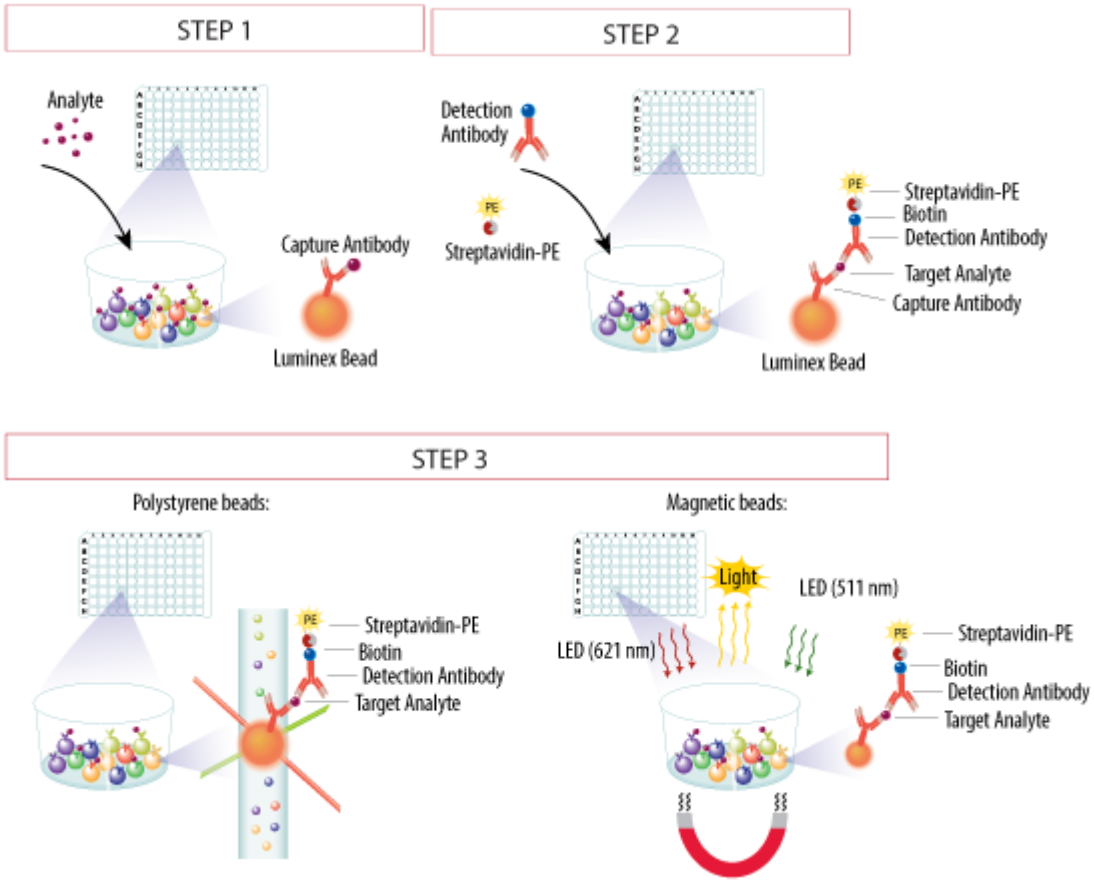


Figure 3.6 Luminex assay principle. In step 1, the sample is added to a mixture of color-coded beads, pre-coated with analyte-specific capture antibodies. The antibodies bind to the analytes of interest. In step 2, biotinylated detection antibodies specific to the analytes of interest are added and form an antibody-antigen sandwich. PE-conjugated streptavidin is added. It binds to the biotinylated detection antibodies. In step 3, polystyrene beads are read on a dual-laser flow-based detection instrument. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Magnetic beads can be read using a different device. A magnet in the MAGPIX analyzer captures and holds the magnetic beads in a monolayer, while two spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and, the second LED determines the magnitude of the PE-derived signal. Each well is imaged with a charge coupled device (CCD) camera. Retrieved from https://www.rndsystems.com/resources/technical/luminex-assay-principle.

Assays were performed according to manufacturer's instructions. In brief, 25 uL of standard or sample were added to the appropriate wells, followed by 25 uL/well of beads and the plate was incubated overnight at 4°C. After washing, we added 25 uL/well of detection antibody and

incubated the plate for 1 hour at room temperature. Plate was processed with 25 uL/well of streptavidin-phycoerythrin for 30 minutes at room temperature and then washed with 150 uL/well of sheath fluid. Finally, signal was detected by Flexmap system.

3.7 Quantitative polymerase chain reaction (qPCR)

To assess gene expression, 100,000-200,000 cells were lysed with RLT buffer (Qiagen) with 1% of β-mercaptoethanol *ex vivo* or after stimulation. β-mercaptoethanol was added to RLT buffer before use to effectively inactivate RNAses in the lysate. Samples were homogenized by vortexing. Cell lysates were stored at –80°C for later use.

RNA extraction was performed using an RNeasy Plus Micro kit (Qiagen, Cat# 74034). First, the lysate was passed through a genomic DNA (gDNA) Eliminator spin column. This column, in combination with the optimized high-salt buffer, efficiently removed contaminating genomic DNA. Ethanol was added to the flow-through to provide appropriate binding conditions for RNA and the sample was then applied to an RNeasy MinElute spin column, where total RNA bound to the membrane and contaminants were washed away. High-quality RNA was then eluted in 14 µl of water.

Isolated RNA was reverse-transcribed into cDNA using the SuperScript First Strand kit (Invitrogen, Cat# 11904018). For each reaction, we combined 1 µl of 10 mM deoxynucleotide triphosphates (dNTP) mix, 1 µl of primer (0.5 µg/µl oligo(dT) 12–18) and diethyl pyrocarbonate (DEPC)-treated water to reach a total volume of 10 µl. The RNA/primer mixture was incubated at 65°C for 5 minutes, then placed on ice. After that, 9uL of a second mix (2 µl of 10X RT buffer + 4 µl of 25 mM $MgCl_{2,}$ + 2 µl of 0.1 M dithiothreitol (DTT) + 1uL of RNaseOUT) were added to each sample. Specimens were incubated at 42°C for 2 minutes. After adding 1 uL of SuperScript II RT, tubes were incubated again at 42°C for 50 minutes. Reaction was terminated at 70°C for 15 minutes and chilled on ice. We finally used 1 uL/tube of RNAse H to eliminate remaining RNA. The reaction was stored at -20°C or used for PCR immediately.

cDNA (<20 ng) was analyzed by real-time PCR with the KAPA SYBR FAST qPCR Master Mix kit (KAPA Biosystems) with integrated antibody-mediated hot start, engineered variant of *Taq* DNA polymerase, SYBR Green I fluorescent dye, $MgCl_2$, dNTPs and stabilizers. The mix was composed of 10 uL KAPA SYBR FAST qPCR Master Mix, titrated amount of 10 uM forward and reverse primer and PCR-grade water to a total volume of 20 uL/reaction. The primers used are reported in the specific experimental method sections of the Results chapters. The cycling parameters in Table 3.2 were used to run the reaction. A melting curve analysis was performed at the end of the run to assess

formation of non-specific products. Assay was run on a 7500 Real-Time PCR System (Applied Biosystems) and analysed with SDS v2.4 software (Applied Biosystems).

Table 3.2 qPCR cycling parameters

STEP	TEMPERATURE	DURATION	CYCLE(S)
Enzyme activation	95 °C	3 minutes	Hold
Denaturation of DNA double chain	95 °C	15 seconds	40
Annealing of primer with DNA template/extension	60 °C	1 minute	40
Dissociation of primer (melting curve)	95 °C 60 °C 95 °C	15 seconds 15 seconds 15 seconds	Hold

3.8 RNA interference (RNAi)

Small (or short) interfering RNA (siRNA) is the most commonly used RNA interference (RNAi) tool for inducing short-term silencing of protein coding genes. siRNA is a synthetic RNA duplex designed to specifically target a particular target mRNA for degradation.

In order to silence *Ceacam-1,* the target gene of our study, we delivered *Ceacam-1* siRNA into PBMCs samples from older donors. Specimens were electroporated with the Neon Transfection System (Invitrogen), an electroporation device that works by using the pipette tip as an electroporation chamber to transfect cells.

PBMCs (3×10^5) were incubated for 18 hours with 10 ng/ml of IL-2 (Peprotech) and 10 ng/ml of IL-15 (Peprotech) to support survival. At the end of incubation, cells were resuspended in 10 µl of buffer T (Neon Transfection kit, Cat# MPK10025, Invitrogen). *Ceacam-1* siRNA (Entrez gene ID 634; detected transcripts NM_001024912.2, NM_001205344.1, NM_001712.4; Ambion) or negative control siRNA (scrambled (Scr); Ambion) were added to the cell suspension at a final concentration of 100 nM. *GAPDH* siRNA (Ambion) was used as a positive control to evaluate efficiency of the silencing. Ten microliters of the suspension were electroporated using optimized electroporation conditions (three pulses at a voltage of 1,700 V for 20 ms).

Cells were then incubated for 24 hours at 37°C and 5% CO_2 and then stimulated in anti-CD16 coated plate (1 µg/ml) for 5 hours at 37°C and 5% CO_2 in the presence of CD107a antibody. Brefeldin A (eBioscience) and Monensin (eBioscience) were added during the last 4 hours of incubation. Surface markers and functions by intracellular staining were assessed by flow cytometry as described above.

3.9 Data analysis

Raw flow cytometry data were analysed using two software packages, namely FlowJo (Treestar) and FACSDiva (BD Biosciences). Both tools are popular and widely used basic flow cytometry softwares for manual and sequential gating of unprocessed data. Outputs are mostly given in the form of unidimensional histograms and biaxial dot plots. FACSDiva only provides data acquisition, machine control, compensation and basic analysis, such as labelling, organization of files and simple gating. FlowJo is a more comprehensive package that offers a broad range of analyses (including cell-cycle, proliferation and kinetics) and several plug-ins to perform data dimension reduction and cluster analysis. Overall, whereas very effective and user-friendly tools for low-dimensional datasets, FlowJo and FACSDiva are limited in their ability to graphically display comparisons for multi-dimensional data present in our study. For these reasons, we used FlowJo and FACSDiva as first-line softwares for acquisition and basic processing of raw low-dimensional data.

The last decade has witnessed significant technical improvements in available cytometry platforms, such that more than 20 parameters can be analysed on a single-cell level by fluorescence-based flow cytometry. The advent of mass cytometry has pushed this limit up to, currently, more than 40 parameters. In manual gating, cell subsets of interest are identified from parent populations via visual inspection of dot plots displaying individual cells' fluorescence intensities. Despite considerable efforts to harmonize immunophenotyping and gating strategies for multicenter studies, this approach suffers from individual user bias when delineating population boundaries and requires prior knowledge of the cell type of interest. Analyses by manual gating focus on specific populations, which often represent only a fraction of the total information contained in a cytometric dataset. Relationships between populations can be overlooked and because biases and *a priori* knowledge dictate analysis, discovery of meaningful, but yet undefined, populations is difficult. Additionally, manual gating is not scalable; as the number of parameters increases, analysing higher-dimensional data by manual gating quickly becomes impractical. For instance, visual analysis of all combinations for a 40-parameter mass cytometry dataset would necessitate examining 780 two-dimensional dot plots. Clearly, the challenge is to build data mining tools that extract relevant information in an objective, precise, reproducible and comprehensive way. Moreover, they should be available to users through intuitive graphical representations and user-friendly interpretation-guided tools.

The first reports on automated cytometry data analysis date back as early as 2007 (Quinn et al., 2007) and opened up the field for future developments (Table 3.3). Early work in analysing high-dimensional cytometry data deployed principal component analysis (PCA), a statistical method for reducing the dimensionality of complex datasets to two or three dimensions. PCA takes all chosen

parameters of the higher dimensional data and calculates a smaller number of variable parameters (called principal components) that best preserve most of the variability of the original data and can be more easily displayed (Genser et al., 2007). PCA performs linear transformations to reduce dimensionality and biological systems can contain many nonlinear relationships. This conflict can result in the production of misleading results by some PCA.

Table 3.3 Overview of the described computational methods for high-dimensional flow cytometry

Tool	Type	Application
PCA	Dimensionality reduction	Visualize linear relationships in multidimensional data
viSNE	Dimensionality reduction	Visualize non-linear relationships in multidimensional data
ACCENSE	Dimensionality reduction	Visualize non-linear relationships in multidimensional data
SPADE	Clustering	Visualize fold differences in expression levels
FlowSOM	Clustering	Cluster cells and visualize marker expression
PhenoGraph	Clustering	Identify unique populations
SPICE	Poly-functionality modelling	Analyse population heterogeneity
Wanderlust	Cell trajectory modelling	Visualize continuous cellular developmental trajectories

To overcome the limitations of linear transformations inherent in PCA, nonlinear dimensionality-reduction techniques can be used. A recently developed tool for nonlinear dimensionality reduction, t-distributed stochastic neighbour embedding (t-SNE), provides a more accurate representation of multidimensional data during the translation to low dimensionality (van der Maaten, 2008). We used t-SNE to obtain unbiased representations of our multi-parameter flow cytometry and mass cytometry data. t-SNE optimally locates cells with similar expression levels near to each other and cells with dissimilar expression levels further apart. t-SNE is the foundational algorithm in two tools for analyzing CyTOF data: viSNE and automatic classification of cellular expression by nonlinear stochastic embedding (ACCENSE). Basically, t-SNE calculates a distance matrix in a high-dimensional space, which is transformed into a similarity matrix. Low-dimensional similarities are calculated using Student's t distribution. viSNE generates a representation of this data that are similar to a biaxial plot and retains the geometry of the populations. The data are represented as cells in high-dimensional data space and do so without relying on traditional gating strategies. viSNE can also discretely and automatically separate cells based on subtype, provided they exist. The cyt feature allows for coloring of cells based on selected expression markers. The data appear as a cloud biaxial

plot with a specific geometry. Differences in populations can be seen as changes in the geometry and events may be coloured to determine which parameter or parameters have changed.

A second group of flow cytometry analysis tools are the clustering-based techniques. Clustering operates by grouping observations into distinct clusters so that similar observations are confined within the same or proximate clusters, and different observations are localized in separate clusters. In a cellular analysis, cells with similar phenotypes are grouped together. One of the most used clustering tools is spanning-tree progression analysis of density-normalized events (SPADE) (Qiu et al., 2011). SPADE depicts cellular populations in a branched tree structure (dendrogram), thus visualizing high-dimensional data in an intuitive, 2D manner. The SPADE tree consists of connected nodes that represent clusters of cells, thereby also providing information about the relationship of cell types.

More recent clustering-based techniques to visualize cytometry data are FlowSOM (flow cytometry data analysis using self-organizing maps) (Van Gassen et al., 2015) and PhenoGraph (Levine et al., 2015). FlowSOM uses self-organizing maps (SOM) to simultaneously cluster and visualize cytometry data in a two dimensional grid of cell type clusters. It provides a similar visualization capacity to SPADE but requires significantly less computation time. PhenoGraph models the high-dimensional space in which each cell is depicted as a node that is connected to its neighbors by edges. In this graph, phenotypically similar clusters of cells will be represented as sets of highly interconnected nodes. These can be seen as “neighborhoods” or “communities” of cells, and can be partitioned in high-dimensional space using similar community-detection algorithms that are being used for the analysis of social networks. The resulting clusters can then be visualized on a t-SNE map, which often corresponds well with the PhenoGraph clustering, or alternatively on a heat map, which will show the expression levels of selected marker across all found clusters.

The software SPICE was used in our work to evaluate the poly-functionality of NK cells (Roederer et al., 2011). SPICE is a data mining software that analyses large FlowJo data sets from poly-chromatic flow cytometry and organizes the normalized data graphically (e.g. pie chart graphs). SPICE was developed specifically to analyse the considerable heterogeneity of some cell populations. In particular, SPICE is ideal for exploring (and quantitatively comparing) the functional and phenotypic profiles of subsets within a complex mixture. The authors used the word “poly-functional” as referred to cells that were able to produce more than one cytokine at the same time.

Developmental trajectory during NK cell maturation was created with the Wanderlust application. Cells are ordered along a trajectory that represents their most likely placement along a developmental continuum (Bendall et al., 2014). In brief, starting from a snapshot of a developmental process at a single time point, the Wanderlust algorithm explores developmental

relationships between cells and constructs a trajectory that resembles the developmental path of a given cellular lineage. It is therefore especially suited to capture and interrogate temporal differentiation processes in the immune system. The Wanderlust trajectory orders the single cells from their most immature to the most mature state, usually using a provisional scale from 0–1, often referred to as a progression or developmental scale. This in situ predicted trajectory can then be used to follow the regulation of all other markers across the developmental path. Similarly, probability state modelling (Bagwell et al., 2015), as implemented in the commercial software Gemstone, has been shown to successfully model cell developmental processes.

3.10 Statistical analysis

Statistical analysis was performed using the statistics software GraphPad Prism (v.6.0c) (GraphPad Software, Inc). Groups of samples were analysed by non-parametric Mann-Whitney U test to compare values. The Wilcoxon matched-pairs signed rank test was used for paired testing of median values of different cell subsets from the same donor. We used the Pearson rank correlation test to compare correlation between anti-HCMV IgG titer and frequency of NK cells subsets. We reported r^2-values and p-values. Analysis with $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were considered significantly different.

Chapter 4: Maturation and function of natural killer cells during persistent immune activation: aging, chronic viral infections and cancer

4.1 Introduction

As part of the innate immune system, NK cells provide important early protection against viruses and tumour cells. The acquisition of effector functions during NK cell differentiation occurs via an intricate series of cellular and molecular events that is finely controlled by transcription factors. T-bet supports NK cells differentiation, with increasing expression as cells become more mature (Townsend et al., 2004). Although T-bet alone can mediate some of the required gene expression changes, coordination with Zinc Finger E-Box binding homeobox 2 (Zeb2) is necessary for complete maturation of NK cells and CD8 T cells (van Helden et al., 2015). Little is known about the role of negative regulators in regulating these processes. A recent study has also highlighted the role of Forkhead box O (Foxo) transcription factors, which play an inhibitory role in terminal NK cell development (Deng et al., 2015).

This transcriptional program is accompanied by a specific tuning of the molecular signature on the surface of differentiating NK cells. As already discussed in the introduction of this thesis, fully mature NK cells are characterized by the surface expression of CD57 (Bjorkstrom et al., 2010; Lopez-Verges et al., 2010) that is best known as a marker for replicative senescence and terminal differentiation in CD8 T cells (Brenchley et al., 2003). Significance of CD57 NK cells in human physiology and persistent immune stimulation such as in aging, cancer and chronic infections has been documented recently by our group (Kared et al., 2016).

One clear example of how infectious stimuli drive NK cell terminal differentiation is HCMV infection. HCMV induces the expansion of a highly mature subset expressing CD57 and the activating receptor NKG2C that may have a crucial role in controlling the infection (Lopez-Verges et al., 2011). $CD57^{pos}NKG2C^{pos}$ NK cells increase in the blood of solid-organ transplant recipients shortly after detection of HCMV viremia and, when the viremia has been controlled, $CD57^{pos}NKG2C^{hi}$ NK cells decrease (Lopez-Verges et al., 2011). Della Chiesa *et al.* described the accumulation of NK cells with the $CD57^{pos}NKG2C^{pos}Siglec\text{-}7^{neg}$ signature upon HCMV reactivation after hematopoietic stem cell

transplantation (Della Chiesa et al., 2014). For these reasons, it has been proposed that $CD57^{pos}NKG2C^{pos}$ NK cells might be memory-like NK cells that expand in response to HCMV reactivation.

Another NK cell maturation marker that is currently receiving much attention is T-cell immunoglobulin– and mucin domain–containing molecule 3 (TIM-3), which marks dysfunctional T cells under chronic immune stimulation. TIM-3 is increasingly considered to be an important target in T cell-based immunotherapy given its important role in immune tolerance and induction of T cell exhaustion in chronic viral infection and cancer (Anderson, 2014). Scarce information is available about the modulation of TIM-3expression by the aging process. In HIV infection, T cell dysfunction and TIM-3 upregulation are driven not only by viral replication but also increased age suggesting that targeting Tim-3 may serve as a novel therapeutic approach to mitigate age-related T cell exhaustion (Tandon et al., 2012). Lee *et al.* found that aging in mice is associated with increased expression of Tim-3 on $CD8^{pos}$ T cells (Lee et al., 2016).

Although TIM-3 is present on several immune cell types, its highest expression is found on NK cells (Gleason et al., 2012; Liu et al., 2010). Tim-3-expressing NK cells have been demonstrated to be highly functional in terms of cytotoxicity and cytokine secretion (Ndhlovu et al., 2012). However, engagement of Tim-3 by one of its ligands (e.g., Galectin-9) or over-expression of Tim-3 during persistent infections, such as HBV (Ju et al., 2010) and HIV (Jost et al., 2013) or in cancer (da Silva et al., 2014) can restrain NK cell function. The discrepancy between these studies might origin from the different experimental layout as well as from the fact that NK cell lines and NK cells from donors have been analysed. Dysfunctional NK cells isolated from the blood of patients with metastatic melanoma exhibited elevated expression of Tim-3 correlating with clinical stage, and subsequent blockade of Tim-3 restored NK cell cytotoxicity and cytokine production (da Silva et al., 2014). While little is known about regulation of Tim-3 and its binding partner on NK cells, Ceacam-1 was recently identified as an important ligand of Tim-3 on T cells, where it is required for the inhibitory function, full maturation and cell surface expression of TIM-3 (Huang et al., 2015). A better understanding of TIM-3 functional role during NK cell maturation and how it relates to an elaborate network of ligands and transcriptional regulators might significantly impact the development of new immunotherapies that harness the inhibitory receptor equipment on the NK cell surface. Additionally, at the best of our knowledge, no studies have yet investigated how age impacts the expression and signalling of TIM-3 and its binding partners.

In this chapter, we compared the phenotype and function of the major NK cell subtypes using three models of persistent immune activation: aging, HCMV infection and hepato-cellular carcinoma (HCC). This study showed that CD57, NKG2C and TIM-3 are hallmarks of NK cell maturation during

aging and that acquisition of these markers correlated with HCMV and inflammation status of the donors. NKG2C^{pos}CD57^{pos} NK cells functionality was shown to be abnormal in older donors and, at least partially, regulated by the TIM-3/Ceacam-1 pathway. Finally, we demonstrated that HCC progression was associated with tumor infiltration of exhausted and cytotoxic-deficient NK cells expressing CD57, TIM-3 and Ceacam-1. Clinical stages of HCC could be segregated according to co-expression of Ceacam-1 and TIM-3, supporting the identification of new immunological targets for checkpoint blockade therapies in order to rescue early innate defense in aging, chronic infections and cancer.

4.2 Results

4.2.1 CD57 is a hallmark of NK cell maturation during aging

4.2.1.1 Imbalance between regulatory and inflammatory NK cells during aging

In order to study heterogeneity of NK cells during aging and to evaluate the influence of chronic CMV infection on NK cells maturation, we first performed deep phenotyping by mass cytometry of young and old healthy donors (n=6). CyTOF data (
Figure 4.1) enabled us to identify several NK cell subsets clustering by specific surface markers (CD94, CD56, CD16), natural cytotoxicity receptors (such as NKp30, NKp44 and NKp46), memory-like/maturation associated markers (NKG2C, CD57), cytokines receptors (CD122, CD127, IL-18R), inhibitory receptors (KLRG1, CD160), transcription factors (Promyelocytic Leukaemia Zinc Finger protein (PLZF), T-bet, Eomes), cycling activity (Ki-67) and granules content (granzyme A/B, perforin).

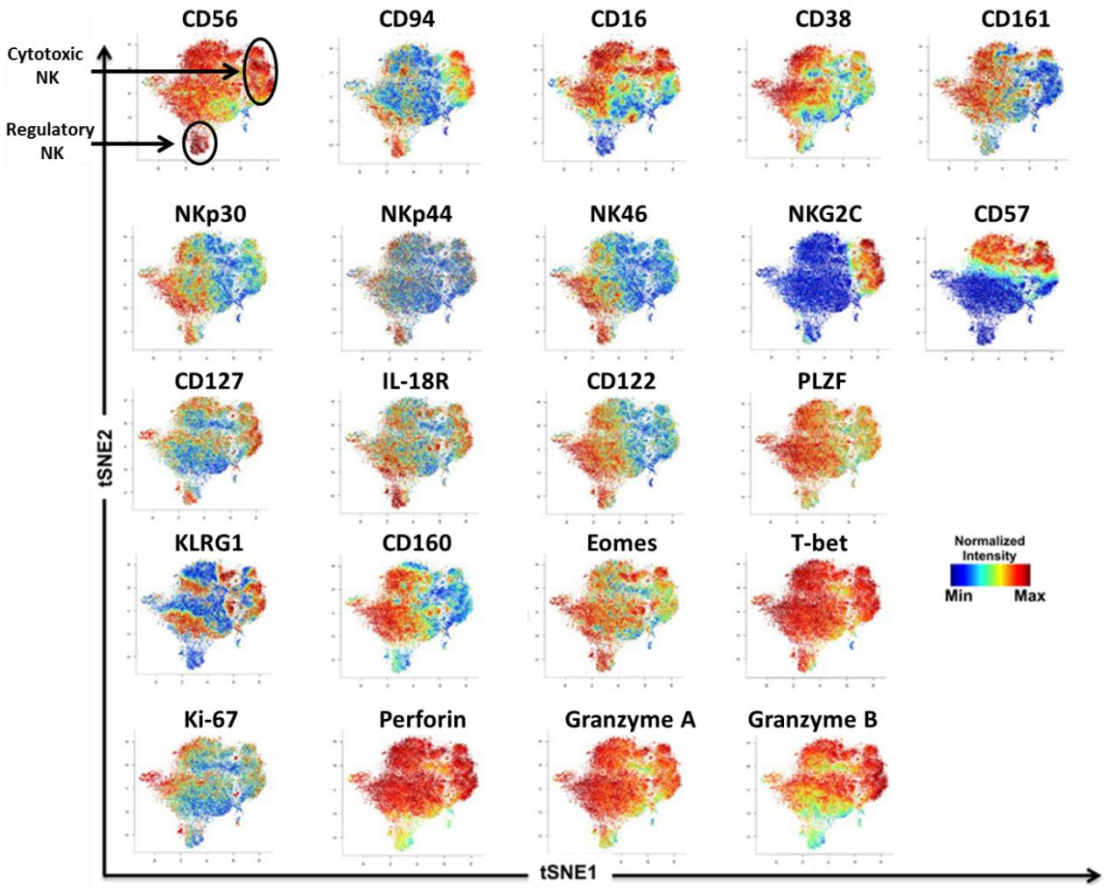


Figure 4.1 Heterogeneity of human NK cells at phenotype, transcriptional and functional levels. PBMCsfrom young and old donors were surface and intra-cellular stained for mass cytometry analysis (CyTOF). Samples were barcoded and acquired simultaneously (n=6). A cold-to-hot heat map represents the normalized intensity of expression for each marker.

Next, we sought to determine the driving force of NK cell cluster diversity through the comparison of NK cells from healthy donors stratified by age and CMV sero-status or during chronic inflammation such as cancer or persistent infections. Flow cytometric analysis of freshly isolated PBMC from 28 young (median age: 20 years old) and 34 older adults (median age: 69 years old) identified the three major populations of NK cells, including regulatory ($CD56^{bright}CD16^{neg}$), cytotoxic ($CD56^{dim}CD16^{pos}$) and inflammatory NK cells ($CD56^{neg}CD16^{pos}$) (Figure 4.2A). $CD56^{dim}CD16^{pos}$ cytotoxic NK cells were the dominant population and did not show any age-related change in frequency (Figure 4.2B). In contrast, we observed an inversion of the frequencies of regulatory and inflammatory subsets, which was associated with age (Figure 4.2B). The median percentage of $CD56^{bright}CD16^{neg}$ regulatory NK cells was reduced from 4.11% in young donors to 2.24% in older donors (p=0.0025) (Figure 4.2B). This was coupled with the expansion of $CD56^{neg}CD16^{pos}$ inflammatory NK cells, usually detected during chronic infections (HIV, HCV) or cancer, which increased from 2.15% in young donors to 5.60% in older donors (p=0.002) (Figure 4.2B). These

innate cells have been characterized for their specific expressions of surface markers and functions. Thus, we investigated the modulation of phenotype in cellular NK populations during aging.

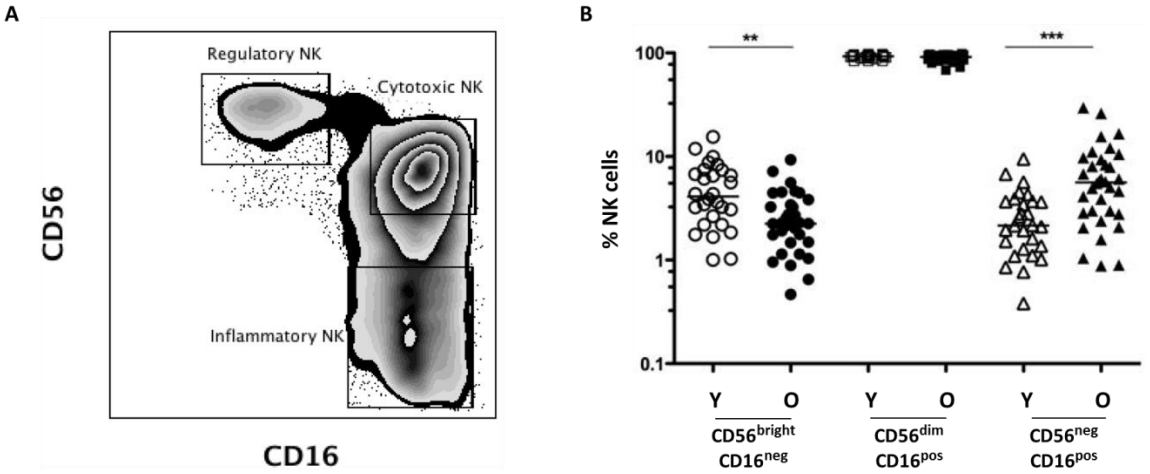


Figure 4.2 Aging affected the distribution of NK cells subsets. (A) Representative density plot of NK cells from an old donor, gated on $CD3^{neg}$ lymphocytes and after exclusion of cells double negative for CD16 and CD56. NK cells were identified as $CD56^{bright}CD16^{neg}$ (regulatory NK cells), $CD56^{dim}CD16^{pos}$ (cytotoxic NK cells) and $CD56^{neg}CD16^{pos}$ (inflammatory NK cells) NK cells. (B) Modulation of NK cell subsets frequency in young (Y) and old (O) donors. Proportions were quantified after exclusion of cells double negative for CD16 and CD56.

4.2.1.2 Acquisition of CD57 and maturation markers by NK cells during lifespan

We then investigated the activation status of the NK cell subsets in the different age groups by measuring co-expression of a range of markers, including the NK activation receptor Nkp46 and the pan-activation marker, CD38. Differentiation stage was assessed through the detection of early markers, such as CD62L and CD27 or the late marker, CD57.

In $CD56^{bright}CD16^{neg}$ regulatory NK cells, the median frequency of CD335 and CD62L was reduced from 73.90% and 98.25% in the young to 17.70% and 40.50% in older donors, respectively ($p<0.0001$) (Figure 4.3A). Expression of CD57 was not detectable in regulatory NK cells from either young or older donors (data not shown). The reduced expression of immature markers in this subset might suggest the acquisition of a mature phenotype during aging.

We noted a reduction in $CD57^{neg}$ frequency in $CD56^{dim}CD16^{pos}$ cytotoxic NK cells from older individuals (data not shown) and within this $CD57^{neg}CD56^{dim}CD16^{pos}$ subset, the frequency of the early maturation markers, CD38, CD62L, and CD335, was decreased from 72.40%, 15.60%, and 27.55% in young donors to 19.70%, 6.58%, and 2.95% in older donors ($p<0.0001$; data not shown). In contrast, the frequency of several subsets of cytotoxic $CD57^{pos}$ NK cells were increased in older

individuals, including CD38^{neg} (1.68% in young vs 13.35% in old, p<0.0001), CD38^{pos} (20.70% vs 57.90%, p<0.0001), CD62L^{neg} (18.20% vs 65.75%, p<0.0001), CD62L^{pos} (2.84% vs 5.415%, p=0.0003), and CD27^{neg} NK cells (22.90% vs 60.00%, p<0.0001) (Figure 4.3B).

The increased frequency of inflammatory NK in the older donors was coupled with acquisition of a mature phenotype (Figure 4.3C). Median frequency of CD335 was reduced from 22.35% in young donors to 2.60% in older donors (p<0.0001), whereas CD57 frequency was increased from 1.02% in young donors to 21.95% in older donors (p<0.0001) (Figure 4.3C).

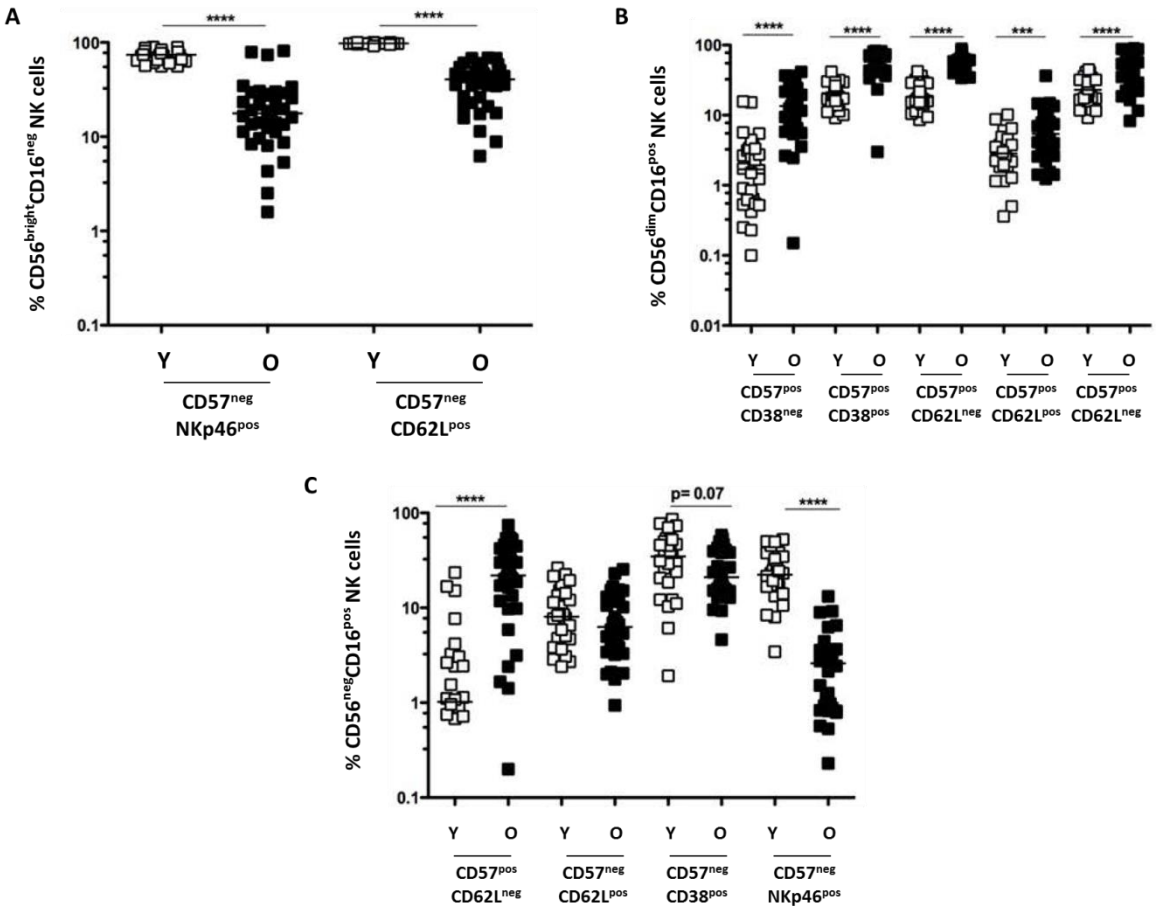


Figure 4.3 Aging was associated with modulation of maturation markers in NK cell subsets. (A) Loss of immature status markers on CD56^{bright}CD16^{neg} NK cells and (B) and (C) upregulation of the maturation marker CD57 on CD56^{dim}CD16^{pos} and CD56^{neg}CD16^{pos} NK cells in old (O) *versus* young (Y) donors.

To study the phenotypic diversity of NK cells independently of the pre-conceived phenotypes, we analyzed clusters of total CD56^{pos} NK cells from the 28 young and 34 older participants by t-Distributed Stochastic Neighbor Embedding (tSNE), a dimensionality reduction tool to map high-dimensional cytometry data onto 2D while conserving high-dimensional structure (van der Maaten,

2008). The combinatorial diversity of NK cell phenotype according to the expression of CD27, CD38, CD57, CD62L, and CD335 was mapped into two dimensions. This analysis identified differential expression of clusters of NK cells in young (green) compared to older adults (red) (Figure 4.4A). Cluster C represented the age-associated up-regulation of CD57 described above. This pattern of phenotypic diversity for all young (n=28) and older adults (n=34) is summarized in Figure 4.4B, representing the differentiation and maturation of NK cells over extended periods of time.

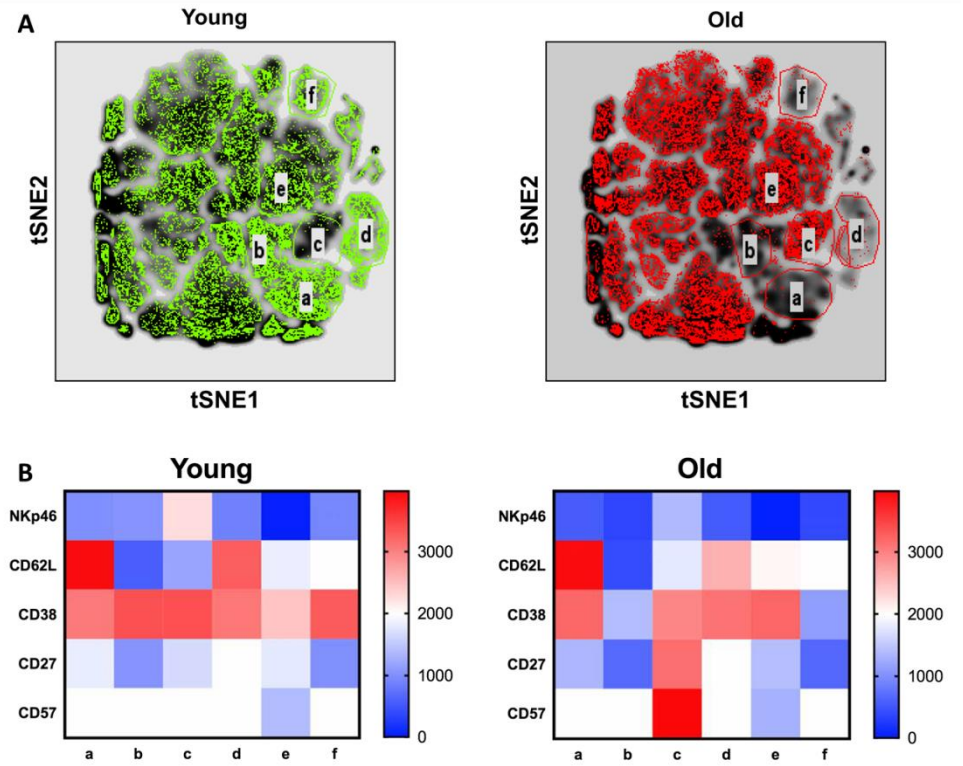


Figure 4.4 Unbiased representation of NK cell repertoire. (A) Overlay of computational analysis of NK cells from young (green, n=28) and old (red, n=34) donors identifies several age-specific clusters (a-f). (B) The heat map represents the intensity of antigen expression in the different clusters of NK cells in young and older donors.

4.2.2 Anti-viral response against CMV and inflammation drive acquisition of CD57 and NKG2C in NK cells during aging

Licensing of NK cells allows recognition and killing of damaged, infected, or transformed cells while preserving tissue integrity. It is thought that persistent viral infection and cancer can modify education and responsiveness of NK cells to activating or inhibitory signals. Additionally, persistent infections such as CMV are confounding factors for T cell differentiation. Hence, we sought to determine if NK cell maturation during aging is associated with manifestation of chronic infections

or systemic inflammation. The frequency of mature cytotoxic CD57^{pos}CD62L^{neg}CD56^{dim} NK cells was correlated with IgG titer against CMV and with pro-inflammatory cytokines present in the plasma. We observed a positive association between the percentage of mature NK cells in the periphery and anti-CMV IgG titers in older donors (r^2=0.3561, p=0.0387, n=34) but not in younger individuals (Figure 4.5A). Moreover, the accumulation of CD57^{pos}CD62L^{neg}CD56^{dim} NK correlated with plasma levels of TNF-α (r^2=0.5223, p=0.0315, n=17) and IL-21 (r^2=0.6733, p=0.0016, n=19) in old people (Figure 4.5B). Plasma concentrations of pro-inflammatory mediators were significantly higher in older donors compared with younger donors (TNF-α: 5.3 pg/ml vs 2.0 pg/ml, p=0.0243; IL-21: 376.9 pg/ml vs 21.5 pg/ml, p<0.0001) respectively. We were unable to detect measurable levels of IL-6 (data not shown), but it is possible that other inflammatory molecules may participate directly or indirectly in the maturation of cytotoxic NK cells during aging.

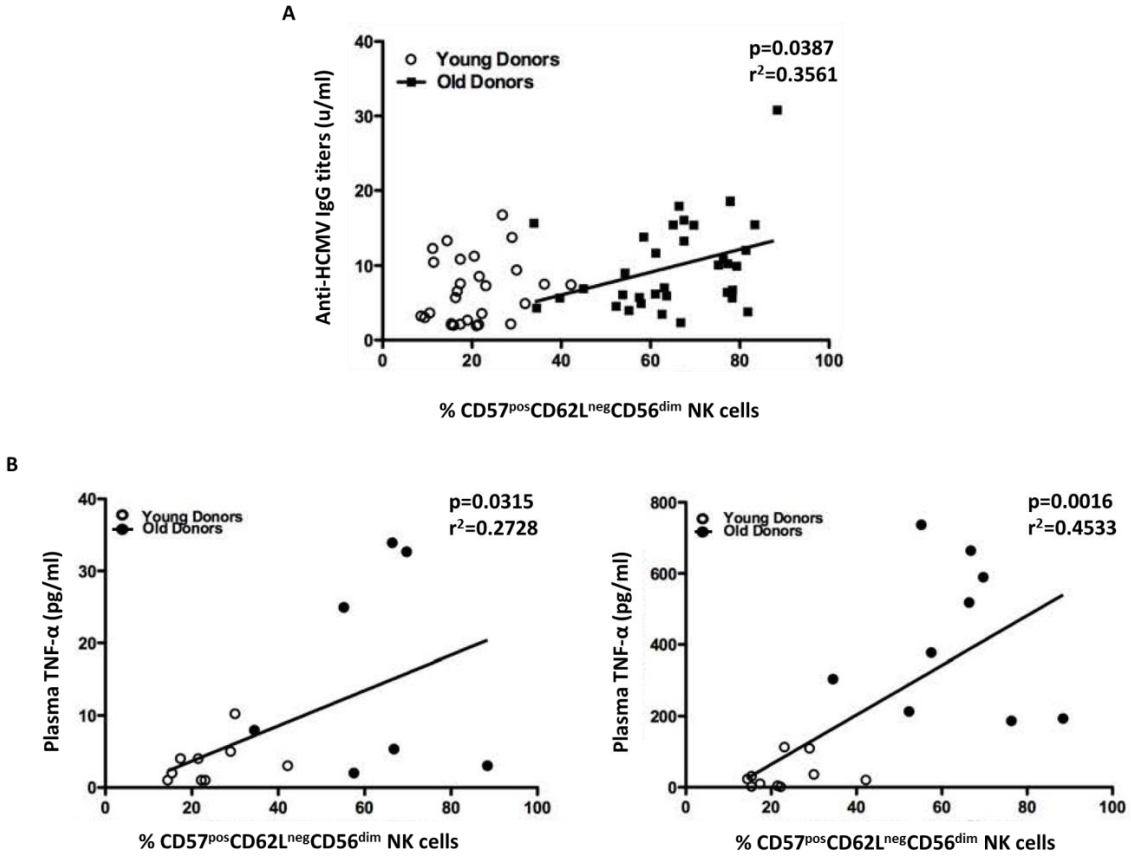


Figure 4.5 HCMV infection and systemic inflammation are associated to NK cell maturation. (A) The frequency of CD57^{pos}CD56^{dim}CD16^{pos} NK cells was positively correlated with the level of IgG antibodies specific to CMV in older donors (closed symbols, p=0.0387 and r^2=0.3561). (B) The frequency of CD57^{pos}CD56^{dim}CD16^{pos} NK cells was positively correlated with the level of pro-inflammatory cytokines in the plasma (p=0.0315, r^2=0.2728 for TNF-α, and p=0.0016, r^2 =0.4533 for IL-21, n=19) in old donors.

As mentioned in the introduction of this chapter, HCMV infection has been shown to promote a specific NK cell signature. There is an accumulation of NK cells with the $CD57^{pos}NKG2C^{pos}Siglec\text{-}7^{neg}$ profile upon HCMV reactivation upon hematopoietic stem cell transplantation (Della Chiesa et al., 2014). Also, HCMV imprint on the NK cell repertoire includes increased expression of CD85j (immunoglobulin-like transcript 2 [ILT2], leukocyte immunoglobulin-like receptor 1 (LIR1), leukocyte immunoglobulin-like receptor B1 [LILRB1]) (Guma et al., 2004). This prompted us to investigate the expression of molecules such as NKG2C, Siglec-7, and CD85j during aging and HCMV infection. An increase of almost two-fold in the frequency of $NKG2C^{pos}Siglec\text{-}7^{neg}CD57^{pos}CD56^{dim}$ and $CD85j^{pos}CD57^{pos}CD56^{dim}$ NK cells was observed in the older donors ($p=0.017$ and $p=0.0007$, respectively; n=20 young and n=18 old donors) (Figure 4.6A). To distinguish the impact of CMV infection from that of aging, we tried to establish a correlation between humoral responses against CMV and the acquisition of these phenotypes in young and older donors. Positive correlations were observed between $CD57^{pos}$ NK cells expressing NKG2C or CD85j and anti-CMV IgG titer but only in young donors ($p=0.0001$ and $p=0.0166$, respectively) (Figure 4.6B).

Analysis of representative concatenated donors according to age and HCMV sero-status confirmed the emergence of a cluster characterized by an up-regulation of CD57 and NKG2C, with limited expression of Siglec-7 and CD85j (Figure 4.6C).

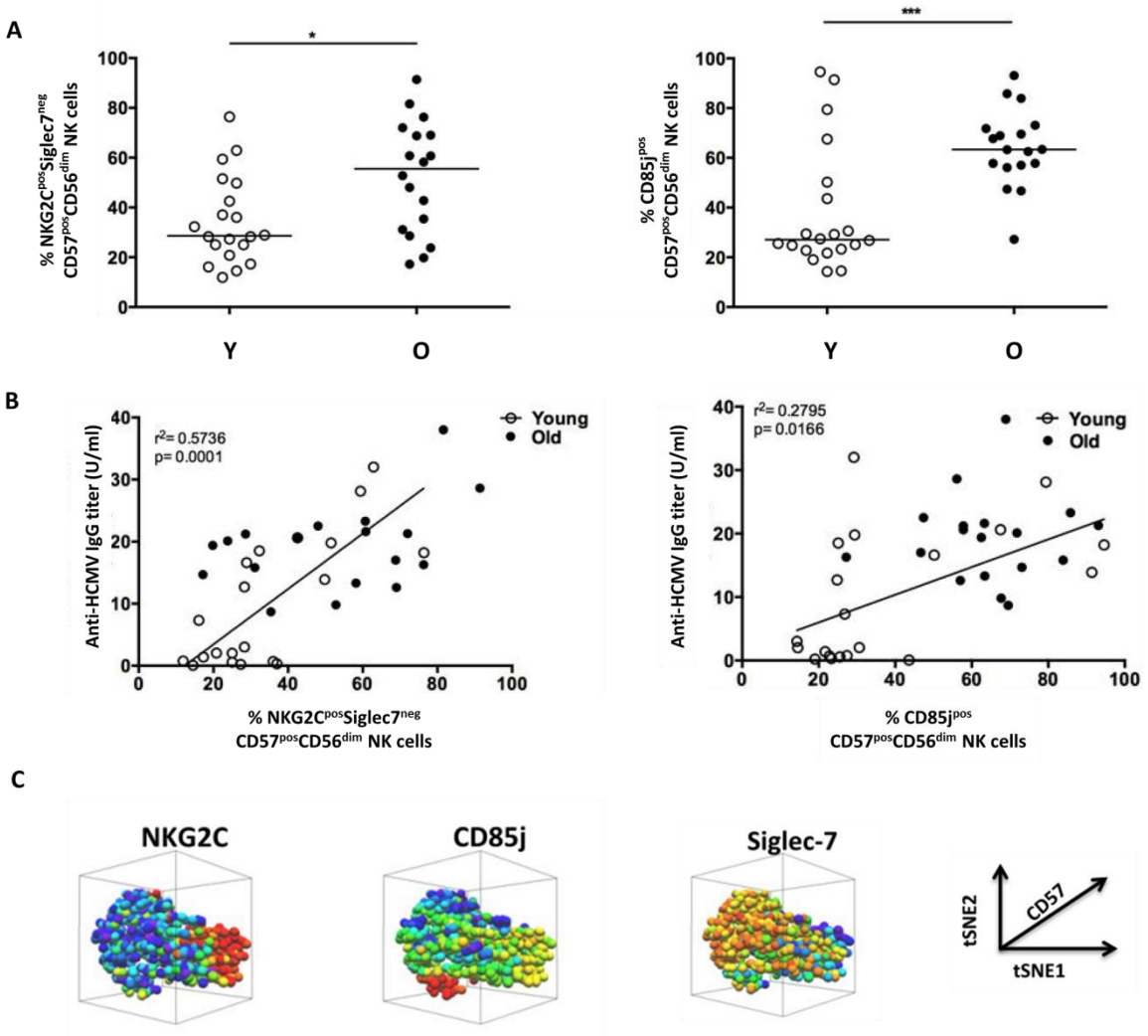


Figure 4.6 Expression of memory-like molecules during aging and HCMV infection. (A) NKG2C and CD85j expression were preferentially increased in CD57^{pos}CD56^{dim}CD16^{pos} NK cells from PBMCs of older donors. (B) The frequency of CD57^{pos}CD56^{dim}CD16^{pos} NK cells that are NKG2C^{pos}Siglec-7^{neg} or CD85j^{pos} were positively correlated with the level of IgG antibodies specific to CMV in young donors (open circle) but not in the elderly (closed circle). (C) NK cell clusters associated with HCMV infection were identified by flow cytometry. Three-dimensional representation of computational analysis of NK cells from donors with elevated levels of anti-CMV IgG. The intensity of expression for each marker was represented by a cold-to-hot heat map.

The impact of persistent infections and aging on T cells due to the up-regulation of inhibitory molecules such as TIM-3 and Ceacam-1 prompted us to investigate if this exhaustion molecule was modulated on NK cells as well. The expression of Tim-3 was increased in CD57^{pos} mature NK cells from old donors (Figure 4.7A). Moreover, a higher co-expression of Tim-3 and Ceacam-1 in NK cells was observed in older people compared with young or CMV seronegative donors (Figure 4.7B).

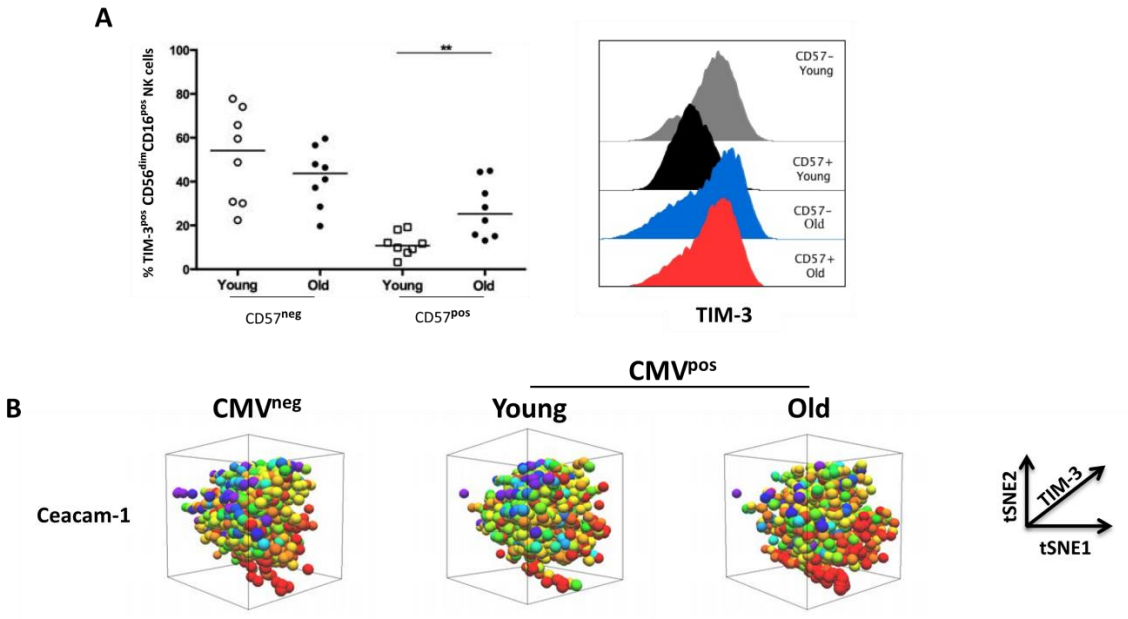


Figure 4.7 Modulation of TIM-3 and Ceacam-1 expression in NK cells with aging and CMV infection. (A) Percentage of TIM-3^{pos} NK cells (left) and representative histogram of TIM-3 expression (right) in CD57^{neg} and CD57^{pos} CD56^{dim}CD16^{pos} NK cells during aging. (B) Three-dimensional bubble plot representation of the co-expression of TIM-3 and Ceacam-1. Flow cytometry staining was performed on gated NK cells from donors grouped according to age or CMV status. The intensity of expression for each marker was represented by a cold-to-hot heat map.

The kinetics of loss or acquisition of phenotypic markers during CMV-driven cell differentiation was analyzed using Wanderlust software. Wanderlust is a graph-based trajectory detection algorithm that receives multiparameter single-cell events as input and maps them onto a one-dimensional developmental trajectory. Cells are ordered along a trajectory that represents their most likely placement along a developmental continuum (Bendall et al., 2014) (Figure 4.8). CD56^{bright}CD16^{neg} NK cells were considered the most immature NK population (starting population) and donors were segregated according to CMV serological status but independent of age, thus establishing an unbiased model that reflects the maturation of NK cells in the absence or presence of CMV infection (Figure 4.8A). The early loss of CD62L and Siglec-7 was coupled with a progressive up-regulation of CD57. The maturation of NK cells was characterized by concomitant expression of CD57 and NKG2C but only in CMV seropositive donors. It is interesting to observe the late divergence of expression between these molecules that could give rise to different daughter cells. Moreover, the trend of acquisition of CD85j, Ceacam-1, and Tim-3 is more interrelated in CMV^{pos} donors compared with CMV^{neg} donors and occurred after up-regulation of CD57 and NKG2C.

We identified four populations according to the single expression or co-expression of CD57 and NKG2C, and compared their Wanderlust score in order to determine maturation status (Figure 4.8B).

We confirmed that NKG2C^{neg}CD57^{neg} (DN) NK cells are the most immature NK subset, while NKG2C^{pos}CD57^{pos} (DP) NK cells represent the most advanced stage of NK cell differentiation. In addition, we were able to demonstrate that NKG2C^{neg}CD57^{pos} (CD57) NK cells display a more differentiated phenotype than the NKG2C^{pos}CD57^{neg} (NKG2C) NK population.

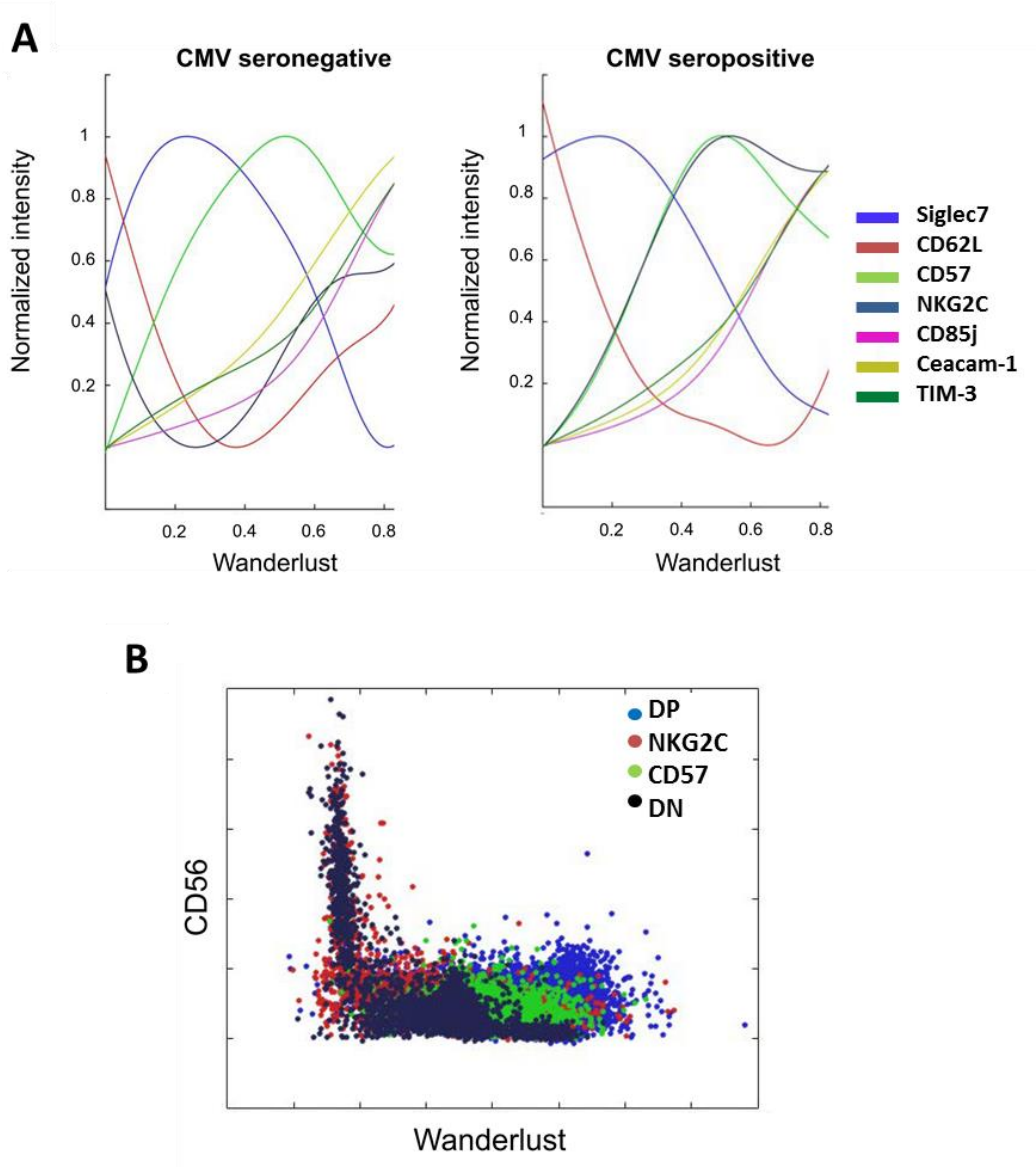


Figure 4.8 CD57 and NKG2C segregate CD56^{dim}CD16^{pos} NK cells in four maturation stages. (A) The Wanderlust trajectory is fixed to an arbitrary scale where the most immature NK cells (CD56^{bright}CD16^{neg}) are at 0 and the most mature at 1. The traces demonstrated the relative expression patterns of Siglec-7, CD62L, CD57, NKG2C, CD85j, Ceacam-1, and TIM-3 across differentiation in HCMV^{neg} and HCMV^{pos} donors. (B) Terminal maturation of CD57^{pos}NKG2C^{pos} NK cells according to Wanderlust trajectory. The differentiation of CD57^{neg}NKG2C^{neg} (DN), CD57^{pos}NKG2C^{neg} (CD57), CD57^{neg}NKG2C^{pos} (NKG2C) and CD57^{pos}NKG2C^{pos} (DP) CD56^{dim}CD16^{pos} NK cells was evaluated according to their Wanderlust score.

4.2.3 Gain of polyfunctionality but loss of cytotoxicity by CD57^{pos} NK cells during aging

We then investigated the impact of the maturation process on NK cell functionality, including cytotoxic activity and cytokine production. CD56^{dim} cytotoxic NK cells were sorted on the basis of maturation according to CD57 expression. Sorted NK cells from six individual young donors and five pools of older subjects (three donors per pool due to low cell counts in our bio-banked samples) were subjected to polyclonal stimulation with PMA and ionomycin to measure cytokine secretion. Mature CD57^{pos} NK cells released higher quantities of IFN-γ and TNF-α although this was not significant (p=0.0579 and p=0.0625, respectively) (Figure 4.9). Mature NK cells from older donors released significantly higher concentrations of TNF-α compared with young donors (580 pg/ml vs 260 pg/ml/M, respectively, p=0.0498). Similar trends were observed for granzyme A (GZA) and granzyme B (GZB) (p=0.0625 and p=0.089, respectively) but not for perforin. The secretion of cytotoxic molecules did not differ between NK subsets in young donors, but was decreased in immature CD57^{neg} NK cells from older donors. We were not able to detect any segregation in the secretory potential of NK cells according to CMV status.

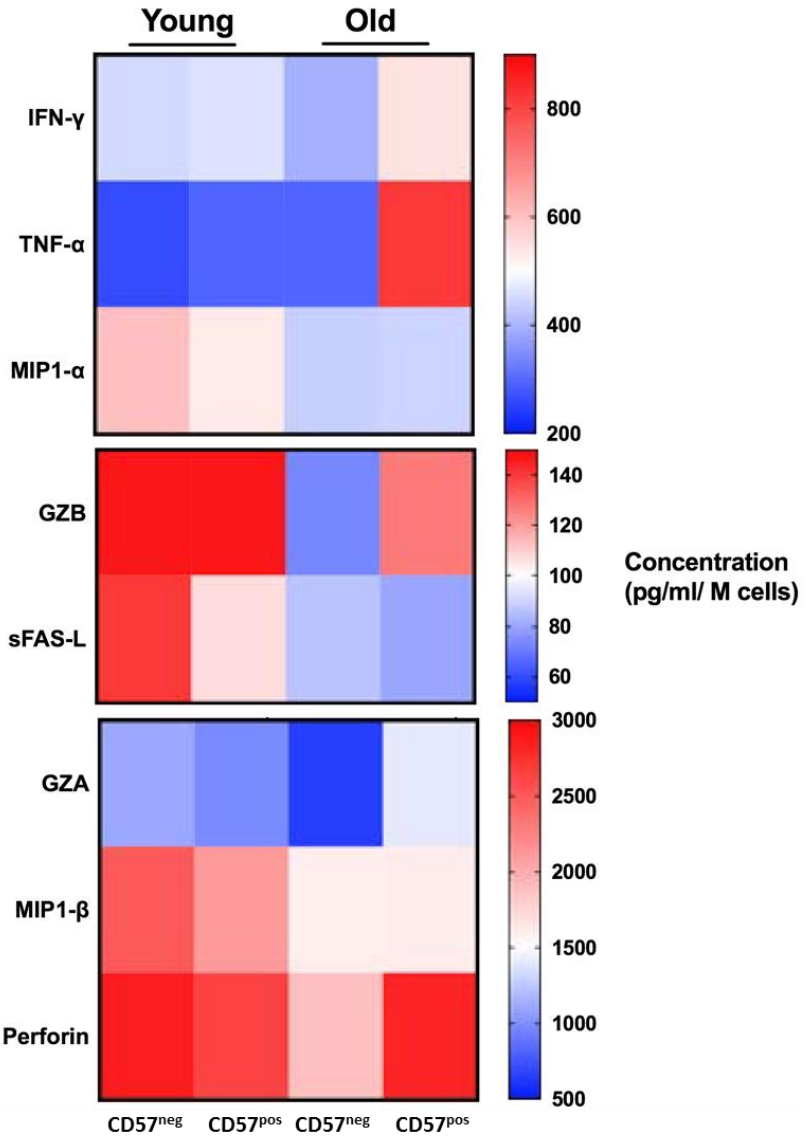
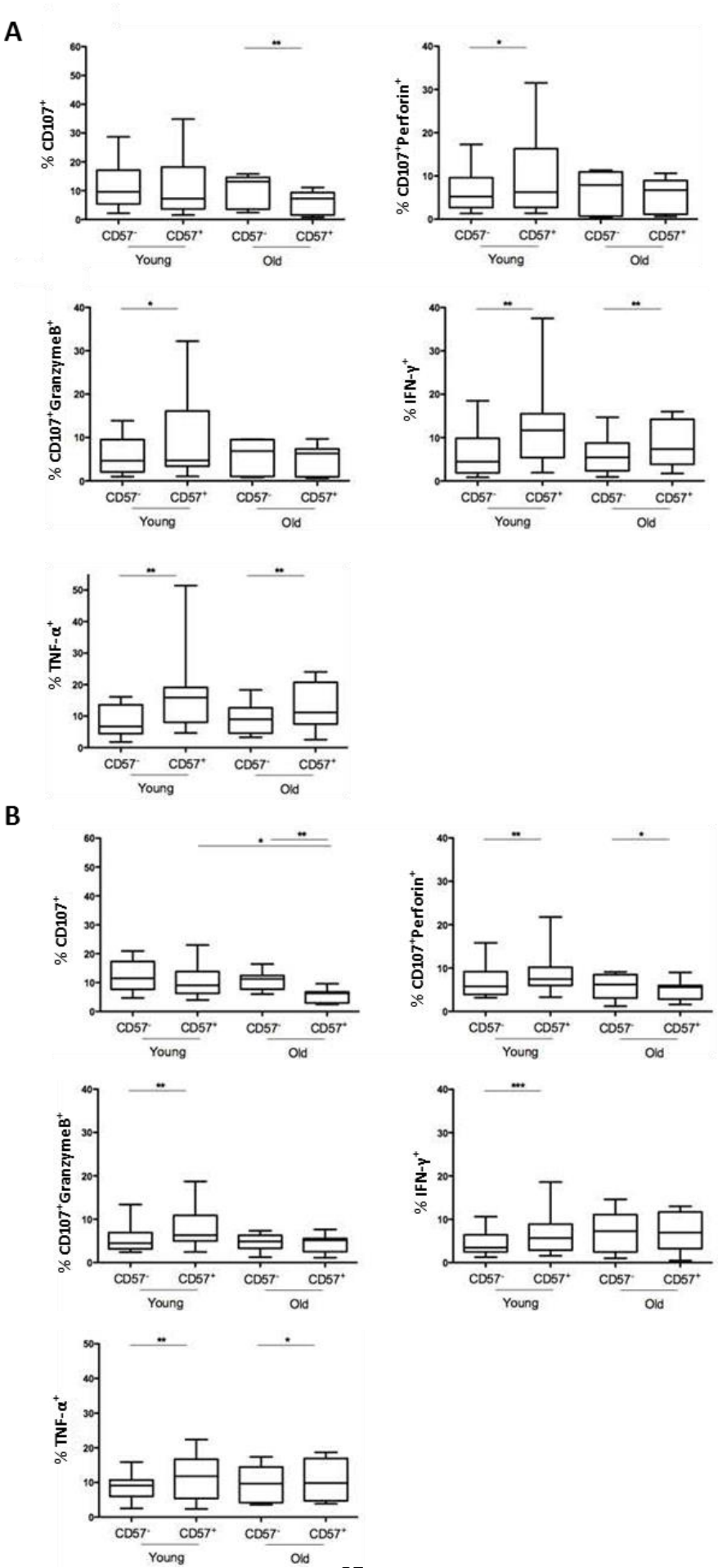


Figure 4.9 Increased secretive potential of cytokines, chemokines and cytotoxic molecules by CD57^{pos} NK cells. CD57^{pos}CD56^{dim}CD16^{pos} NK cells from young and old individuals were challenged with polyclonal stimulation (PMA/Ionomycin), detected by Luminex and represented by heat map (pg/ml/M cells).

To evaluate the physiological functionality of NK cells, we quantified the ability of immature and mature NK cells to secrete cytokines, degranulate (CD107a /LAMP-1) and release cytotoxic molecules by intracellular flow cytometry and using more specific challenges. Stimulations included ligation of CD16 (Figure 4.10A), which mimics antibody dependent-cell-mediated cytotoxicity (ADCC), and the gold standard assay for NK stimulation, which involves pulsing innate lymphocytes with the K562 erythro-leukemia cell line (Figure 4.10B) that lacks surface MHC class I expression and thus represents a target for NK cells. The degranulation ability of NK cells was reduced in mature CD57^{pos} NK cells from older individuals compared with younger donors after CD16

engagement (p=0.002) or pulsing with K562 cells (p=0.0078). CD107a expression was also decreased in mature NK cells from older donors compared with younger donors after pulsing with K562 cells (p=0.0287). The release of cytotoxic granules was examined by co-staining of CD107a and perforin or granzyme B. As expected, mature NK cells from young donors were highly cytotoxic; however, $CD57^{pos}$ NK cells from older donors displayed lower (after pulse with K562 for $CD107a^{pos}perforin^{pos}$, p=0.0231) or equivalent (after CD16-mediated stimulation) cytotoxic properties than $CD57^{neg}$ NK cells, despite the preservation of enhanced cytokine secretion by mature NK cells during aging.


A
% CD107+
% CD107+Perforin+
% CD107+GranzymeB+
% IFN-γ+
% TNF-α+
CD57- CD57+ CD57- CD57+
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B
% CD107+
% CD107+Perforin+
% CD107+GranzymeB+
% IFN-γ+
% TNF-α+
CD57- CD57+ CD57- CD57+
Young
Old

Figure 4.10 CD57 expression affected the physiological functionality of NK cells during aging. PBMCs were stimulated by CD16 ligation (A) and K562 cell line (B) and functions of NK cells were assessed by flow cytometry.

We investigated if the loss of cytotoxicity in $CD57^{pos}$ NK cells was associated with or independent of cytokine secretion. Poly-functionality of $CD57^{pos}$ NK cells during aging was defined using Spice software (Figure 4.11). Mature NK cells expressing CD107a displayed a greater proportion of polyfunctional activity in older donors compared with younger donors. Notably, levels of perforin were preserved during aging and an increase in granzyme B, IFN-γ and TNF-α was observed in $CD107^{pos}CD57^{pos}$ mature NK cells under all tested conditions.

Taken together, these results suggest that reduction of cytotoxicity in mature NK cells is counter-balanced by an increase in poly-functionality during aging.

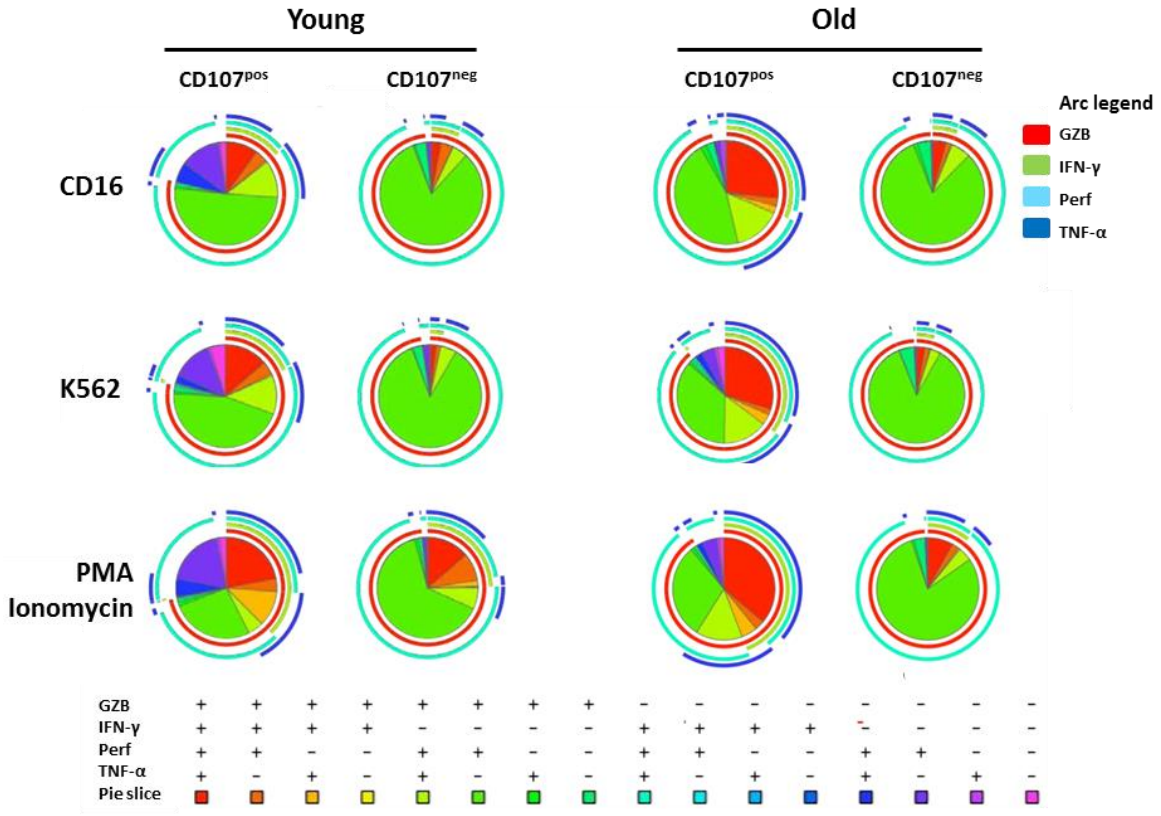


Figure 4.11 Poly-functionality of cytotoxic NK cells after specific stimulation was increased in aged donors. The functions of $CD107a^{neg}$ and $CD107a^{pos}$ $CD57^{pos}$ NK cells were analyzed by Spice and the pie chart arc identified the relative frequency of each molecule. The different pie charts represent unique combinations of molecules.

4.2.4 Increased expression of Zeb2 in poly-functional CD57^{pos}NKG2C^{pos} NK cells in HCMV infection

To further characterize mature NK cells, we sorted mature NK cells from HCMV-seropositive donors according to CD57 and NKG2C expression. After excluding doublets and dead lymphocytes, we identified NK cells as CD3^{neg}HLA-DR^{neg}; then CD56^{dim}CD16^{pos} NK cells were subdivided into three populations based on CD57 and NKG2C expression (NKG2C^{neg}CD57^{pos} (CD57), NKG2C^{pos}CD57^{neg} (NKG2C) and NKG2C^{pos}CD57^{pos} (DP)) (Figure 4.12).

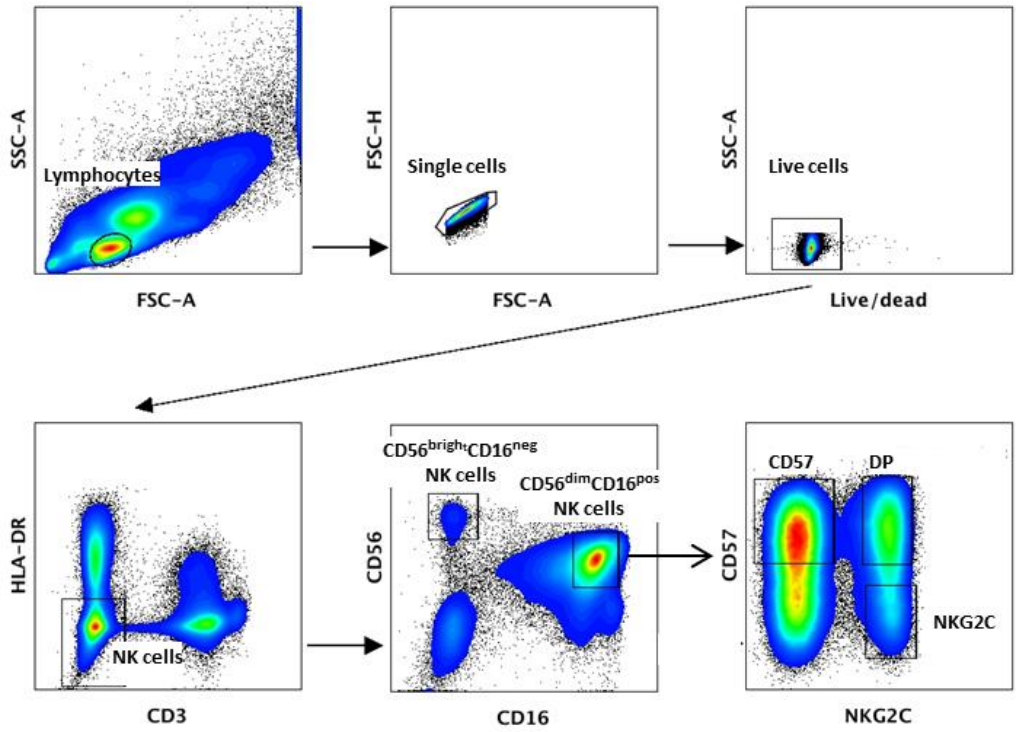


Figure 4.12 Sorting gating strategy of mature NK cells. CD56^{dim}CD16^{pos} NK cells were sorted according to CD57 and NKG2C expression in order to assess functionality and gene expression of HCMV-induced NK cells. Sorted subsets were identified as: NKG2C^{neg}CD57^{pos} (CD57), NKG2C^{pos}CD57^{neg} (NKG2C) and NKG2C^{pos}CD57^{pos} (DP).

Analysis of supernatants from CD16-stimulated NK cells revealed heterogeneity in the CD57 subset based on NKG2C expression, including differential release of MIP-1α/β, IFN-γ, TNF-α, granzyme A/B, and perforin by NKG2C^{neg}CD57^{pos}, NKG2C^{pos}CD57^{neg} and NKG2C^{pos}CD57^{pos} NK cells (Figure 4.13).
Gene expression of the sorted NK populations was also compared with regulatory NK cells (CD56^{bright}) with reference to naïve T cells (data not shown) and terminal effector T cells (CD57^{pos}CD45RA^{pos} T cells were considered as the last stage of T cell differentiation) in order to normalize individual variations between donors and to compare the intrinsic modulation of gene

expression during NK and T cell maturation. We focused our interest on transcription factors involved in the negative (Foxo3) or positive (Zeb2/T-bet) regulation of NK cell maturation.

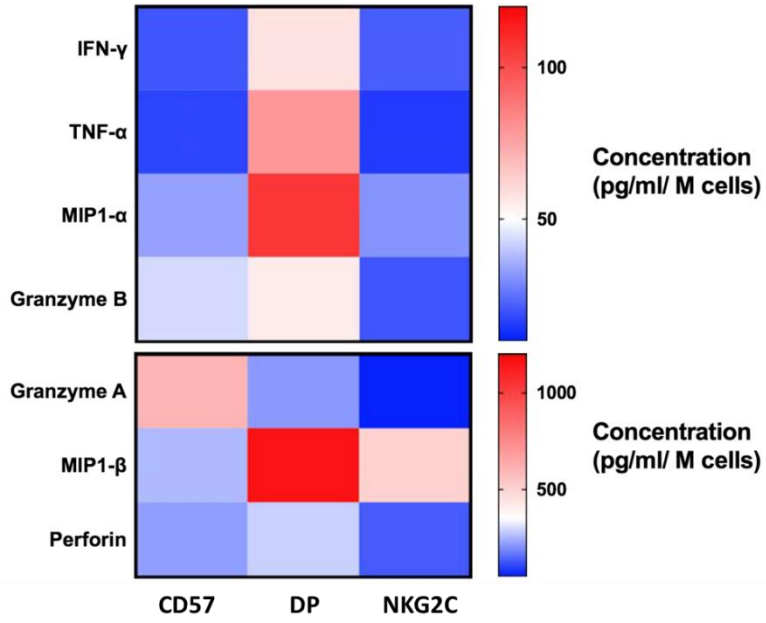
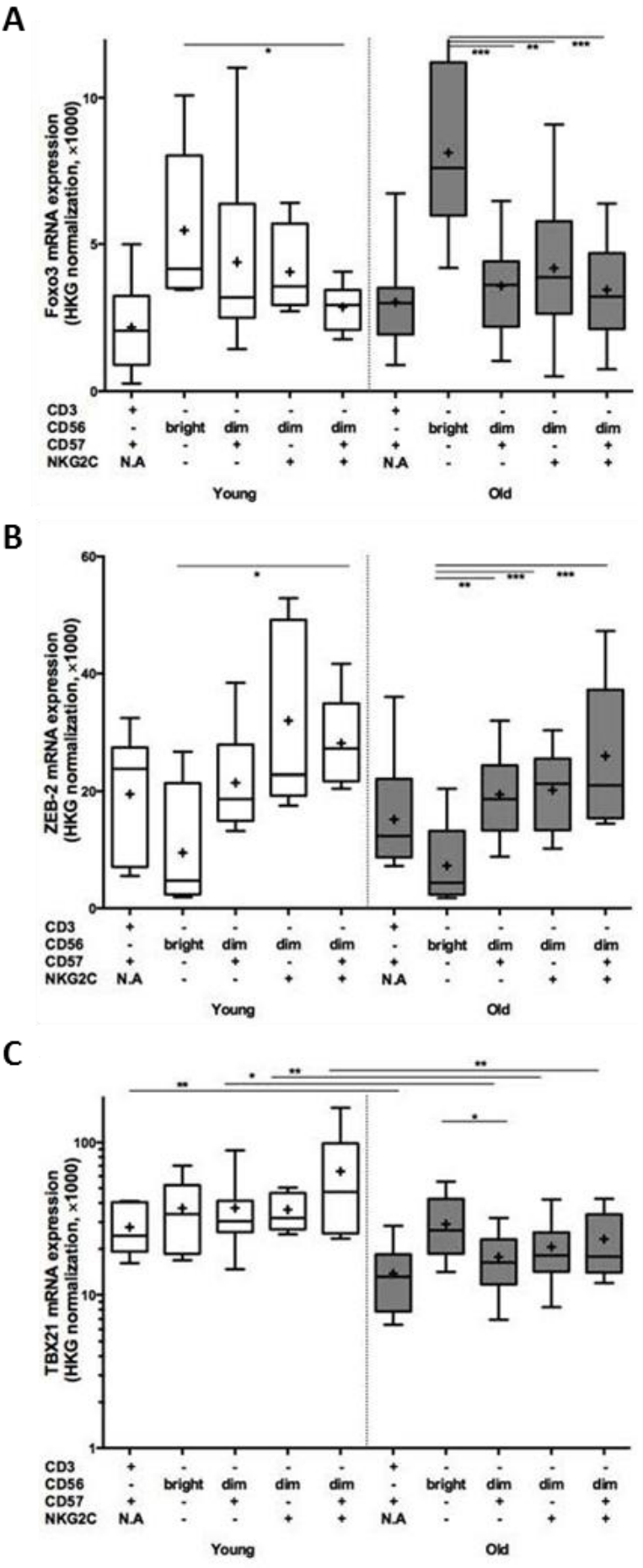


Figure 4.13 Identification of poly-functional NK cells using CD57 and NKG2C expression. $CD56^{dim}CD16^{pos}$ NK cells from frozen PBMCs were sorted according to CD57 and NKG2C expression and stimulated with CD16 antibody. Supernatants were analyzed by Luminex and the heat map represented the concentration of each molecule (pg/ml/M cells).

The highest level of Foxo3 expression was found in regulatory NK cells and progressively decreased during NK maturation (p=0.0013 for $NKG2C^{pos}CD57^{neg}$, p=0.0001 for $NKG2C^{neg}CD57^{pos}$, and p=0.0006 or p=0.0152 for $NKG2C^{pos}CD57^{pos}$ NK cells in older and young donors, respectively). Stratification of donors revealed an early down-modulation of Foxo3 expression in NK cells from older individuals (Figure 4.14A). In contrast, Zeb2 expression was enhanced in terminal effector T cells as reported and in all NK cells in comparison to naïve T cells. The level of Zeb2 was progressively upregulated during NK cell differentiation in comparison to regulatory NK cells (p=0.0252 for $NKG2C^{pos}CD57^{pos}$ from young donors, p=0.001, p=0.0015 and p=0.0005 for $NKG2C^{neg}CD57^{pos}$, $NKG2C^{pos}CD57^{neg}$ and $NKG2C^{pos}CD57^{pos}$ NK cells from older donors, respectively). Early acquisition of Zeb2 thus characterized differentiation of NK cells in older individuals (Figure 4.14B). The stability of *TBX21* mRNA in NK cells from young donors differed from the transient decrease during NK cell maturation in older donors (p=0.0129 in $NKG2C^{neg}CD57^{pos}$ NK cells). Finally, *TBX21* expression was also lower in $NKG2C^{neg}CD57^{pos}$, $NKG2C^{pos}CD57^{neg}$ and $NKG2C^{pos}CD57^{pos}$ NK cells from older donors (p=0.0145, p=0.0059 and p=0.0097 respectively) (Figure 4.14C). NK cells expressing CD57 and NKG2C thus

represent the final stage of maturation of NK cells during aging, characterized by specific functions and a specific expression pattern of transcription factors i.e., Foxo3^{low}Zeb2^{high}T-bet^{low}.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Foxo3 mRNA expression (HKG normalization, ×1000)
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Young
Old

Figure 4.14 Alteration of transcription factors expression during NK cell maturation in aged donors. Sorted NK and T cell subsets from young and older donors were immediately lysed. Gene expression of Foxo3 (A), ZEB-2 (B) and *TBX21* (C) was analyzed by RT-PCR and normalized according to the expression of housekeeping genes (β-actin, GlycerAldehyde 3-Phosphate DeHydrogenase (GAPDH)).

4.2.5 Regulation of CD57^{pos}NKG2C^{pos} NK cells polyfunctionality by Foxo3/T-bet and TIM-3/Ceacam-1 expression

The role of TIM-3 as a co-inhibitory molecule in T cells prompted us to investigate its relationship with NKG2C and CD57 in the modulation of NK function. NKG2C^{pos}CD57^{neg}, NKG2C^{neg}CD57^{pos}, and NKG2C^{pos}CD57^{pos} NK cells were sorted, pre-incubated with neutralizing anti-TIM-3 or control IgG mAbs, and stimulated with CD16 antibody. TIM-3 blockade did not significantly change the secretory profile of NKG2C^{pos}CD57^{neg} or NKG2C^{neg}CD57^{pos} NK cells (data not shown). In comparison, functionality of NKG2C^{pos}CD57^{pos} NK cells was enhanced after neutralization of TIM-3 (Figure 4.15). Increased levels of TNF-α, MIP1-α/β, and granzyme B were detected after TIM-3 blockade (p=0.0313 for all molecules and p=0.0625 for granzyme A and perforin secretion).

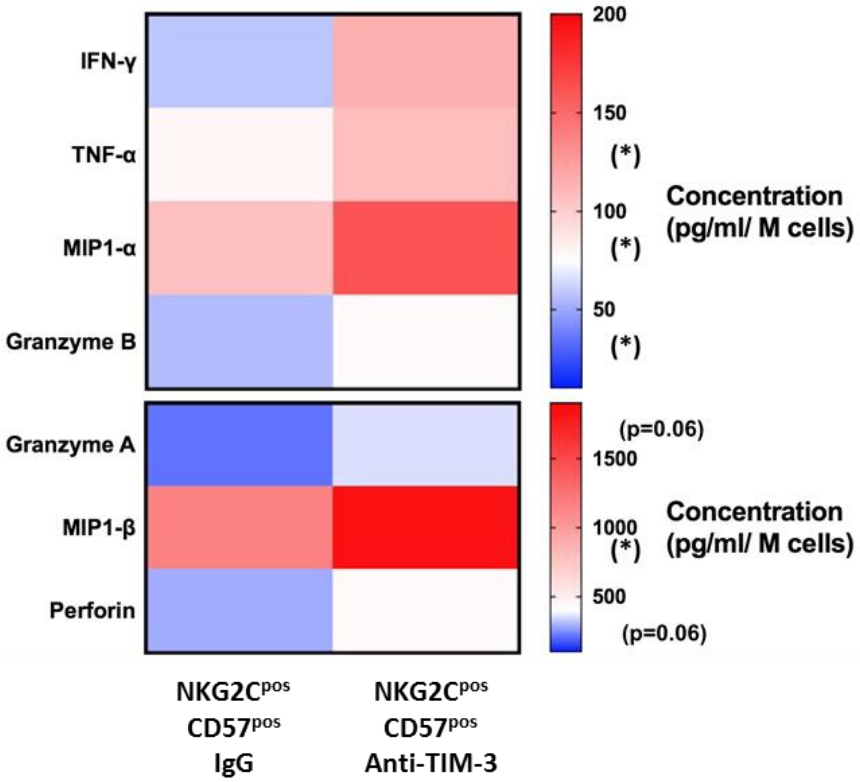


Figure 4.15 Reversion of CD57^{pos}NKG2C^{pos} NK cells exhaustion by TIM-3 blockade. Sorted NK cell subsets were pre-incubated with anti-TIM-3 or IgG control before overnight stimulation with CD16-coated antibodies. Supernatants were analyzed by Luminex and the concentrations of analytes were represented by heat map.

We sought to determine if this modulation in cytokine, chemokine, and cytotoxic molecule secretion was coupled with any change in transcription factor expression. Interestingly, Foxo3 expression was significantly increased after TIM-3 blockade in NKG2C^{neg}CD57^{pos} and NKG2C^{pos}CD57^{pos} NK cells compared to *ex vivo* (p=0.0159 and p=0.019, respectively) (Figure 4.16A). Moreover, T-bet mRNA (*TBX21*) expression was upregulated in NKG2C^{pos}CD57^{pos} NK cells after the neutralization of TIM-3 compared to Ig control (p=0.0313) (Figure 4.16B). These results suggest a direct inhibition of Foxo3 and T-bet by TIM-3 in NK cells expressing CD57. Zeb2 expression was unaltered after TIM-3 neutralization (Figure 4.16C), thus excluding a direct interaction between TIM-3 and Zeb2.

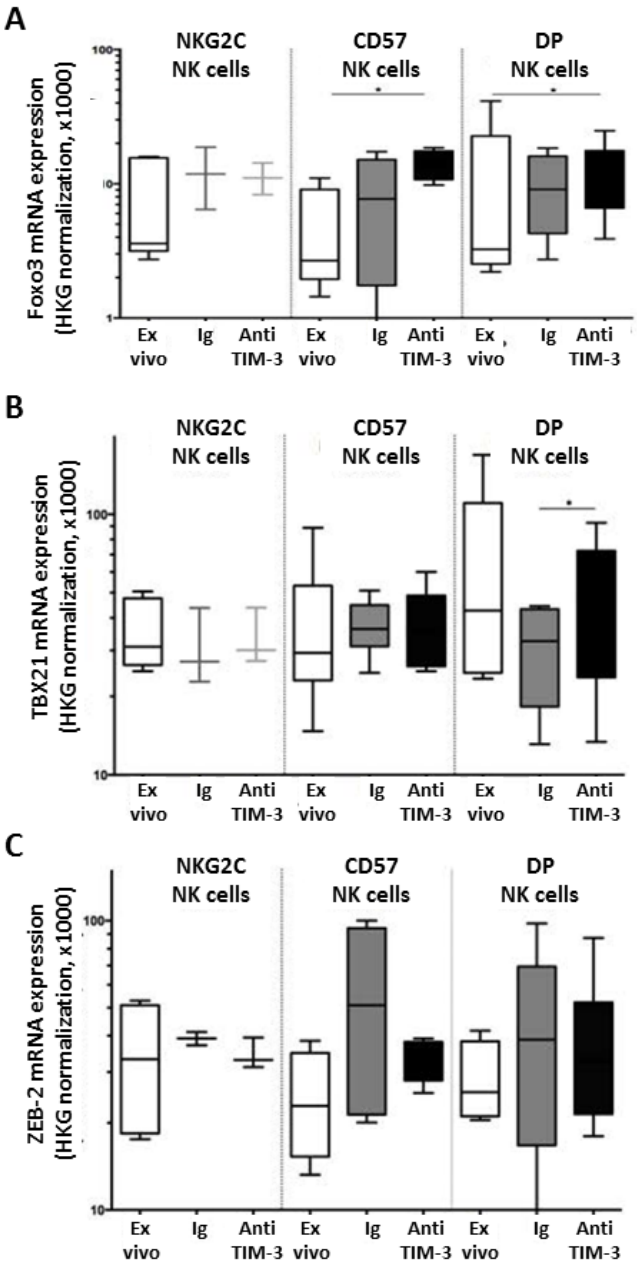


Figure 4.16 Induction of Foxo3 and *TBX21* expression in CD57^{pos}NKG2C^{pos} NK cells after TIM-3 inhibition. Gene expressions of Foxo3 (A), *TBX21* (B) and ZEB-2 (C) in NK cell subsets were analyzed directly *ex vivo* and after *in vitro* stimulation with CD16-coated antibodies.

Whether or not TIM-3 overexpression negatively affects NK cell function remains controversial. To address this question, we decided to target Ceacam-1 because of its relationship with CD57 and NKG2C in NK cells and its recently discovered involvement in the regulation of TIM-3-mediated exhaustion in T cells (Huang et al., 2015). The role of Ceacam-1 in TIM-3-mediated exhaustion of NK cells was evaluated by siRNA-mediated silencing using PBMCs from older donors, followed by CD16 stimulation and assessment of NK functionality by flow cytometry. Ceacam-1 silencing

restored NK cell proliferation potential as measured by Ki-67 expression (p=0.0313 for $CD57^{neg}$ and $CD57^{pos}$ NK cells) and induced expression of T-bet in $CD57^{pos}$ NK cells (p=0.0313) (Figure 4.17).

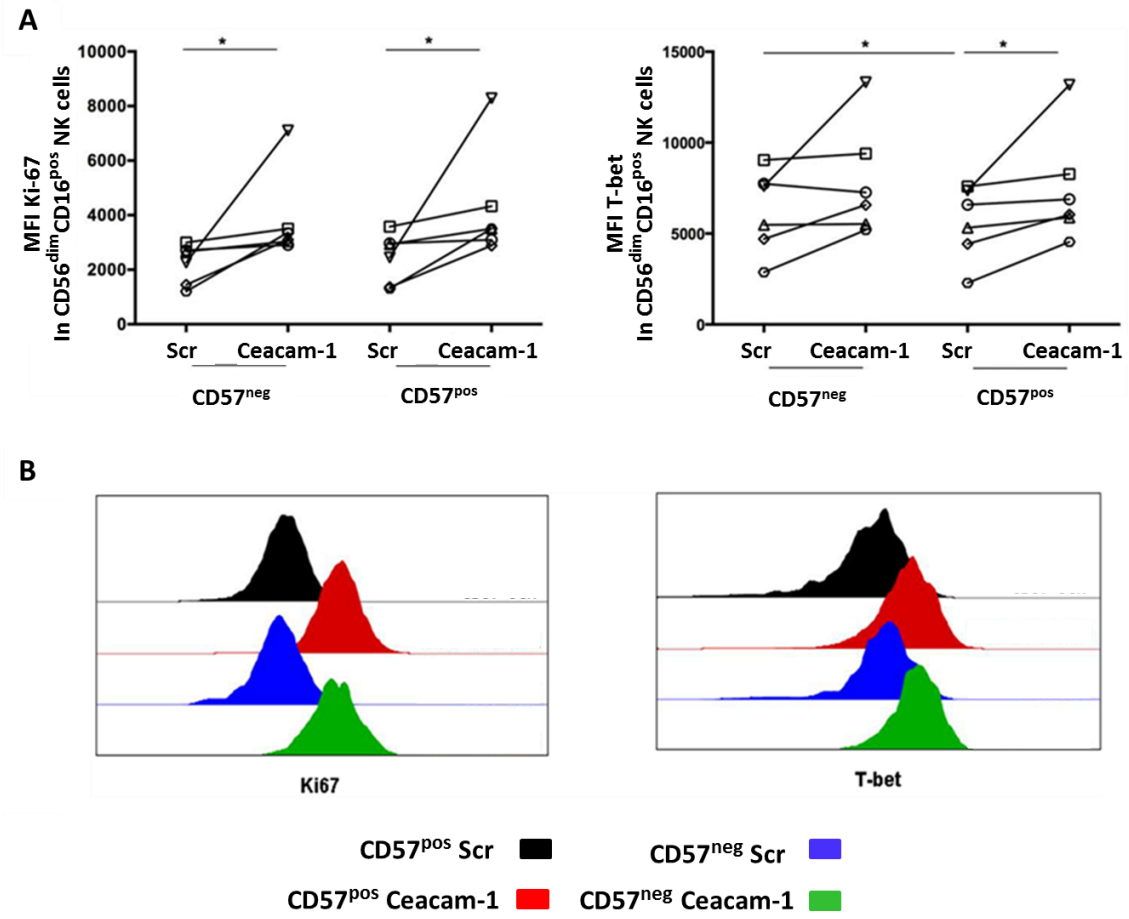


Figure 4.17 Ceacam-1 silencing restored NK cell proliferation potential and induced expression of T-bet in $CD57^{pos}$ NK cells. (A) Frozen PBMCs from six older donors were pre-activated overnight with IL-2 and IL-15 before transfection with scrambled (Scr) or specific Ceacam-1 siRNA. After resting, transfected cells were stimulated with CD16-coated antibodies. Ki-67 and T-bet expression were measured by intracellular staining on fixed cells. (B) Representative histogram of Ki-67 and T-bet expression in $CD57^{neg}$ and $CD57^{pos}$ NK cells from scrambled and Ceacam-1 siRNA-transfected PBMCs.

However, Ceacam-1 silencing did not restore cytotoxicity and cytokine secretion (as observed with Tim-3 blockade) (Figure 4.18). This discrepancy may be explained by the use of total PBMCs instead of sorted NK subsets (as in Figure 4.15). In conclusion, the data indicates that Tim-3 plays a pivotal role as a co-stimulatory molecule in immature NK cells and as a co-inhibitory molecule in association with Ceacam-1 in mature $NKG2C^{pos}CD57^{pos}$ NK cells.

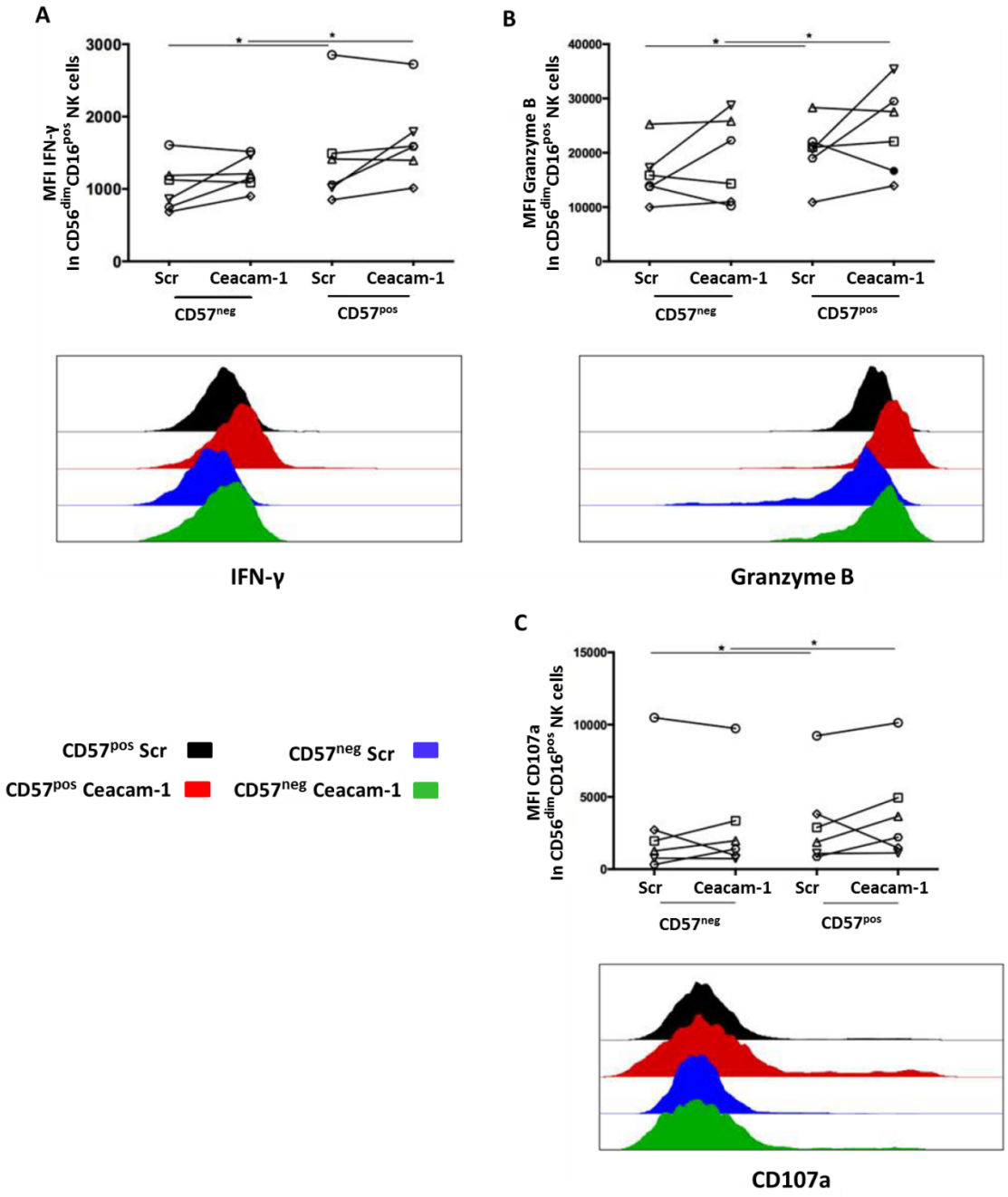


Figure 4.18 Preservation of IFN-γ and cytotoxic molecules release after siRNA silencing of Ceacam-1. Statistics and representative histograms of effector functions from NK cells after silencing with scrambled or Ceacam-1 siRNA. Frozen PBMCs from six older donors were pre-activated overnight with IL-2 and IL-15 before transfection with scrambled (Scr) or specific Ceacam-1 siRNA. After resting, transfected cells were stimulated with CD16-coated antibodies. IFN-γ, granzyme B and CD107a expression were measured by intracellular staining on fixed cells.

4.2.6 Identification of intratumoral cytotoxicity^{low} NK cells in advanced HCC stages by the expression of Ceacam-1 and CD57

To determine the relevance of our earlier results in a pathophysiological environment, we investigated CD57-expressing NK cell functionality in patients with different stages of hepato-cellular carcinoma (HCC) (Table 4.1). We compared phenotype and function of NK cells in paired samples collected from blood, tumor-infiltrating lymphocytes (TILs), or non-infiltrating lymphocytes residing in the liver (NILs). The composition of NK cells differed in the liver and peripheral blood. A significant enrichment of CD56^{bright}CD16^{neg} regulatory NK cells was detected in NILs and TIL associated with a reduction in the frequency of CD56^{dim} cytotoxic NK cells in TILs (Figure 4.19).

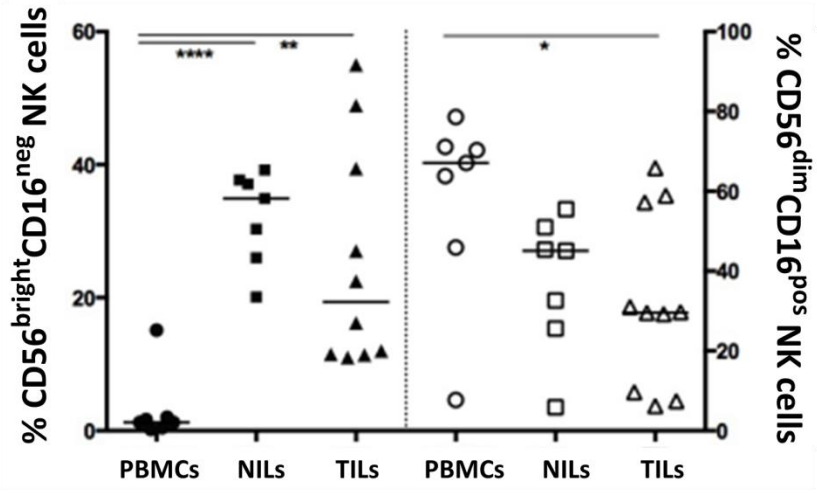


Figure 4.19 The composition of NK cell subsets differed in peripheral blood, healthy liver and tumoural liver. Percentage of CD56^{bright}CD16^{neg} and CD56^{dim}CD16^{pos} NK cell subsets were measured on paired PBMCs, NILs and TILs by flow cytometry.

Analysis of CD56^{dim}CD16^{pos} NK cells from TILs revealed a higher intensity of CD57 expression in the advanced stages of HCC (Figure 4.20A). Moreover, transcription factors, such as T-bet and Eomes, and exhaustion molecules, including PD-1, Ceacam-1, and TIM-3, were upregulated *ex-vivo* in patients with stage 2 to 4 HCC (Figure 4.20A). Functions of NK cells were also strongly dependent on clinical staging. The potential cytotoxicity of liver-resident NK cells (degranulation) was dramatically reduced in advanced stages of HCC while TNF-α secretion was increased (Figure 4.20B). Thus, the maturation of intra-tissue NK cells was associated with an exhausted phenotype and loss of cytotoxicity as previously observed in the peripheral blood of older adults.

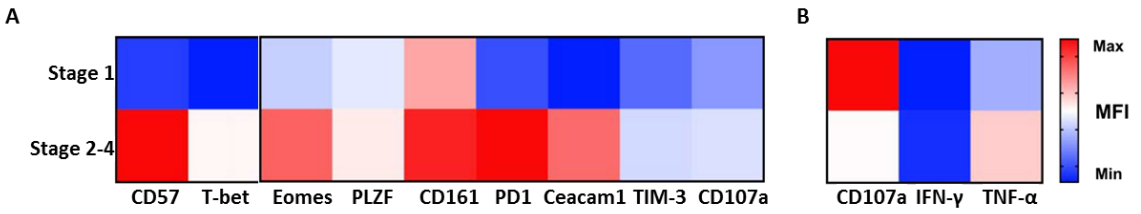


Figure 4.20 Liver infiltration of Ceacam-1^{pos}CD57^{pos} degranulation-deficient NK cells during progressive hepato-cellular carcinoma. (A) Phenotypic analysis of tumor-infiltrating lymphocytes in the liver was performed by surface and intracellular staining. HCC patients were grouped according to clinical diagnosis and classified as non-progressive (stage 1, n=4) and progressive cancer (stage 2–4, n=6). The median values of MFI for each group were represented by heat map. (B) Functions of NK cells were evaluated by flow cytometry after polyclonal stimulation of TILs and represented by heat map.

Whether or not CD57 expression identifies the same population (in terms of phenotype and function) in peripheral blood, tissue, and tumour is still unclear. To address this point, we compared paired samples from a patient diagnosed with advanced HCC (stage 4) according to CD57 expression. Firstly, this analysis indicated that the distribution of transcription factor was discriminatory; T-bet was associated with CD57^{pos} NK cells while Eomes and PLZF were coupled with CD57^{neg} NK cells in all tissues (Figure 4.21A). Secondly, CD161 and PD1 were also differentially segregated according to CD57 expression (Figure 4.21A). Finally, TIM-3 expression was enhanced in the liver (in particular in CD57^{neg} NK cells from the TILs) (Figure 4.21A). The secretion of IFN-γ and TNF-α was globally increased in the tumor with predominance in the CD57^{pos} NK cell subset (Figure 4.21B). Cytotoxic granules containing granzyme B were preserved, but the degranulation (and potential cytotoxicity) was decreased in CD57^{pos} NK cells (Figure 4.21B).

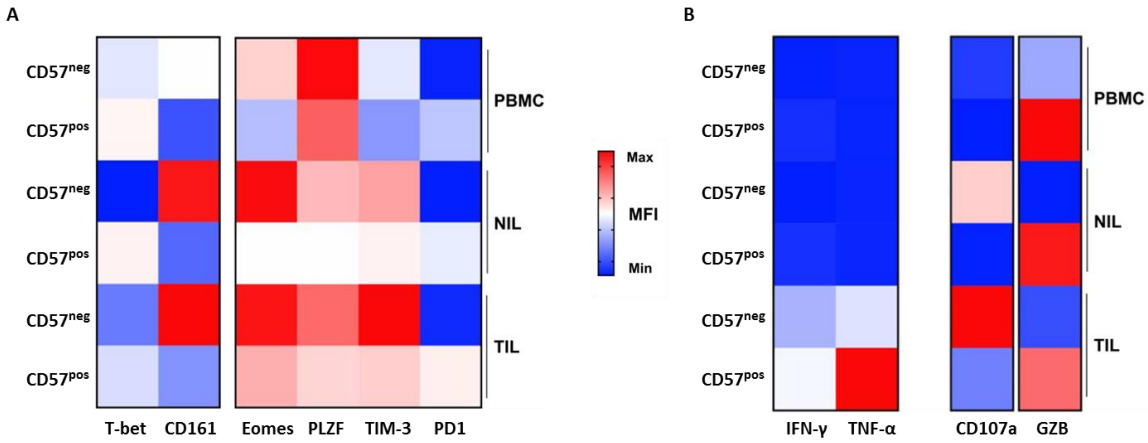


Figure 4.21 Profiling of intra-tumor NK cells based on CD57 expression. (A) Paired samples of an HCC patient with progressive pathology (stage 4) were phenotyped by flow cytometry and represented according to CD57

expression using a heat map. (B) Functions of NK cells were evaluated by flow cytometry after polyclonal stimulation of PBMCs, NILs or TILs and represented by heat map.

To decipher the relationship between TIM-3 expression in liver NK cells and cancer progression, we examined the co-expression of Ceacam-1 and Tim-3 in $CD56^{dim}$ cytotoxic NK cells (Figure 4.22). We were able to efficiently segregate stages of HCC progression according to co-expression of Ceacam-1 and TIM-3. Advanced liver cancer was indeed associated with NK cells presenting an exhausted phenotype and deficient cytotoxic functions. This data requires validation at a larger scale to evaluate if the phenotype of intra-tumoural NK cells could be used as a reliable diagnostic or prognostic biomarker. Further investigations are also necessary to evaluate the consequences of *in vivo* TIM-3/Ceacam-1 blockade as a potential new innate and adaptive immune checkpoint inhibitor.

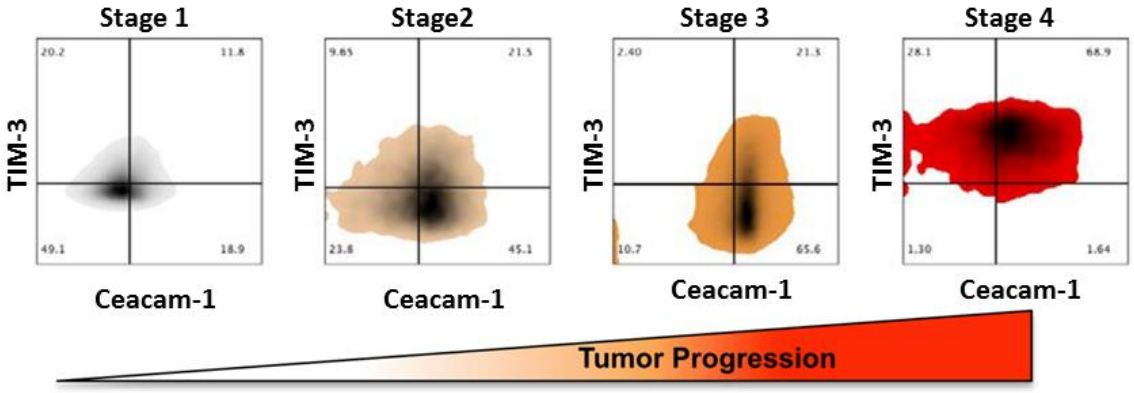


Figure 4.22 Stratification of cancer progression by TIM-3 and Ceacam-1 expression in NK cells from TILs. Phenotype of NK cells infiltrating the tumor from patients at different cancer progression stage was evaluated by flow cytometry.

4.3 Discussion

The switch in the partitioning between $CD56^{bright}$ NK cells and $CD56^{neg}CD16^{pos}$ NK cells represents a hallmark of NK cell development during aging. These results differed from the increased frequency of activated cytotoxic NK (Borrego et al., 1999) or the reduction of absolute count of regulatory NK cells (Chidrawar et al., 2006) observed by other groups. Differences of methodology, ethnicities, CMV sero-status and systemic inflammation may explain the variation in results.

As occurs for other immune cells, the expansion of inflammation-prone NK cells is also observed during persistent infections, such as HIV or HCV, that are coupled with high-grade inflammation. Inflammaging was defined here by a peripheral increase of IL-21 and TNF-α in older donors (Figure

4.5B). It could be envisaged that these pro-inflammatory cytokines constitute a driving force for the maturation of cytotoxic $CD56^{neg}CD16^{dim}$ NK cells and the expansion of $CD56^{neg}CD16^{pos}$ NK cells. However, the source of these cytokines remains to be identified. Putative candidates include several subsets of CD4 T cells including Th1, Th17, and follicular helper T cells. Dysregulation of these populations during aging are under scrutiny, but the increase of cytokines could also be explained by higher bioavailability due to lower utilization (due possibly to reduced IL-21R expression). IL-21, along with IL-2, represents the major homeostatic cytokines sustaining the survival and function of T cells, notably during chronic infection (e.g., LCMV, HIV, HCV) (Chevalier et al., 2011; Elsaesser et al., 2009; Kared et al., 2013). Hypo-responsiveness to IL-21, similar to the decreased sensitivity of NK cells to IL-12, IL-15, and IL-18, might explain the reduction of target cells for IL-21 (Frohlich et al., 2009). Furthermore, even if the frequency of mature $CD57^{pos}$ NK cells was correlated with inflammatory and CMV status (as observed for T cells (Kared et al., 2016), no association was found between inflammation and immune response against CMV. Loss of control of CMV replication and low-grade inflammation thus participate independently in the maturation of NK cells. Even though we detected an accumulation of NKG2C and CD85j expression at the surface of $CD57^{pos}$ NK cells, correlations between these markers and non-control of CMV replication were only established in younger donors (Figure 4.6).

The mechanisms governing maturation of NK cells are dependent on the TIM-3 pathway and, in particular, on the cis/trans-expression of TIM-3 with its ligand Ceacam-1 at the surface of late-differentiated NK cells. The computational model generated with Wanderlust analysis enabled us to chronologically classify the acquisition of different antigens for NK cell differentiation using an unbiased approach, which confirmed the early and specific acquisition of NKG2C, Ceacam-1, and TIM-3 in CMV-seropositive donors. CD57 and NKG2C expression are sufficient to discriminate the maturation stages of cytotoxic NK cells that are characterized by differential expression of transcription factor such as Foxo3, T-bet, or Zeb2. Although the link between TIM-3 and T-bet has been documented in the exhaustion of T cells during chronic infection and cancer (Buggert et al., 2014; Jones et al., 2008), the regulatory role of T-bet for Tim-3 is still debated.

Cooperation with other molecules, such as STAT3-NFIL3 (Zhu et al., 2005) or Zeb2, is crucial to induce TIM-3 in T cells and in NK cells. We observed in our HIV cohort a strong positive correlation between TIM-3 and T-bet, TIGIT and CD160 (data not shown). Moreover, Foxo3 could induce Eomes expression and drive the differentiation of murine pathogenic Th1 cells (Stienne et al., 2016). The concerted regulation of TIM-3/T-bet and Foxo3-Eomes pathway in NK cells may request further investigations in the future. The dysregulation of Zeb2 during NK cell maturation and decreased expression of T-bet in $NKG2C^{pos}CD57^{pos}$ NK cells from older donors suggests an altered propensity

to acquire TIM-3 (and probably Ceacam-1) during aging. Moreover, the functions of NK subsets are finely regulated and additionally dependent on the expression of TIM-3 and Ceacam-1. While neutralization of TIM-3 in NKG2C^{pos}CD57^{pos} NK cells induced an increased release of cytokines, chemokines, and cytotoxic molecules, blockade of Ceacam-1 in CD57^{pos} NK cells affected only the proliferation potential and T-bet expression but not cytotoxicity or cytokine secretion. We could not exclude that the role of TIM-3 is distinct in different NK subsets and is dependent on Ceacam-1 expression. The impact of other TIM-3 ligands, such as phosphatidylserine, Galectin-9 and HMGB1, require further investigation (Anderson et al., 2016).

We demonstrate here the role of TIM-3/ Ceacam-1 pathway as negative regulator of innate NK immunity during aging and these findings were extended to liver cancer. The participation of supplementary transcription factors, such as PLZF and Eomes, is also plausible in blood and tissue. The reduction of PLZF observed here in CD57^{pos} NK cells from tumor-infiltrating lymphocytes has already been demonstrated as responsible for the promoter DNA hypermethylation of genes coding for FcεRγ, SYK, and EAT2 (Schlums et al., 2015). Loss of these molecules by CD56^{dim} NK cells is attributed to HCMV infection and defined as a specific phenotype of “adaptive” NK cells. The stratification of TIM-3/Ceacam-1 expression in intra-tumoral NK cells according to cancer progression requires validation in a larger cohort but could constitute a promising biomarker during hepato-cellular carcinoma and possibly in other cancers or infectious diseases. It is interesting to observe that the loss of cytotoxicity (here marked by CD107a) is coupled with an increase of TNF-α in CD57^{pos} NK cells from TILs. This change is similar to the decrease of cytotoxicity and the upregulation of cytokine secretion in CD57^{pos} NK cells from aged participants observed here and by others (Strauss-Albee et al., 2015). Whether the increased poly-functionality of NK cells during aging is sufficient to compensate for the degranulation defect in pathogen elimination or cancer immune-surveillance is unclear.

The mechanisms driving the expansion of exhausted CD57^{pos} NK cells remain to be identified. IL-6 and CCL2 could transform and recruit NK cells with a phenotype associated with tumor-infiltrated lymph nodes (Ali et al., 2014). In the context of autoimmune disease, IFN-β therapy promotes Tim-3 expression at the surface of T cells from the peripheral blood of multiple sclerosis patients (Ottoboni et al., 2012). IL-27 could also drive the expression of TIM-3 on CD4 T cells. A similar induction of TIM-3 may occur for NK cells (Zhu et al., 2015). Finally, if the controversial topic of human innate memory was shown to directly participate in the expansion of mature NK cells, the human equivalent of murine Ly49H (ligand of the CMV antigen m157) (Sun et al., 2009) would require identification for viral infections. NK diversity could be induced by acute and chronic infections, such as HIV-1 (Reeves et al., 2015) or West-Nile virus (Strauss-Albee et al., 2015). During

aging, we could speculate that successive infections induce a footprint on partially exhausted NK cells (Zeb2^{high}Tbet^{low}), leading to NK cell diversity but rendering NK cells less responsive to neo-infections, vaccination, or anti-tumour activity. The increased heterogeneity of the NK cell repertoire was indeed associated with a higher susceptibility to HIV infection, reflecting an NK cell deficiency in the antiviral response (Strauss-Albee et al., 2015).

The preservation of functional innate immunity during chronic infections, cancer and aging thus requires either limited acquisition of exhaustion molecules such as Ceacam-1 and TIM-3 in mature NK cells coupled with stable transcription factor expression or the neutralization of these inhibitory molecules by interventional therapy.

4.4 Experimental procedures

4.4.1 Donors

Blood was collected from participants of the Singapore Longitudinal Aging Study (SLAS) cohort and from young controls. The study on the SLAS cohort has been approved by the National University of Singapore-Institutional Review Board 04–140. The study on the young control cohort has been approved by the Ethics Committee of the NUS-IRB 09-256. All study participants provided informed written consent. For details about SLAS and young control cohorts and blood processing please refer to Chapter 2.

Immune cells were isolated from hepato-cellular carcinoma (HCC) tumors, adjacent non-tumor tissues, as well as peripheral blood mononuclear cells (PBMCs) collected from the same patients (n=11) at the National Cancer Centre, Singapore. Patient consent was obtained with institutional review board (IRB) approval (2009/524/B). Clinical characteristics of the patients are listed in Table 4.1. Details about liver tissue processing can be found in Chapter 2.

4.4.2 Phenotyping

Cell phenotyping was performed by flow cytometry on 28 fresh PBMC samples from young donors and 34 fresh PBMC samples from older donors. For each staining, 1×10^6 PBMCs were used. Lymphocytes were gated based on FSC/SSC profile and doublets/dead cell exclusion. T cells were excluded by CD3 expression, followed by NK cell identification on CD3^{neg} lymphocytes using CD16 and CD56 expression. The antibodies are listed in Table 4.2. Flow cytometry was performed on an LSR Fortessa Cell Analyzer (BD Biosciences) and automatic compensation was applied.

Three million frozen PBMCs were stained for mass cytometry analysis. Data were acquired on a CyTOF instrument (DVS Sciences). The antibodies are listed in Table 4.3. Detailed information on this assay is reported in Chapter 3.

4.4.3 Flow cytometry functional assay

Assessment of cytokine release and cytotoxic molecule degranulation was performed by flow cytometry on ten PBMC samples from young donors and ten PBMC samples from older donors. For each staining, 1×10^6 PBMC were used. Cell stimulation with phorbol 12-myristate 13-acetate (PMA)/Ionomycin (50 ng/ml of PMA and 500 ng/ml of Ionomycin), K562 cell line (at a 1:10 ratio), or an anti-CD16 purified antibody-coated plate (1 µg/ml and 10 µg/ml) was performed. Unstimulated and PMA/Ionomycin activated cells were used as negative and positive controls, respectively. Cells were incubated for 5 h at 37°C and 5% CO2 in the presence of CD107a antibody (BD Biosciences). Brefeldin A (eBioscience) and Monensin (eBioscience) were added during the final 4 h of incubation. For the list of antibodies used, refer to Table 4.2. Flow cytometry was performed on an LSR Fortessa Cell Analyzer (BD Biosciences).

Table 4.1 Clinical features of HCC patients

ID	Sex	Age	Ethnicity	Stage	Grade	Tumor size (cm)	α-feto protein (ng/ml)	Treatment	Diabetes	HBV
94	M	77	Chinese	3	3	11	3.7	No	1	0
947	M	53	Chinese	2	3	5.2	N.A.	No	0	1
2050	M	66	Chinese	3	3	7.6	N.A.	No	1	0
6889	M	73	Chinese	1	2	5	N.A.	No	0	1
HEP0152	M	60	Chinese	3	3	15	17349	No	1	0
491	M	56	Chinese	3	3	13	171	Yttrium-90	0	1
HEP0174	M	66	Chinese	1	N.A.	6.5	7.2	No	0	1
HEP0026	M	73	Indonesian	4	N.A.	3.5	8.4	Surgery	1	0
HEP0178	M	64	Chinese	3b	N.A.	20	4072	TACE	1	0
HEP0185	M	76	Chinese	1	2	1.5	3.6	No	0	0
HEP0186	M	63	Chinese	1	2	4.1	N.A.	No	1	1

N.A.=not applicable

4.4.4 CMV ELISA

Frozen plasma samples were thawed and diluted 1:100 in the appropriate buffer. Seropositivity to CMV was tested by ELISA (Genesis Diagnostics) according to the manufacturer's instructions. The protocol used is reported in Chapter 3.

Table 4.2 List of antibodies and relative assays

ANTIGEN	FLUOROCHROME	CLONE	COMPANY	ASSAY
CD3	FITC	UCHT1	BioLegend	Phenotyping Sorting
CD3	APC-Cy7	SK7	BD Biosciences	Functional
CD3	BV786	UCHT1	BD Biosciences	Phenotyping
CD56	PE-Cy5	HCD56	BioLegend	Phenotyping Functional Sorting
CD56	FITC	HCD56	BioLegend	Phenotyping
CD16	AF700	3G8	BioLegend	Phenotyping Functional
CD16	APC-Cy7	3G8	BioLegend	Sorting
CD16	Purified	3G8	BioLegend	Stimulation
CD62L	APC-Cy7	DREG-56	BioLegend	Phenotyping
CD27	BV650	L128	BD Biosciences	Phenotyping
CD57	PB	HCD57	BioLegend	Phenotyping
CD57	BV570	HNK-1	BioLegend	Functional
CD335	APC	9E2	BioLegend	Phenotyping
CD38	PE	HB7	eBioscience	Phenotyping
IFN-γ	BV605	B27	BD Biosciences	Functional
TNF-α	PE-Cy7	Mab11	BioLegend	Functional
Granzyme B	PECF594	GB11	BD Biosciences	Functional
Perforin	BV421	B-D48	BD Biosciences	Functional
CD107a	BV786	H4A3	BD Biosciences	Functional
T-bet	PE	4B10	BioLegend	Functional
T-bet	BV421	4B10	BioLegend	Functional
Ki-67	AF647	Ki-67	BioLegend	Functional
TIM-3	Purified	F38-2E2	BioLegend	TIM-3 blockade
Ig control	Purified	MOPC-21	BioLegend	TIM-3 blockade
TIM-3	BV650	F38-2E2	BioLegend	Phenotyping
TIM-3	PE	344823	R&D	Phenotyping
NKG2C	PE	134591	R&D	Phenotyping
CD66a	BV421	B1.1/CD66	BD Biosciences	Phenotyping
Siglec-7	APC	6-434	BioLegend	Phenotyping
CD85j	PE-Cy7	GHI/75	BioLegend	Phenotyping
Eomes	PE-Cy7	WD1928	eBioscience	Phenotyping
PLZF	APC	6318100	R&D	Phenotyping
CD161	PE-Cy5	DX12	BD Biosciences	Phenotyping
PD-1	FITC	MIH4	BD Biosciences	Phenotyping
HLA-DR	BV605	L243	BioLegend	Sorting

Table 4.3 List of antibodies for mass cytometry (CyTOF)

ANTIGEN	Label (Atomic mass)
CD45	89
CD14	112
CD57	115
HLA-DR	142
CD69	145
CD8	146
CD4	147
CD45RO	148
CD49a	149
KLRG1	150
CD27	151
CD122	152
CD103	153
T-bet	155
Granzyme A	157
CD56	158
CD161	159
NKp44	160
CD38	161
Ki-67	162
CD127	163
Granzyme B	164
IL-18R	165
NKp46	166
TIM-3	167
CD3	168
CD25	169
NKG2C	170
Eomes	171
CD94	172
NKp30	173
Cd160	174
Perforin	175
DNA	191/193
Cisplatin	195
CD16	209

4.4.5 Multiplex analyte assays

A multi-analyte flow assay kit (LegendPlex Human Th Cytokines, BioLegend) was used for simultaneous quantification of TNF-α, IL-21, and IL-6 cytokine plasma levels from blood samples of eleven young donors and nine older donors. The assay was performed according to the manufacturer's instructions. The protocol used is reported in Chapter 3.

Cell sorting was performed with a FACSAria III (BD Biosciences) on 6 PBMC samples from young donors and 6 PBMC samples from older donors according to CD57 and NKG2C expression. For the list of antibodies used for sorting, refer to Table 4.2. After 18-h incubation with anti-CD16 (Biolegend; 1 µg/ml), supernatants were collected and tested by Luminex assay. The Milliplex

HCD8MAG-15K (Millipore) was used according to manufacturer's instructions (as explained in Chapter 3).

4.4.6 TIM-3 blockade

TIM-3 receptor blockade on NK cell function was assessed by pre-incubating PBMCs or sorted NK cell subsets in the presence of anti-TIM-3 purified antibody (Biolegend; 10 µg/ml) or Ig control (Biolegend; 10 µg/ml) for 1 h prior to anti-CD16 stimulation (Biolegend; 1 µg/ml; 5 h or 18 h at 37°C and 5% CO2). For the list of antibodies used, refer to Table 4.2.

4.4.7 Quantitative real-time PCR

NK cells sorted according to CD57 and NKG2C expression were lysed with RLT buffer with 1% of β-mercaptoethanol *ex vivo* or after 18-h anti-CD16 stimulation (Biolegend; 1 µg/ml). RNA extraction was performed using an RNeasy Plus Micro kit (Qiagen) and reverse transcribed into cDNA using the SuperScript First Strand kit (Invitrogen). cDNA was analyzed by real-time PCR with the KAPA SYBR qPCR Master Mix kit (KAPA Biosystems) and the following primers: *ZEB2* (Entrez Gene ID 9839; detected transcripts NM_001171653, NM_014795, XM_006712881, XM_006712882; Qiagen), *TBX21* (Entrez Gene ID 30009; detected transcript NM_013351; Qiagen) *and FOXO3* (Entrez Gene ID 2309; detected transcripts NM_001455, NM_201559, XM_005266867, XM_005266868, Qiagen).

4.4.8 RNA-mediated interference

Seven frozen PBMC samples from older donors were electroporated with a Neon transfection kit and device (Invitrogen). Cells (3×10^5) were incubated for 18 h with 10 ng/ml of IL-2 (Peprotech) and 10 ng/ml of IL-15 (Peprotech) and resuspended in 10 µl of buffer T (Neon kit, Invitrogen). Ceacam-1 siRNA (Entrez gene ID 634; detected transcripts NM_001024912.2, NM_001205344.1, NM_001712.4; Ambion) or negative control siRNA (scrambled (Scr); Ambion) were added to the cell suspension at a final concentration of 100 nM. Ten microliters of the suspension were electroporated (1,700 V, 20 ms, three pulses). GAPDH siRNA (Ambion) was used as a positive control to evaluate efficiency of the silencing. Cells were incubated for 24 h at 37°C and 5% CO2 and then stimulated in anti-CD16 coated plate (1 µg/ml) for 5 h at 37°C and 5% CO2 in the presence of CD107a antibody. Brefeldin A (eBioscience) and Monensin (eBioscience) were added during the last 4 h of incubation. Surface markers and functions by intracellular staining were assessed by flow cytometry as described above.

4.4.9 Data analysis

Flow cytometry analysis: data were analyzed using FlowJo (Treestar) and FACSDiva (BD Biosciences). Samples were compared using GraphPad Prism software (v.6.0c). The software SPICE (Roederer et al., 2011) was used to evaluate the polyfunctionality of NK cells. Developmental trajectory during NK cell maturation was created with the Wanderlust algorithm (Bendall et al., 2014). **tSNE analysis of flow and mass cytometry data:** unbiased representations of multi-parameter flow cytometry data were obtained using the t-distributed stochastic neighbor embedding (tSNE) algorithm (van der Maaten, 2008). **Statistical analysis:** groups of young and elderly donors were analyzed by Mann-Whitney U test to compare values. The Wilcoxon matched-pairs signed rank test was used for paired testing of median values of different subsets from the same donor. We used the Pearson rank correlation test to compare correlation between anti-CMV IgG titer and frequency of NK cells subsets. We reported r^2-values and p-values. Analysis with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significantly different between the groups.

Chapter 5: Maturation, functions and gene regulation of murine natural killer cells are modulated by aging

5.1 Introduction

A hallmark of immunosenescence is the increased susceptibility of the elderly population to infections (Akbar et al., 2004) and cancer (Campisi, 2003). Knowing that NK cells play a critical role in coordinating tumour immune-surveillance and the immune response to virally-infected and transformed cells, it becomes clear that the understanding of NK cell maturation and function during aging may lead to novel insights into age-related immune dysfunctions.

NK cells become functionally competent during a complex multi-step process of development, maturation and education that is associated with progressive acquisition or down-regulation of a series of cell surface markers. $CD122^{pos}$ NK precursors (NKPs) arise from the common lymphoid progenitors (CLPs) and their definitive commitment to the NK lineage is accompanied by the acquisition of NK1.1 and CD27. As these early NK cells mature, they upregulate CD11b, CD49b (recognized by mAb DX5), CD62L, Ly6C, CD43 and TIM-3, while expression of the CXC-chemokine receptor 3 (CXCR3) and CD127 dims (Di Santo, 2006). At this step, NK cells leave the bone marrow and start seeding various lymphoid and non-lymphoid peripheral tissues, especially lymph nodes, blood, spleen and lungs. After migrating to the periphery, downregulation of CD27 and upregulation of KLRG1 mark the most mature NK subset. During maturation, NK cells also undergo a process of education (licensing) with acquisition of a high level of the MHC-I-binding Ly49 receptors, that are the counterparts of the human killer cell immunoglobulin-like receptors (KIRs) (Rahim et al., 2014). In particular, Ly49H expression has been associated to the so-called MCMV-induced memory NK cells (Sun et al., 2011). These cells are able to proliferate, expand and become highly activated during acute MCMV infection, a potent driver of NK differentiation. According to the expression of CD27 and CD11b, Hayakawa and Smyth proposed a linear differentiation model from $CD11b^{neg}CD27^{pos}$ (CD27) to $CD11b^{pos}CD27^{pos}$ (double positive [DP]) to $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells (Hayakawa and Smyth, 2006). Some researchers reported a fourth maturation stage defined as $CD11b^{neg}CD27^{neg}$ (double negative [DN]) and preceding the CD27 subset (Chiossone et al., 2009). However, DN NK cells remain poorly described.

This profound phenotypic variation reflects changes to cell functionality, influencing immunomodulatory and proliferative capacity, cytotoxic arsenal and trafficking machinery. CD27 NK cells are the most potent cytokine producers (especially INF-γ, TNF-α and GM-CSF) and can

proliferate extensively; conversely, the CD11b population is composed of professional cytotoxic cells with poor proliferative and cytokine secretion ability (Di Santo, 2006).

An intricate network of transcription factors manages NK maturation. Nuclear factor interleukin 3 regulated (Nfil3) (Kamizono et al., 2009), thymocyte selection-associated high mobility group box protein (TOX) (Aliahmad et al., 2010) and ETS1 (Ramirez et al., 2012) are regulators of the early stages. Final maturation is mainly shaped by T-box containing protein (T-bet, encoded by *Tbx21*) (Townsend et al., 2004), zinc finger E-box–binding protein 2 (ZEB2, encoded by *Zeb-2*) (van Helden et al., 2015) and B-lymphocyte-induced maturation protein 1 (Blimp-1, encoded by *Prdm1*) (Kallies et al., 2011). T-bet drives terminal NK cell maturation by reducing proliferation (Townsend et al., 2004), up-regulating the expression of sphingosine-1 phosphate receptor 5 (S1pr5) mRNA (Jenne et al., 2009), GZB and KLRG1 (Simonetta et al., 2016) and promoting the transition to the mature CD11b stage through repression of CD27 (Soderquest et al., 2011). Part of these effects seems to be mediated by T-bet induction of Blimp-1 and ZEB2. Blimp-1 expression in NK cells requires T-bet and is upregulated during development, when it functions to restrict proliferation (Kallies et al., 2011). During NK maturation, also ZEB2 expression is induced by T-bet, and T-bet and ZEB2 together control the expression of the same genes in a concerted manner in order to irreversibly induce terminal differentiation (van Helden et al., 2015). The transcription factor zinc finger and BTB domain containing 32 (Zbtb32, encoded by *Zbtb32*) has been shown to be dispensable for NK maturation but regulates the proliferative burst of Ly49H^{pos} NK cells in response to MCMV infection, that is required for NK cell–mediated protection against lethal viral challenge; Zbtb32 has been found to promote proliferation by antagonizing the anti-proliferative Blimp-1 (Beaulieu et al., 2014).

Previous studies showed that differentiation of human and murine natural killer cells is deeply influenced by aging. A decreased number and percentage of total NK cells and mature NK cells in most tissues of aged mice has been reported (Beli et al., 2014; Fang et al., 2010). NK cells from aged mice present several maturation defects such as lower expression of KLRG1 (Beli et al., 2014). Nair et al. recently confirmed and further expanded previous data showing reduced proliferation *in vivo*, dysregulated expression of Eomes and altered expression of integrins (Nair et al., 2015). They also demonstrated that this array of defects in NK differentiation is the result of deficient maturation signals provided by bone marrow stromal cells during aging. However, whether defective NK cell maturation in aging significantly influences their functional competence remains poorly understood.

Based on the literature above, we sought to expand the knowledge on how aging impacts murine NK cell maturation and function. In this study, we report that NK cells are reduced in frequency and

numbers and exhibit an altered phenotype in the blood and spleens of aged mice. Investigating the expression of a variety of cell surface markers associated with the maturation process, we demonstrate that aging is characterized by an accumulation of immature NK cells coupled with a reduction in the late differentiated CD11b subset. This phenotypic immaturity reflects a relevant functional immaturity. Our results show that cytokine secretion, cytotoxicity and gene expression of NK cells are modulated by the aging process along a maturation pathway defined by CD11b and CD27 and, in some cases, LY49H and KLRG1.

5.2 Results

5.2.1 Aged mice had reduced total and mature NK cells in spleen and blood

First of all, number and percentage of total NK cells (defined as $CD3^{neg}NK1.1^{pos}$) were measured across the two ages. When we compared the spleens and blood of young (4 months old) and aged (21 months old) naive mice, we observed that total NK cells proportion was significantly diminished in aged mice (Figure 5.1A). Median percentages decreased with aging from 2.7% to 0.9% in spleen and from 11.9% to 1.4% in blood. Number of total NK cells was also found to be lower in both spleen (where it halved from a median of $2x10^6$ to $1x10^6$) and in blood (from a median of 5575 to 920) in older mice (Figure 5.1C).

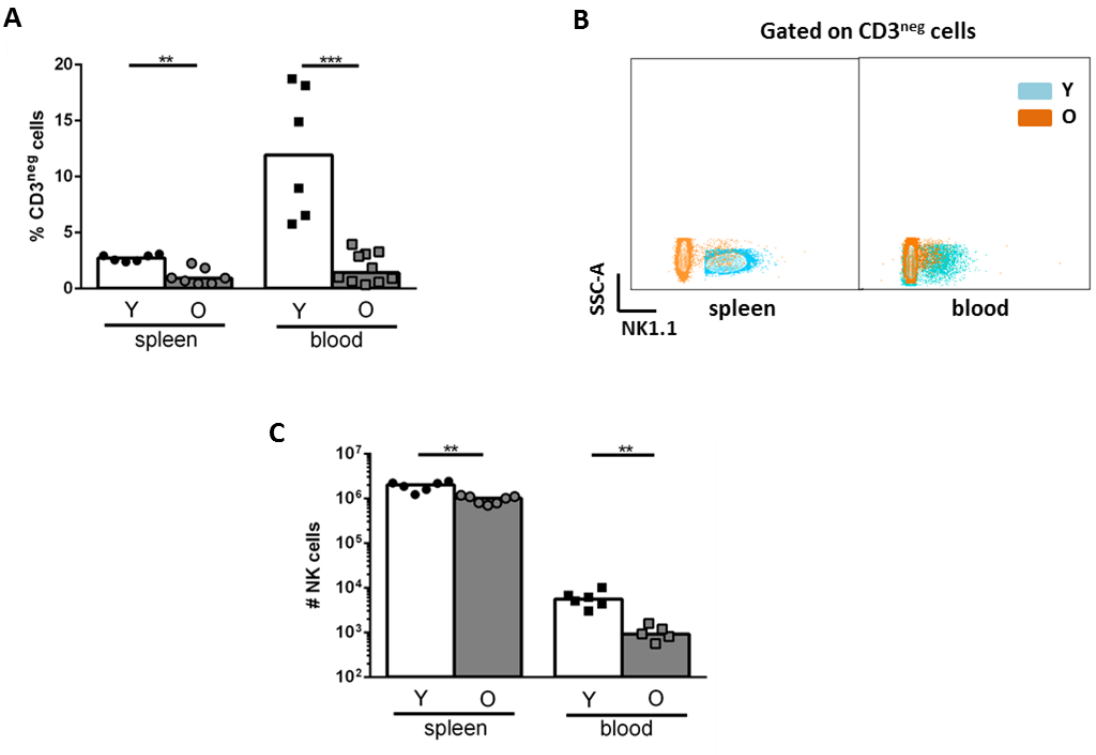


Figure 5.1 Aged mice had reduced total NK cells in spleen and blood. (A) NK cell percentage statistics and (B) representative pictures in spleen and blood. (C) NK cell numbers in whole spleen and blood (per 150 uL of blood). (C) Percentage of $CD27^{pos}$ and $CD11b^{pos}$ NK cells in spleen and blood.

When we examined the distribution of NK cell maturation stages, we observed that aging affects not only the overall quantity of NK cells but also their differentiation process. A plethora of surface markers was used to investigate this phenomenon. Initially, the expression of the immature status marker CD27 and the late maturation marker CD11b was measured independently. Percentage of NK cells positive for CD27 and the relative MFI resulted higher in spleen and blood of old mice while there was a decrease of both measurements regarding CD11b (Figure 5.2), suggesting that aging is characterised by phenotypic immaturity in the tissues studied.

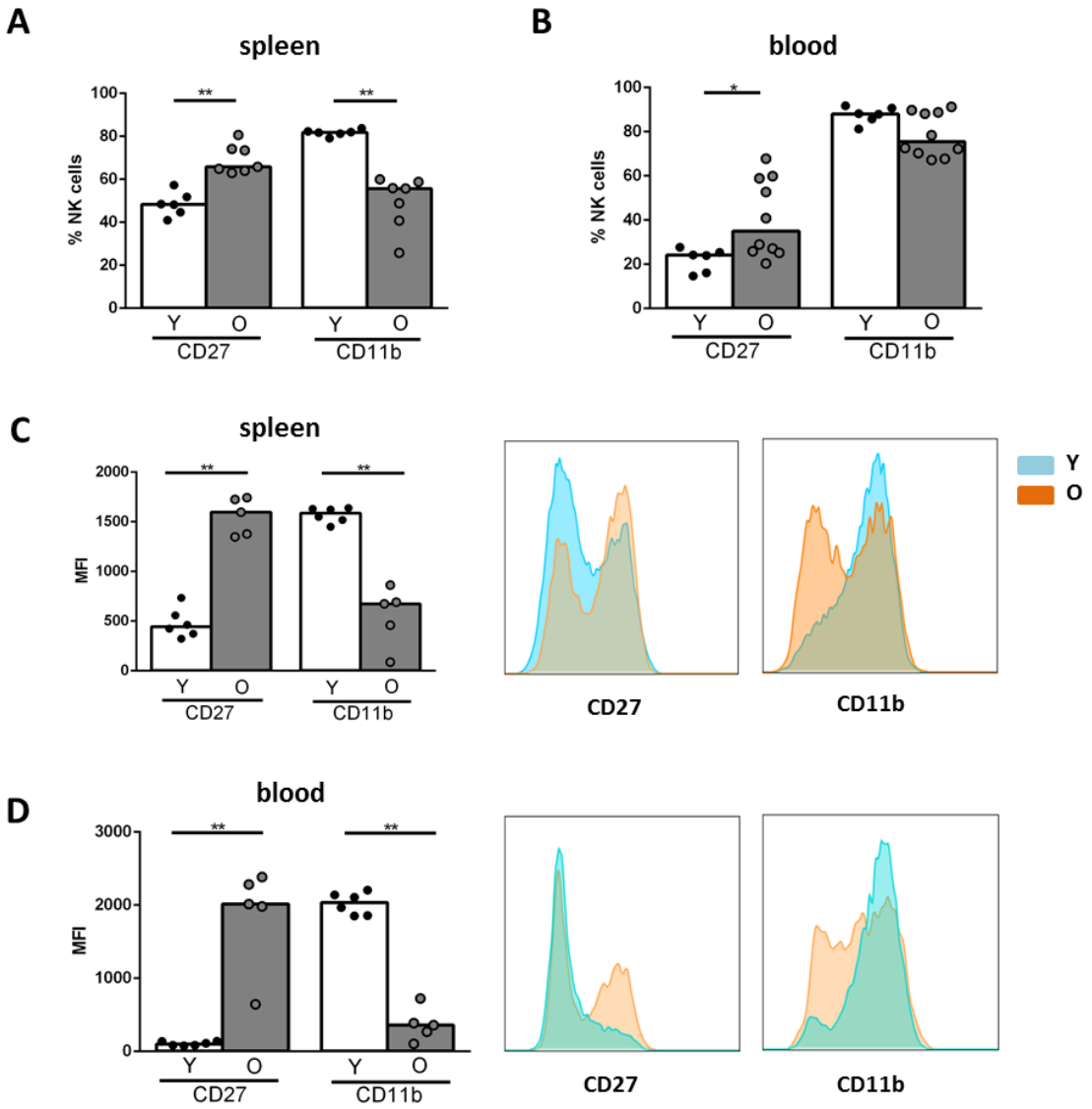


Figure 5.2 Aged mice had altered expression of maturation markers on NK cells in spleen and blood. (A) Percentage of NK cells expressing CD11b or CD27 in spleen and (B) blood in young (Y) and old (O) mice. (B) Expression levels of CD11b or CD27 in spleen and (C) blood in young (Y) (n=6) and old (O) mice (n=5).

These data were confirmed using the CD27/CD11b model of NK cell differentiation that allowed us to subdivide the total NK population into four subsets: $CD11b^{neg}CD27^{neg}$ (double negative [DN]), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (double positive [DP]), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells, listed from the least mature to the most mature (Figure 5.3). Older animals showed an accumulation of immature CD27 NK cells (from 13.8% to 36.3% in spleen; from 5% to 19.1% in blood) coupled with a reduction in the CD11b late differentiated subset (from 49.1% to 18% in spleen; from 72.4% to 62.9% in blood). Additionally, we described a significantly higher proportion of DN NK cells in spleen whereas no difference was found regarding the intermediate DP population in both tissues.

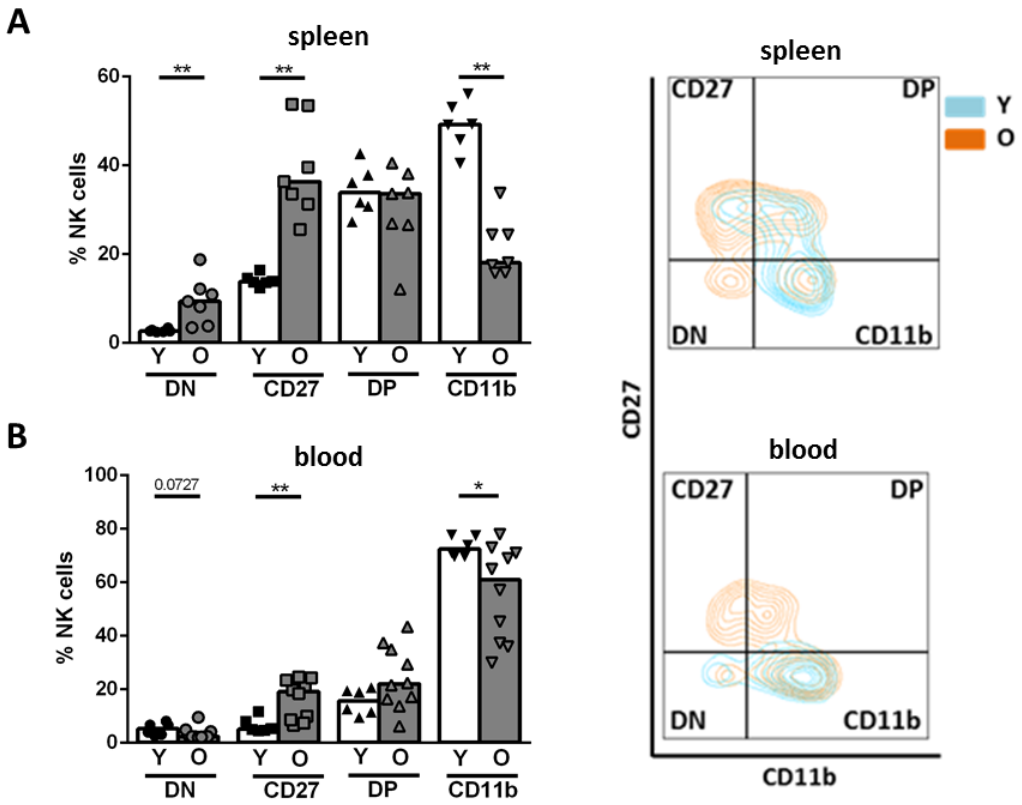


Figure 5.3 Aged mice had reduced total NK cells in spleen and blood. (A) Statistics and representative histograms of NK subset distribution according to CD27 and CD11b expression in spleen and (B) blood in young (Y) and old (O) mice. $CD11b^{neg}CD27^{neg}$ (double negative [DN]), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (double positive [DP]), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells.

5.2.2 Age-associated deficits in NK cell maturation correlated with systemic inflammation

Human aging is accompanied by an increase in the levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in the serum and tissues, a condition of persistent low-grade inflammation that has been termed “inflamm-aging” (Franceschi and Campisi, 2014). This phenomenon has been associated with the onset and progression of age-related diseases, including type 2 diabetes mellitus, cardiovascular disease, cancer and neurodegeneration (Franceschi and Campisi, 2014). However, data regarding this inflammatory status in the aged murine model are poor.

To address this question, serum level of some pro-inflammatory molecules were measured. Concentrations of TNF-α, IL-1β and MIP-3α were significantly higher in older mice compared with younger ones (Figure 5.4).

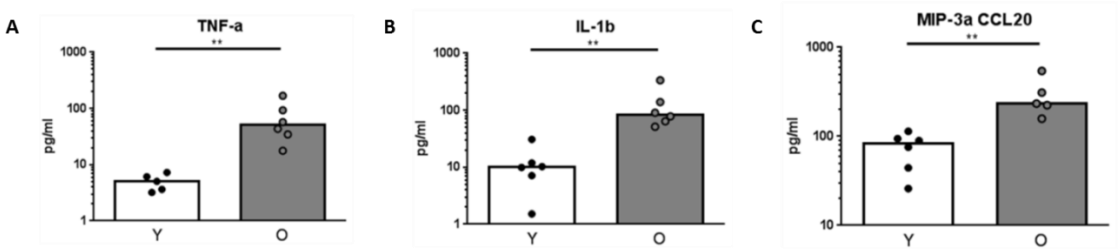


Figure 5.4 Aged mice showed signs of systemic inflammation. Concentration of (A) TNF-α, (B) IL-1β and (C) MIP-3α (CCL20) were tested in the serum of young (Y) and old (O) mice via Luminex technology (TNF-α: 50.7 pg/ml vs 5.06 pg/ml, p=0.0043; IL-1β: 84.47 pg/ml vs 10.1 pg/ml, p<0.0022; MIP-3α: 232.1 pg/ml vs 82.59 pg/ml, p=0.0043).

Additionally, we sought to determine if altered NK cell maturation during aging is associated with systemic inflammation. The frequency of immature CD27 or mature CD11b NK cells in spleens (Figure 5.5) and blood (data not shown) correlated significantly with the concentration of pro-inflammatory cytokines present in the serum of older animals. We were unable to detect measurable levels of IL-6 (data not shown).

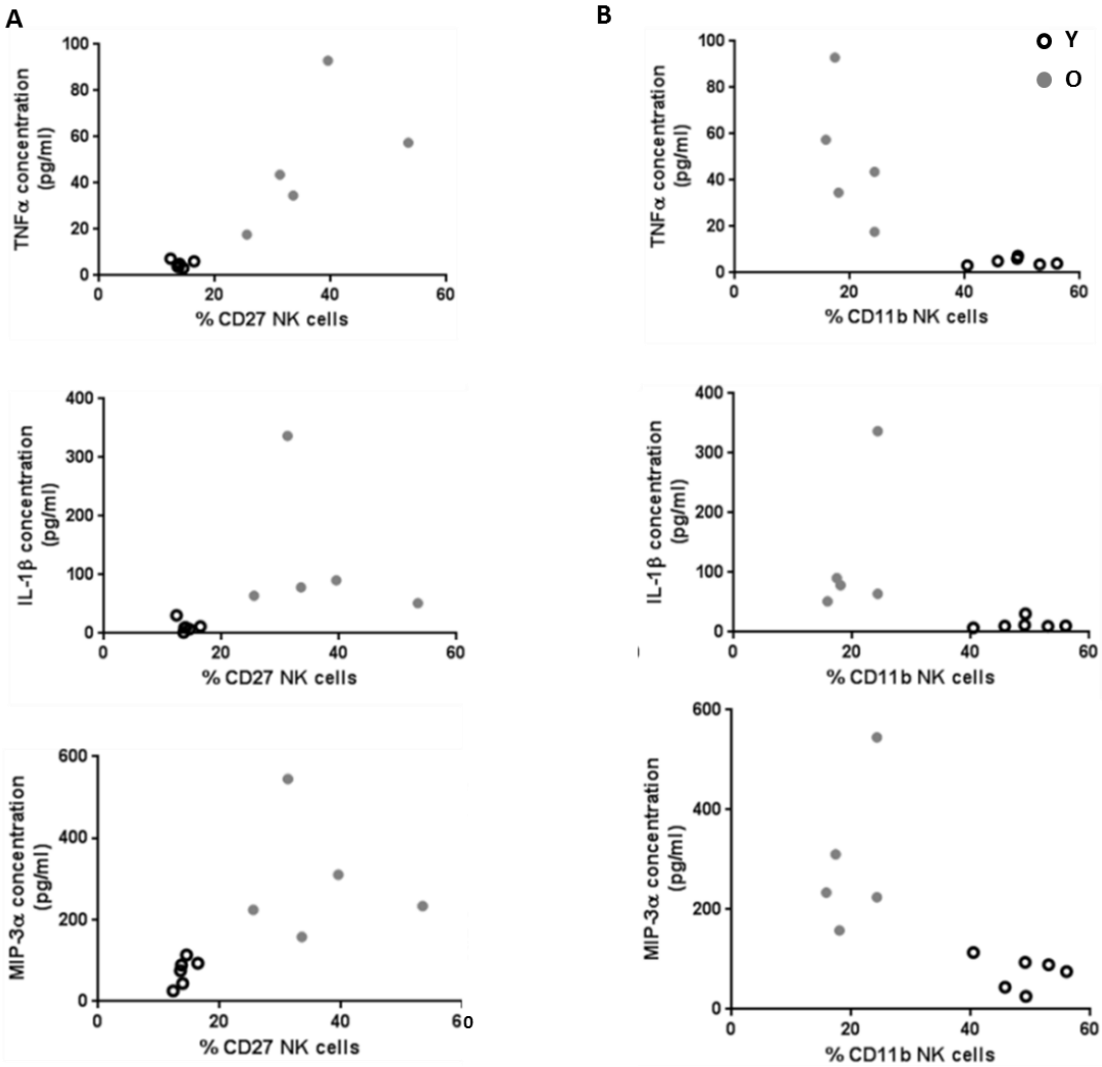


Figure 5.5 Age-associated systemic inflammation correlated with altered NK cell differentiation. (A) The percentage of CD27 NK cells present in the spleen was positively correlated with the level of pro-inflammatory cytokines in the serum of older animals (TNF-α: r=0.7818, p=0.0064; IL-1β: r=0.7182, p=0.0162; MIP-3α: r=0.8818, p=0.0007). (B) The percentage of CD11b NK cells present in the spleen was negatively correlated with the level of pro-inflammatory cytokines in the serum of older animals (TNF-α: r=-0.7927, p=0.0051; IL-1β: r=-0.6515, p=0.0339; MIP-3α: r=-0.8064, p=0.004).

5.2.3 Total NK cells from aged mice showed additional signs of phenotypic immaturity

To extend and detail the aforementioned results, we stained NK cells from spleen and blood of young and old mice with further antibodies to molecules whose levels are regulated along the NK cell differentiation pathway (Figure 5.6). Percentages of NK cells expressing KLRG1, CD62L and DX5, which are normally expressed in the most mature NK cells, and relative MFI were reduced on total

NK cells in the spleen of aged mice while Ly49H showed a significant decline in spleen and blood. No difference was found for other two maturation markers, namely Ly6C and TIM-3.

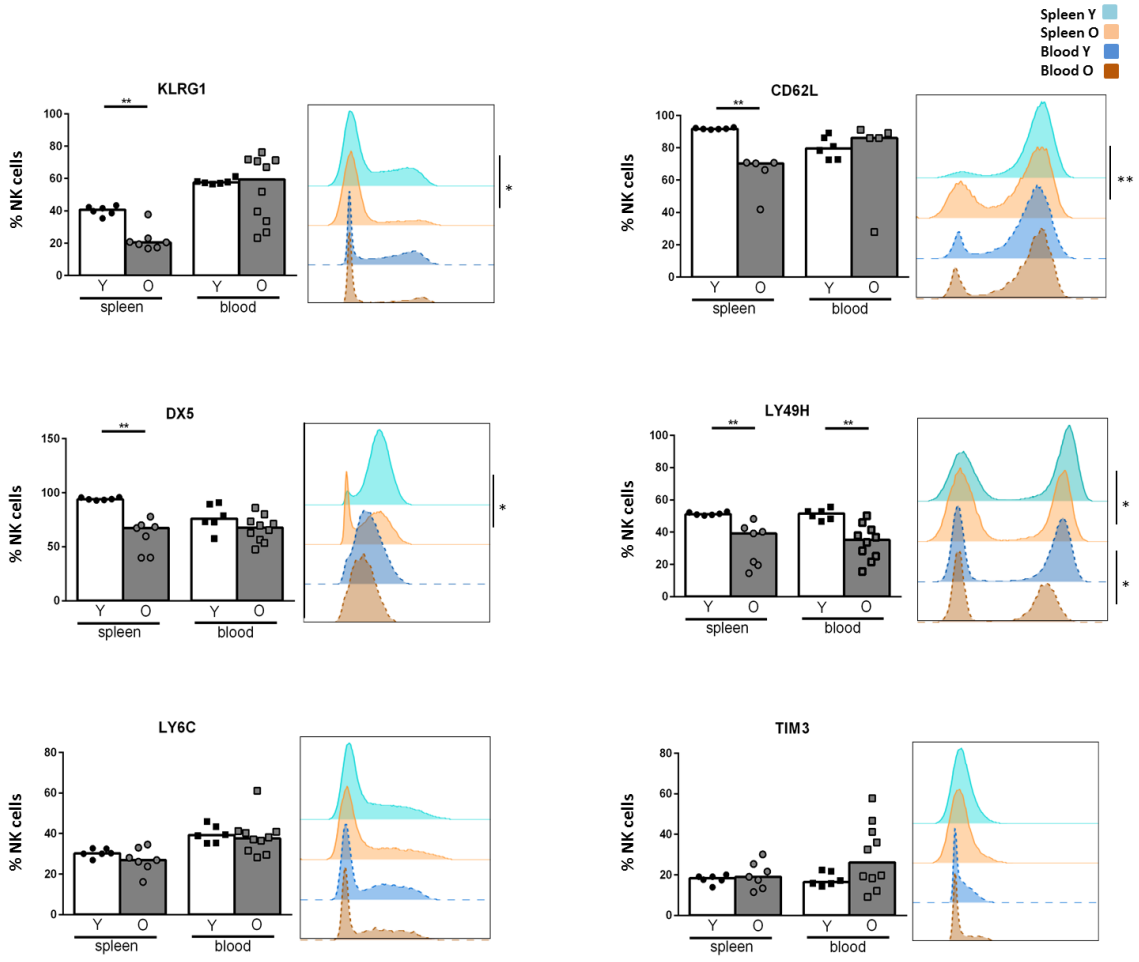


Figure 5.6 NK cells from aged mice showed additional sign of phenotypic immaturity. Percentage of NK cells positive for the indicated markers and related histograms of the MFI in spleen and blood of young (Y) (n=6) and old (O) mice (n=7-10).

5.2.4 Age modulated acquisition of maturation markers on NK cell subsets in spleen and blood

The observation that normal maturation at the total NK cell population level was impaired in old animals prompted us to analyse at which differentiation stage/s this abnormality occurs. In spleen, the most generalised alteration involves CD62L^{pos} NK cells and DX5^{pos} NK cells of older mice whose proportion was lower in all maturation subsets (Figure 5.7). The MFI of CD62L and DX5 also decreased with age across the whole differentiation process (Figure 5.9). Additionally, the expected acquisition of KLRG1 and Ly6C appeared hampered in all phases but one (CD11b cells for KLRG1 and

DP cells for Ly6C) (Figure 5.7 and Figure 5.9). The decline in the percentage of cells possessing the receptor Ly49H and in its level on the surface was limited to the immature DN cells and the late differentiated CD11b population (Figure 5.7 and Figure 5.9). TIM-3 was the most preserved of the molecules investigated between young and old animals since we showed that the MFI and the proportion of TIM-3^{pos} cells differed in CD11b NK cells only, with higher measurements in aged mice (Figure 5.7 and Figure 5.9).

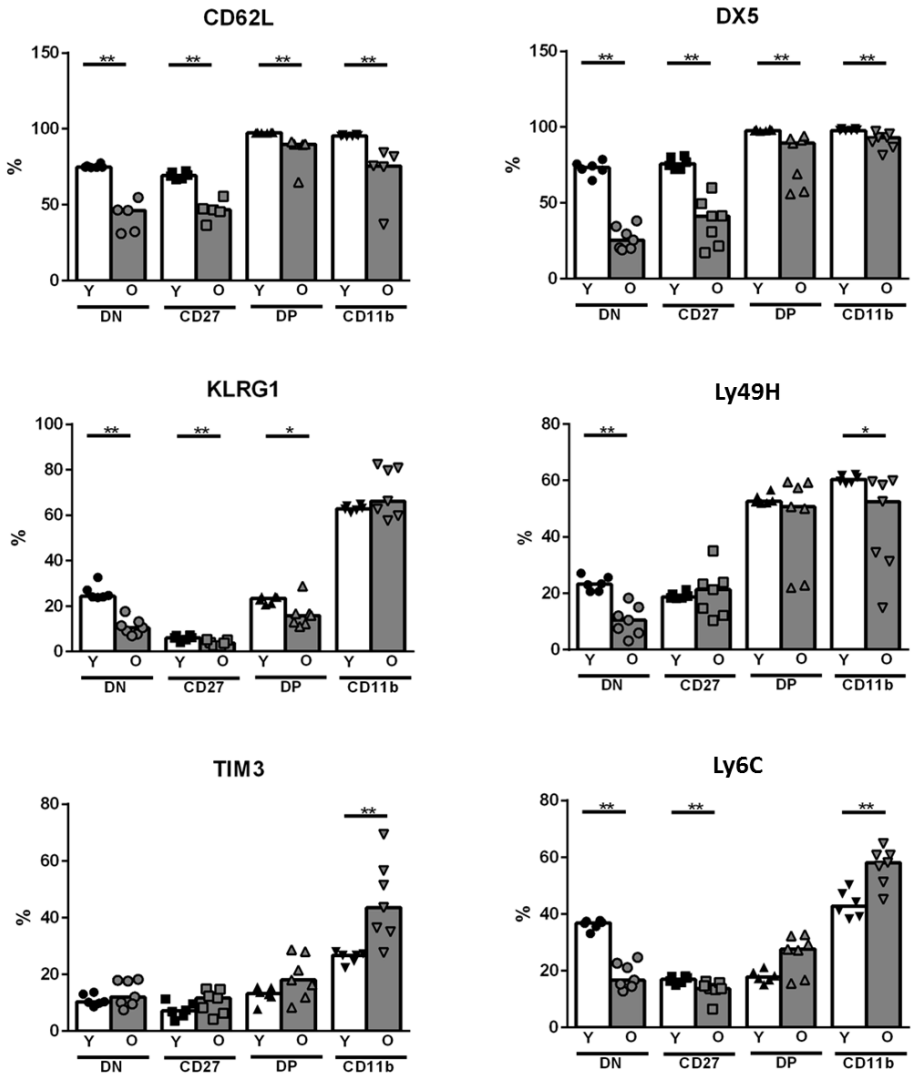


Figure 5.7 Age modulated acquisition of maturation markers on NK cell subsets in spleen. Percentage of NK cells positive for the indicated markers in spleen in the different maturation subsets in young (Y) and old (O) mice. CD11b^{neg}CD27^{neg} (double negative [DN]), CD11b^{neg}CD27^{pos} (CD27), CD11b^{pos}CD27^{pos} (double positive [DP]), CD11b^{pos}CD27^{neg} (CD11b) NK cells.

In blood, NK cell maturation appeared to be mostly unaltered or even driven by aging. Old animals have higher expression of some maturation markers on certain NK cell subpopulations (DN cells for KLRG1, CD27 cells for CD62L, DN and CD27 cells for Ly6C and DP cells for TIM-3) (Figure 5.8 and Figure 5.9). More consistent with the spleen data were the results related to Ly49H and DX5 in blood (Figure 5.8 and Figure 5.9). With aging, the proportion of Ly49H^{pos} immature CD27 NK cells in blood increased whereas it was diminished in intermediate DP and in mature CD11b cells. Percentage of cells expressing the molecule DX5 differed at the late stage of maturation, with a lower value in 21-month old mice (Figure 5.8 and Figure 5.9). Thus, our results and those of others ((Beli et al., 2014; Fang et al., 2010; Nair et al., 2015)) demonstrate that aged animals had fewer mature NK cells in the blood and spleen and our deep phenotypic characterization revealed that they also showed altered expression of the investigated NK cell maturation markers, with different effects in the two tissue types analysed here.

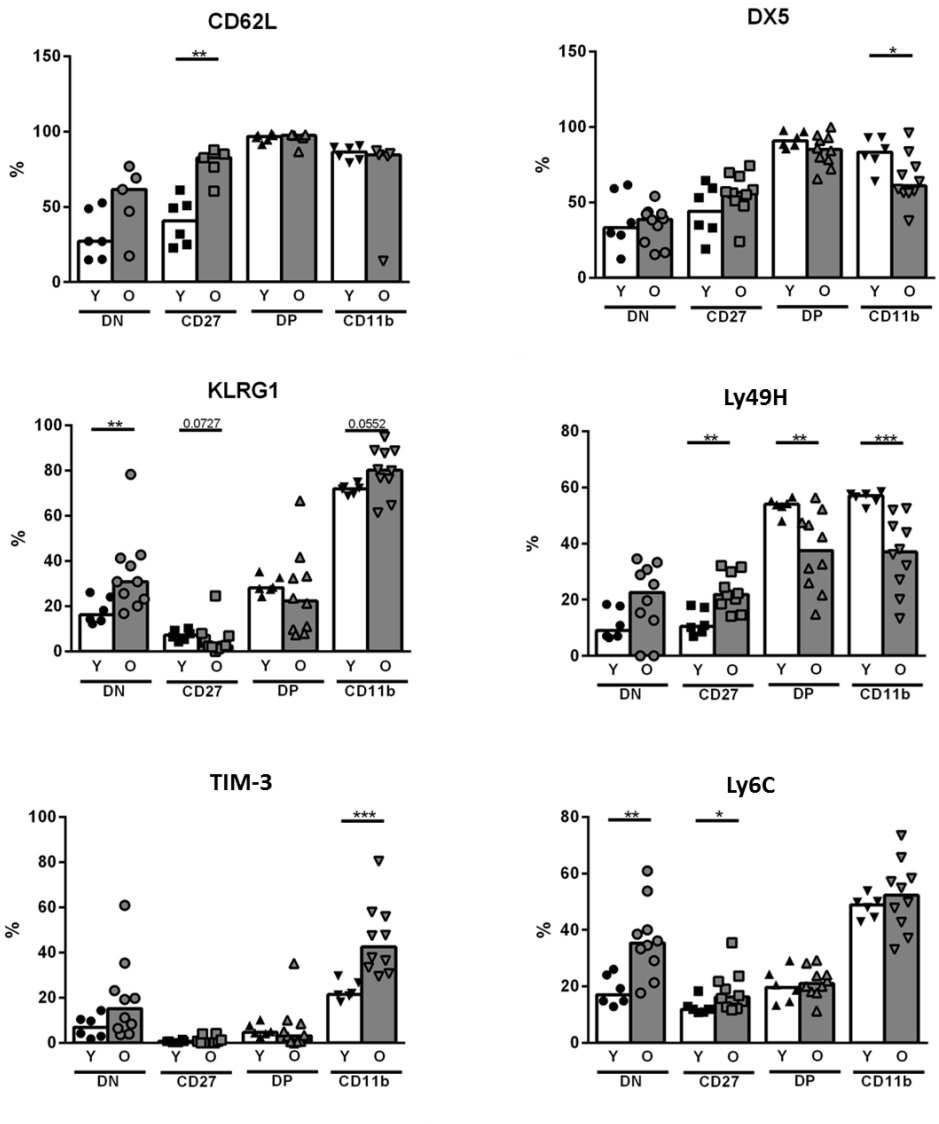


Figure 5.8 Age modulated acquisition of maturation markers on NK cell subsets in blood. Percentage of NK cells positive for the indicated markers in blood in the different maturation subsets in young (Y) and old (O) mice. $CD11b^{neg}CD27^{neg}$ (double negative [DN]), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (double positive [DP]), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells.

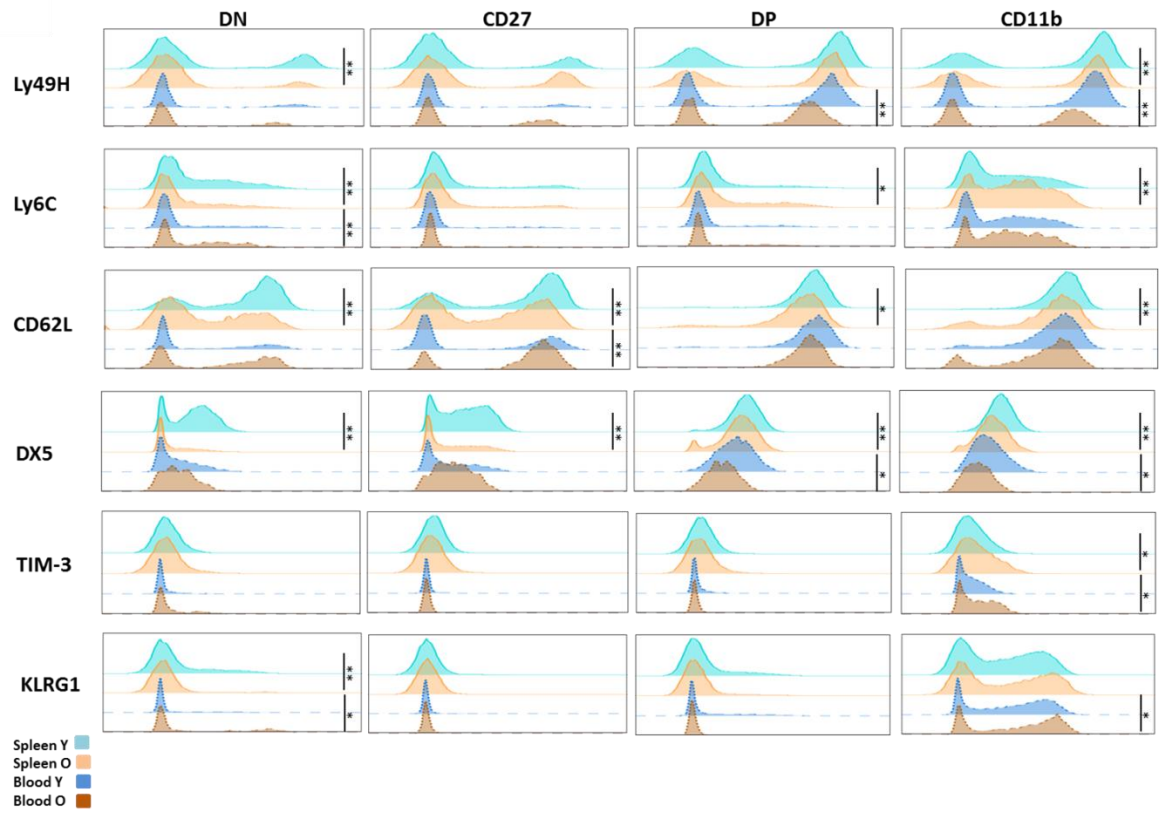


Figure 5.9 Expression levels of maturation markers were affected by aging on NK cell subsets in spleen and blood. Histograms of the MFI of the indicated markers in spleen and blood are representative of the mean value of young (Y) and old (O) groups (n=6-10). $CD11b^{neg}CD27^{neg}$ (double negative [DN]), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (double positive [DP]), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells.

5.2.5 Functional abilities and gene expression of NK cells were modulated by age along a maturation pathway defined by CD11b, CD27, LY49H and KLRG1

NK cell function relies on the expression and signalling balance of many activating and inhibitory receptors which are acquired at different phases during cell differentiation. As reported above, we observed a significant decline in the memory-associated activating receptor Ly49H and the inhibitory receptor KLRG1 on NK cells isolated from spleens of old animals. Thus, we focused our investigation on how the co-expression of Ly49H and KLRG1 affects NK cell functions and genetic regulation along the aging process. In order to accomplish this task, NK cells of different maturation stages and isolated from spleens of 6 young and 6 old mice were sorted based on the expression of CD11b, CD27, Ly49H and KLRG1 into six populations: $CD11b^{neg}$ $CD27^{pos}$ $Ly49H^{neg}$ $KLRG1^{neg}$, $CD11b^{pos}$ $CD27^{pos}$ $Ly49H^{pos}$ $KLRG1^{neg}$, $CD11b^{pos}$ $CD27^{pos}$ $Ly49H^{neg}$ $KLRG1^{neg}$, $CD11b^{pos}$ $CD27^{neg}$ $Ly49H^{pos}$ $KLRG1^{neg}$,

CD11b^{pos} CD27^{neg} Ly49H^{pos} KLRG1^{pos}, CD11b^{pos} CD27^{neg} Ly49H^{neg} KLRG1^{pos}. The gating strategy used is represented in Figure 5.10.

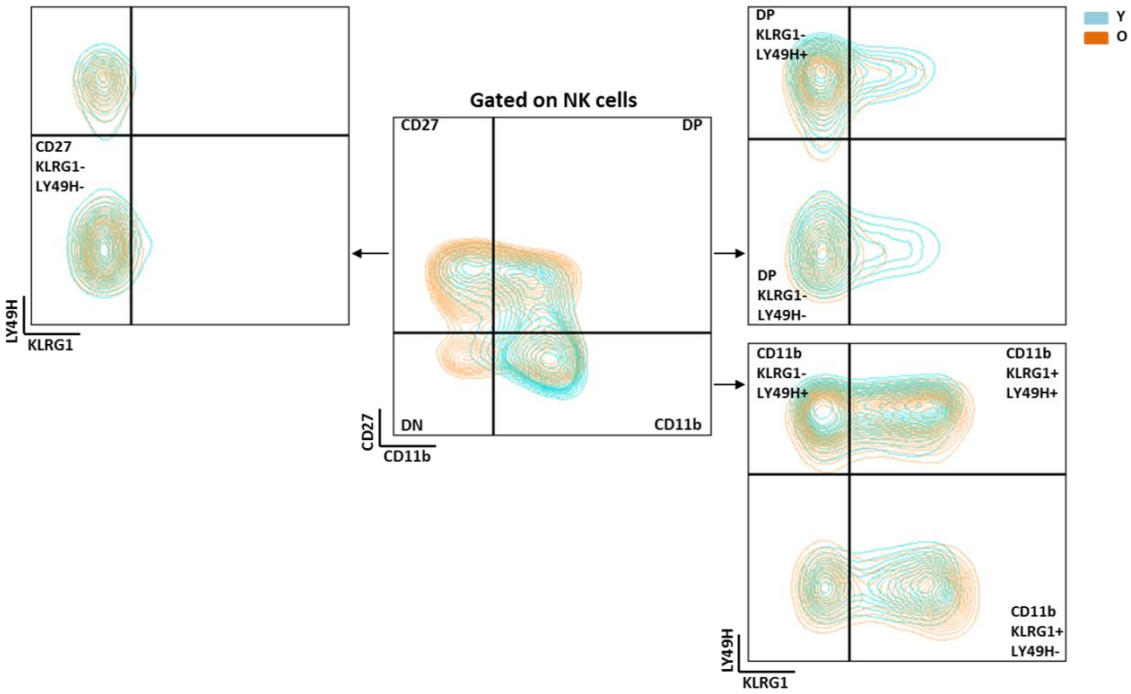


Figure 5.10 Sorting gating strategy to assess NK cell functionality and gene expression. NK cells isolated from spleens of 6 young and 6 old mice were sorted according to the expression of CD11b, CD27, LY49H and KLRG1 into six populations as indicated in this figure.

NK cells exert their effector function by producing various cytokines and chemokines as well as by lysing the target cell, depending on the nature of the stimulation. Supernatants of PMA/ionomycin-stimulated sorted NK cell subsets were tested through Luminex and ELISA to assess the release of the main cytokines known to be secreted by NK cells and the cytotoxic molecule GZB. In both young and old mice, the highest level of IFN-γ secretion was found in immature CD27 NK cells and progressively decreased during NK maturation (Figure 5.11A). However, while the decline was gradual in NK cells from young animals, there was a steep drop between DP and CD11b NK cells in the older ones. Additionally, the three CD11b subsets secreted more IFN-γ in young than in old mice. It is also worthy to highlight that IFN-γ production was modulated not only by expression of CD27 and CD11b as expected, but also by that of Ly49H and KLRG1. Ly49H^{pos} DP cells were indeed more able to secrete IFN-γ than Ly49H^{neg} DP cells in young mice; moreover, among young and old CD11b NK cells, those expressing Ly49H only showed to be more functional in this regard than cells expressing KLRG1 only (and than Ly49H^{pos}KLRG1^{pos} cells in young). Secretion of IL-2 was also characteristic of the least mature subpopulation independently of age with no relevant differences in the other maturation stages. As in the case of IFN-γ, all three subsets of late differentiated NK

cells partially lost their ability to secrete IL-2 with aging (Figure 5.11A). Immature CD27 NK cells and Ly49H^{neg}KLRG1^{neg} DP NK cells from old mice presented a higher release of the pro-inflammatory cytokines TNF-α, GM-CSF and IL-6 compared to the same subset from younger mice (Figure 5.11A). At both ages, there was a declining pattern in TNF-α and GM-CSF secretion; few significant differences in the other maturation stages were observed. Regarding IL-6, while we found a consistently low release across differentiation in young animals, the high value at the CD27 level dropped with maturation at older age. Similarly, the ability to secrete IL-10 declined as CD27 mature in old but not in young animals (Figure 5.11A). In addition, CD27 NK cells and Ly49H^{neg}KLRG1^{pos} CD11b NK cells from aged mice released more IL-10.

Sorted NK populations were also tested for the secretion of GZB, the most thoroughly characterized of the cytotoxic molecules used by NK cells (Figure 5.11B). It is striking from our observations that aging is accompanied by a significant loss of GZB release in all maturation stages. Going beyond the classic CD27/CD11b categorization, we deduced that, at young age, the expression of marker Ly49H is associated with a more elevated value among DP and CD11b NK cells. The trend appeared similar in older mice but significance could not be reached.

A series of transcription factors play a critical role in directing NK cell functional maturation. To determine whether the age-related defects we found in NK cell maturation and function were associated with a dysregulation in key transcription factors, their expression was evaluated by qPCR (Figure 5.12). *Tbx21* is a positive regulator of NK differentiation. As expected, in 4-month old animals, its expression increases with maturation with a relevant rise at the DP-CD11b passage. The situation differs at older age with an early decline and subsequent increment that failed to reach the same level observed in the young group. Also *Zeb-2* and *Prdm1* are known to drive maturation. As in the case of *Tbx21*, there was a steady increase as NK cells mature with no association with Ly49H and KLRG1 expression. However, this rise happened earlier, at the transition between immature CD27 cells and intermediate DP cells. During aging, expression of these regulators failed to upregulate as in the young counterparts leading to significant differences (in the three CD11b subsets for *Zeb-2*; in CD27 cells and Ly49H^{neg}KLRG1^{pos} CD11b for *Prdm1*). *Zbtb32* regulates the proliferative burst of Ly49H^{pos} NK cells in response to MCMV infection but it has been found to be is dispensable for NK maturation. In our results from young mice, *Zbtb32* expression remains relatively low in all subsets tested after an early decline. At older age, CD27, the two DP populations and Ly49H^{pos}KLRG1^{neg} CD11b showed a higher expression of *Zbtb32*. Thus, the declining pattern became clearer with aging. Altogether, the abnormalities observed in both functionality and gene expression were consistent with the phenotypic data reported above, confirming that aging profoundly affects the NK cell maturation process at different levels.

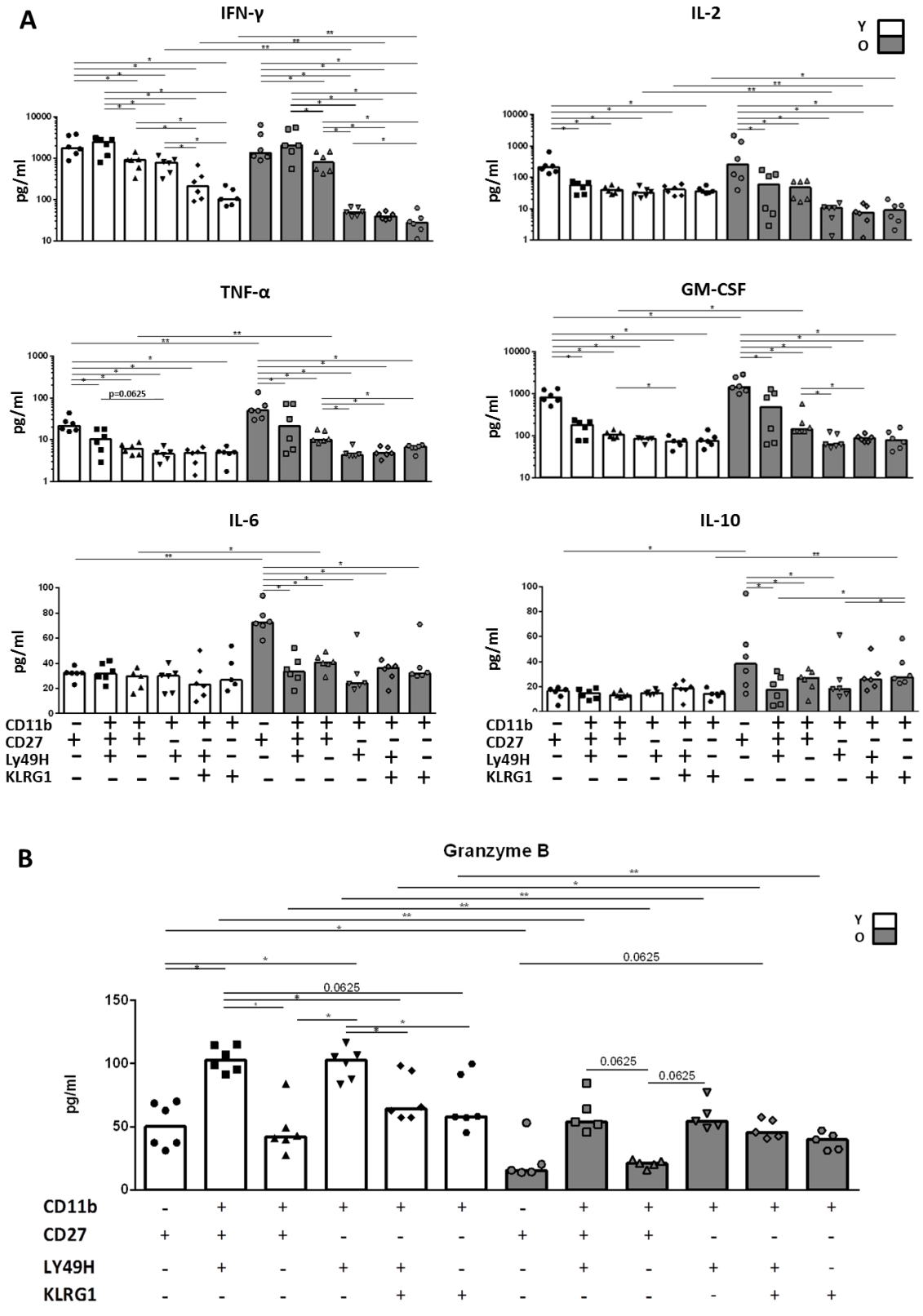


Figure 5.11 Cytokine secretion and cytotoxicity of NK cells are modulated by age along a maturation pathway defined by CD11b, CD27, Ly49H and KLRG1. (A) Supernatants of PMA/ionomycin-stimulated sorted NK cell subsets were tested through Luminex to assess the release of cytokines. (B) GZB production in supernatants of PMA/Ionomycin-stimulated sorted NK cell subsets by ELISA.

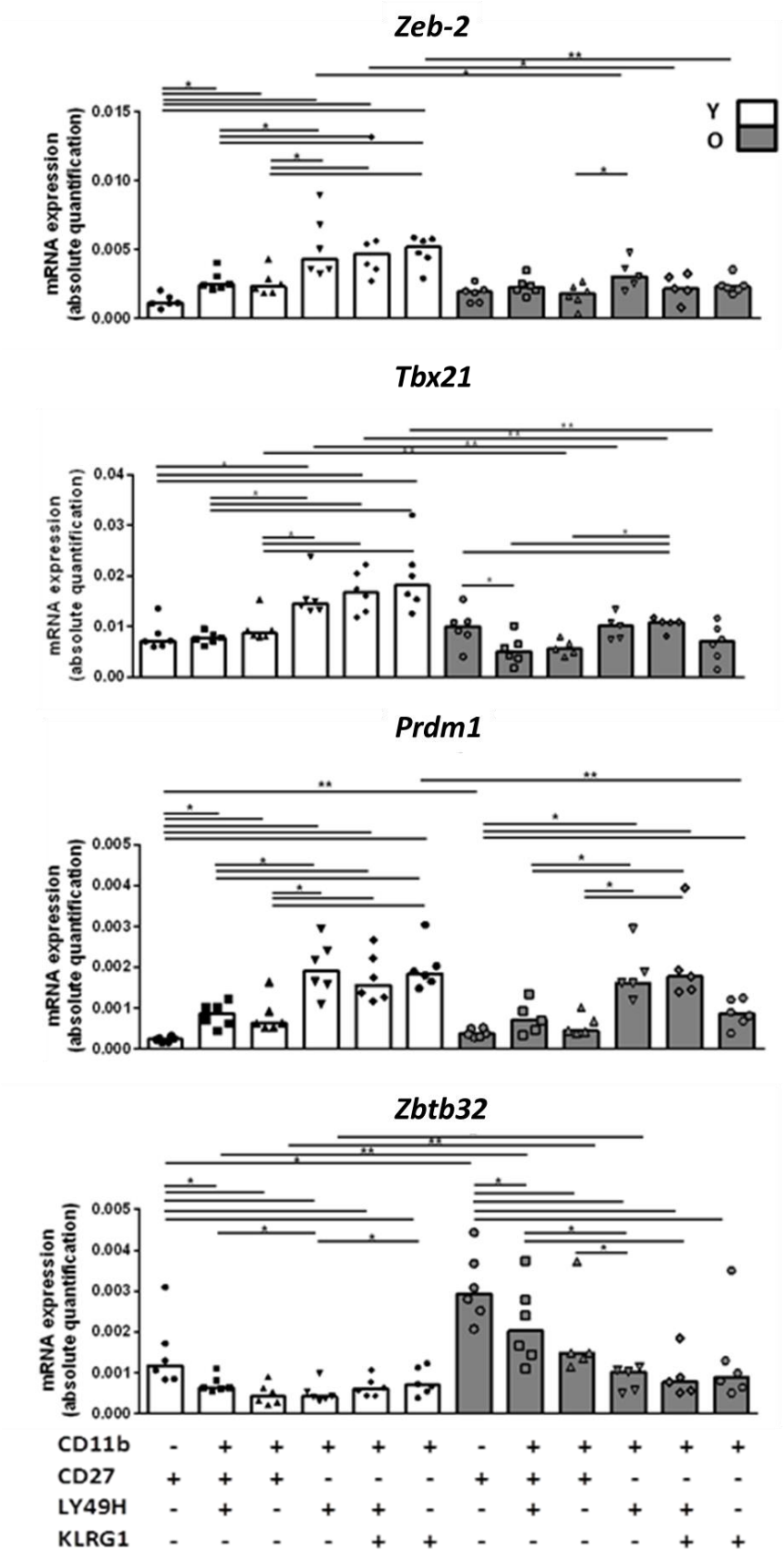

Zeb-2
Tbx21
Prdm1
Zbtb32
mRNA expression (absolute quantification)
Y
O
CD11b
CD27
LY49H
KLRG1

Figure 5.12 Gene expression of NK cells are modulated by age along a maturation pathway defined by CD11b, CD27, Ly49H and KLRG1. Expression of indicated transcriptional regulators of NK cell maturation was analyzed by RT-PCR and normalized according to the expression of housekeeping genes.

5.3 Discussion

As shown here and in previous literature (Almeida-Oliveira et al., 2011; Beli et al., 2014; Fang et al., 2010; Hazeldine and Lord, 2013; Nair et al., 2015; Shehata et al., 2015), aging significantly influences the maturation and the functional capacity of human and murine NK cells. Our study showed that NK cells from aged animals were characterised by a profound phenotypic immaturity that comprised activating and inhibitory receptors, adhesion molecules and other maturation markers. This was accompanied by a functional immaturity, including declining IFN-γ and GZB release. Secretion of IFN-γ and GZB was modulated by the aging process along a maturation pathway defined by CD11b, CD27, Ly49H and KLRG1. Age-related failure of maturation at the phenotypic and functional levels was associated to an abnormal genetic regulation.

First, we observed that old animals showed reduced percentage and numbers of NK cells both in spleens and blood. Reduction of NK cells in different tissues has been previously reported (Beli et al., 2014; Fang et al., 2010). However, others did not find such age-related differences (Nogusa et al., 2008; Plett and Murasko, 2000) and human studies published discordant data, with most of them reporting increased NK cells (Borrego et al., 1999; Gayoso et al., 2011; Hazeldine and Lord, 2013; Solana et al., 2012b). Thus, the influence of the aging process on NK cell distribution needs further consideration. Aging affected not only the overall quantity of NK cells but also their differentiation process. As documented here and elsewhere (Beli et al., 2014; Nair et al., 2015), the reduced proportion of NK cells in spleen and blood was due to decreasing mature CD11b NK cell subset. This reduction has been linked to loss of resistance to lethal mousepox (Fang et al., 2010). Additionally, we observed a significant increase of the least differentiated DN (in spleen) and CD27 NK cell populations (in spleen and blood) with aging. The situation in the human model appears to be completely different. It has been demonstrated that older adults have greater proportion of mature $CD57^{pos}$ NK cells in the blood (Hazeldine and Lord, 2013; Kared et al., 2016; Le Garff-Tavernier et al., 2010). With regards to NK cell subsets, the proportions and/or number of $CD56^{dim}$ NK cells increase with age while the elderly possess significantly fewer $CD56^{bright}$ NK cells (Almeida-Oliveira et al., 2011; Hazeldine and Lord, 2013). However, it is note-worthy that human studies are restricted to peripheral blood samples. Also, discrepancies between human and mice study outcomes may be explained by the fact that what is observed in the elderly people is not the result of aging alone. Genetic variability and environmental factors, such as history of

infections/vaccinations and diet, shape the human immune system. Conversely, mice are housed in specific pathogen-free conditions and fed a standard diet.

Elderly humans show higher levels of pro-inflammatory cytokines such as TNF, IL-1β and IL-6 in the serum and tissues (Franceschi and Campisi, 2014). This phenomenon, termed inflammaging, is a highly significant risk factor for both morbidity and mortality because almost all age-related diseases share an inflammatory pathogenesis. Murine data regarding this persistent low-grade inflammation are still inadequate. The aforementioned redistribution of NK cells across the maturation pathway correlated significantly with serum levels of some pro-inflammatory cytokines. Aged animals were characterized by more elevated concentration of TNF-α, IL-1β and MIP-3α in serum compared to younger mice. However, the source of these cytokines remains to be identified. Further investigation is necessary to understand whether inflammaging is (at least partially) responsible for the alterations seen in NK cell maturation during aging or the accumulation of cytokine-producing immature NK cells is involved in this systemic rise in pro-inflammatory molecules.

To enrich our study on NK cell phenotypic immaturity in aging, we sought to analyse the expression of surface markers (beyond the conventional CD27/CD11b classification) that have been linked to a normal NK cell differentiation program. Expression of activating and inhibitory receptors was also altered with aging. The prominent and most used hallmark of mature NK cells is the inhibitory receptor KLRG1. The ligands for this receptor are classical cadherins, (E-, N- and R-cadherins). These cadherins are expressed in healthy, solid tissues and therefore may have a role in the prevention of lysis of healthy cells (Huntington et al., 2007a). The expected acquisition of KLRG1 with maturation failed in total splenic NK cells and all maturation stages except for the last one, confirming results of previous studies (Beli et al., 2014; Nair et al., 2015). Chiu *et al.* found that this impairment in the upregulation of KLRG1 along maturation had severe effect on functionality of NK cells. Indeed, NK cells in aged mice failed to expand and acquire KLRG1 in response to pathogen-derived products (Chiu et al., 2013). In the spleen and blood, total and mature NK cells of aged mice had decreased expression of Ly49H, a MHC-I-binding activating receptor that associate with murine MCMV-encoded m157 protein. It is interesting that lack of Ly49H in C57BL/6 mice resulted in high viral replication in the spleen and dramatically enhanced pro-inflammatory cytokine secretion in the serum and spleen, suggesting that Ly49H^{pos} NK cells work to limit MCMV-induced pathology (Fodil-Cornu et al., 2008). More recently, a surprising new role emerged for NK cells bearing Ly49H. These cells recognize MCMV-infected cells expressing m157 in an antigen-specific manner, then undergo a clonal-like expansion and mediate a protective response (Sun et al., 2011). This discovery paved the way to the investigation of the so-called innate memory. MCMV-specific memory NK cells have

been described as mature CD11b NK cells that are positive for Ly49H and Ly6C (Sun et al., 2009). Ly6C shows distinct expression patterns in different developmental stages of NK cells but also other lymphocytes, monocytes/macrophages, granulocytes and endothelial cells (Omi et al., 2014). Our results evidenced that, while there was no age-related difference in total NK cells, aged mice had altered expression of Ly6C in some maturation stages, with an increase in CD11b NK cells. Thus, further study is compelling to describe how the drop in Ly49H and the abnormality in Ly6C observed here with aging could impact the innate immune response of old mice against CMV and other infections.

As for Ly6C, more mature CD11b NK cells expressed another maturation marker, TIM-3. On NK cells, TIM-3 can act as an activating co-receptor, since exposure to its ligand Gal-9 enhances IFN-γ production by TIM-3^{pos} NK cells (Gleason et al., 2012) but it can also deliver inhibitory signals, given that the ability of NK cells to kill target cells is decreased upon TIM-3 cross-linking (Ndhlovu et al., 2012). Results are contradictory and little is still known about TIM-3 regulation on NK cells and it would be intriguing to investigate the implications of the alteration seen here on NK functionality during aging.

Upon infection or sterile inflammation, NK cells redistribute from the bone marrow to peripheral tissues in order to exert their effector functions locally at the pathological sites. Several NK cell–expressed adhesion molecules, such as including selectins, chemokine receptors and integrins, are responsible for guiding NK cell homing. The selectin CD62L (L-selectin) and the collagen-binding integrin α2 (CD49b, recognized by mAb DX5) are responsible for the recruitment and localization of NK cells into draining lymph nodes. In our study, splenic total NK cells and all maturation phases showed a relevant loss of both CD62L and CD49b (DX5), suggesting a decreased ability of NK cells from aged animals to migrate to lymph nodes. Indeed, NK cells in old mice fail to move to the lymph nodes following ectromelia virus infection (Fang et al., 2010). Additionally, Coombes et al. observed that CD49b regulates the localization of NK cells in lymph nodes during Toxoplasma gondii infection (Coombes et al., 2012).

A fine balance of several activating and inhibitory receptors acquired during differentiation is crucial for the normal functionality of NK cells. As mentioned above, we reported a significant decline in the memory-associated activating receptor Ly49H and the inhibitory receptor KLRG1 on NK cells isolated from spleens of old animals. Thus, we sought to analyse how this age-associated abnormal expression of Ly49H and KLRG1 relates to NK cell functions and genetic regulation. An essential function of NK cells is to produce cytokines, such as IFN-γ and TNF-α, that recruit inflammatory cells and regulate dendritic cells, T cells and B cells. Thus, cytokine secretion by NK cells influences both innate and adaptive branches of the immune system. The decline in IFN-γ production during

maturation was gradual in young animals whilst there was a steep drop at CD11b stage in the older mice. This led to a lower IFN-γ secretion by the mature subsets with aging. Therefore, aging is characterised by less mature NK cells that secrete less IFN-γ. Also, IFN-γ production was shaped not only by expression of CD27 and CD11b, but also by that of Ly49H and KLRG1. No data exist so far regarding secretion of cytokines by Ly49H^{pos} NK cells in absence of infections. Importantly, blockade of KLRG1 signalling was reported to significantly recover the impaired IFN-γ production by NK cells from HCV-infected subjects (Wang et al., 2013). We also observed that immature NK cells from old mice released more pro-inflammatory cytokines TNF-α, GM-CSF and IL-6 compared to younger mice, possibly participating to systemic inflammaging. Indeed, aged animals possess more immature NK cells secreting more pro-inflammatory molecules.

Along with cytokine production, NK cells can directly kill abnormal cells via secretion of cytotoxic molecules, namely perforin, granzymes and granulysin. Here, we show that all differentiation stages experienced a significant loss in their ability to release GZB during aging. This outcome is in line with past literature that reported a relevant decline of cytotoxicity by NK cells from aged mice (Fang et al., 2010; Shehata et al., 2015) and consistent with the phenotypic immaturity observed. Similarly to IFN-γ production, the highest amount of GZB was secreted by intermediate and mature NK cell bearing the activating receptor LY49H and lacking the inhibitory molecule KLRG1. Thus, Ly49H expression appeared to be an important marker of functional NK cells in the steady state.

Finally, modulation of some transcriptional factors during maturation and in relation to Ly49H and KLRG1 expression was investigated. In accordance with phenotypic and functional immaturity, positive regulators of NK cell maturation including *Tbx21*, *Zeb-2* and *Prdm1* failed to upregulate in aged mice as in the younger counterparts. Recently, Shehata et al. documented by flow cytometry the reduction in the expression of *Eomes* and *Tbx21* but not *Gata-3* or *Prdm1* in aged NK cells and their subsets isolated from bone marrow. Interestingly, *Tbx21* and *Eomes* downregulation in aged mice was associated with the loss of cytotoxicity and was induced by the aged environment, pointing to a cell extrinsic induction of a senescent phenotype (Shehata et al., 2015). *Zbtb32* is a member of the Broad complex, Tramtrack, Bric á brac and Zinc Finger (BTB-ZF) proteins that have an essential and non-redundant role in the regulation of lineage commitment, development and effector function in lymphocytes (Beaulieu and Sant'Angelo, 2011). On this basis, Beaulieu et al. sorted Ly49H^{pos} NK cells from MCMV-infected and uninfected animals and identified *Zbtb32* as a crucial regulator of the proliferative burst of MCMV-specific memory NK cells responding to viral infection *in vivo* (Beaulieu et al., 2014). Here we report, for the first time at the best of our knowledge, the analysis of the modulation of *Zbtb32* expression during NK cell differentiation, highlighting a declining pattern in young and old mice. Most of the maturation phases presented

higher expression in old animals, especially the immature subpopulation. Since so far no data are available regarding the role of Zbtb32 in Ly49H^{neg} NK cells, there is the need to investigate how this transcriptional regulator impacts NK cell functional at more immature stages.

In conclusion, the reduced presence of NK cells in circulation and in the spleen and their altered maturation status can have severe implications on the immune responses of aged mice to viral infections and cancers. The declined expression of genetic drivers of NK maturation and of Ly49H (that are associated with IFN-γ and GZB release) may be, at least partially, responsible for the loss of functionality seen with aging.

5.4 Experimental procedures

5.4.1 Mice and sample preparation

C57BL/6 mice were used at two ages: 4 months (young) and 21 months (old). We chose to use 21 month old mice as old group members as most previous literature about the aging process in mice did. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and local Animal Welfare and Ethics Review Board (AWERB) in the UK and Singapore. Blood and spleen tissue processing is detailed in Chapter 3.

5.4.2 Phenotyping

Cell phenotyping was performed by flow cytometry on 6 fresh spleen and blood samples from young mice and 6 fresh spleen and blood samples from old mice. For each staining, 2×10^6 splenocytes and 150 uL of whole blood were used. Lymphocytes were gated based on FSC/SSC profile and doublets/dead cell exclusion. T cells were excluded by CD3 expression, followed by NK cell identification on CD3^{neg} lymphocytes using NK1.1 expression. The antibodies are listed in Table 5.1. Flow cytometry was performed on an LSR Fortessa Cell Analyzer (BD Biosciences) and automatic compensation was applied.

5.4.3 Sorting

NK cells isolated from spleens of 6 young and 6 old mice were sorted according to the expression of CD11b, CD27, LY49H and KLRG1 into the following six populations:

CD11b^{neg} CD27^{pos} Ly49H^{neg} KLRG1^{neg},

CD11b^{pos} CD27^{pos} Ly49H^{pos} KLRG1^{neg},

CD11b^{pos} CD27^{pos} Ly49H^{neg} KLRG1^{neg},

$CD11b^{pos}\,CD27^{neg}\,Ly49H^{pos}\,KLRG1^{neg}$,

$CD11b^{pos}\,CD27^{neg}\,Ly49H^{pos}\,KLRG1^{pos}$,

$CD11b^{pos}\,CD27^{neg}\,Ly49H^{neg}\,KLRG1^{pos}$.

The antibodies used are listed in Table 5.1. Cell sorting was performed on a BD Influx 3 lasers (BD Biosciences). Compensation was performed using single colour controls prepared from BD Comp Beads (BD Biosciences).

Table 5.1 List of antibodies used for sorting and phenotyping

Antigen	Fluorochrome	Clone	Company
CD3	AF-700	17A2	BioLegend
NK1.1	BV-421	PK136	BioLegend
CD27	PEC-y7	LG7F9	eBioscience
CD11b	APC-Cy7	M1/70	BioLegend
KLRG1	PE/Dazzle-594	2F1/KLRG1	BioLegend
Ly6C	BV-605	HK1.4	BioLegend
CD62L	BV-786	MEL-14	BD Biosciences
TIM-3	PE	RMT3-23	eBioscience
Ly49H	AF-647	3D10	BioLegend
CD49b (DX5)	FITC	DX5	BioLegend

5.4.4 NK cell stimulation

Sorted NK subsets were stimulated with a cocktail of phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (250 ng/ml) at 0.5 million cells/ml in RPMI + 10% FBS, for 18 hours at 37 °C. Supernatants were collected after incubation, cell debris were removed by centrifugation and stored for future use at -80°C.

5.4.5 Granzyme B ELISA

Supernatants from sorted NK cell subsets and plasma samples were tested with a Mouse Granzyme B ELISA Ready-Set-Go (eBioscience, Cat# 88-8022-22) according to the manufacturer's instructions. Refer to Chapter 3 for details on this assay.

5.4.6 Multiplex analyte screening

Supernatants from sorted NK cell subsets and serum samples were tested with a Milliplex MAP Mouse Th17 kit (Millipore, Cat# MTH17MAG-47K) according to manufacturer's instructions. Refer to Chapter 3 for details on this assay.

5.4.7 Quantitative real-time PCR

Sorted NK cells were lysed with RLT buffer with 1% of β-mercaptoethanol after 18 hours of PMA/ionomycin stimulation. Total RNA extraction was performed using an RNeasy Plus Micro kit (Qiagen) and reverse transcribed into cDNA using the SuperScript First Strand kit (Invitrogen). cDNA was analyzed by real-time PCR with the KAPA SYBR qPCR Master Mix kit (KAPA Biosystems). Primers are listed in Table 5.2.

Table 5.2 List of primers used for qPCR

Gene	Company	Forward sequence	Reverse sequence
Actb	Qiagen QuantiTect Primer Assay	Proprietary sequence	Proprietary sequence
Zeb2	Qiagen QuantiTect Primer Assay	Proprietary sequence	Proprietary sequence
Tbx21 (Tbet)	Qiagen QuantiTect Primer Assay	Proprietary sequence	Proprietary sequence
Zbtb32	Custom	5'-TCCAGATACGGTGCTCCCTTCT-3'	5'-CCAGAGAGCTTTGGAGTGGTTC-3'
Prdm1 (Blimp1)	Custom	5'-ACAGAGGCCGAGTTTGAAGAGA-3'	5'-AAGGATGCCTCGGCTTGAA-3'

5.4.8 Data analysis

Flow cytometry data were analyzed using FlowJo (Treestar) and FACSDiva (BD Biosciences). Samples were compared using GraphPad Prism software (v.6.0c). Groups of young and old mice were compared by Mann-Whitney U test. The Wilcoxon matched-pairs signed rank test was used for paired testing of median values of different subsets from the same mouse. Comparisons with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant.

Chapter 6: TIMP-3 knock-out mice show deficits in NK and T cell maturation and function during aging

6.1 Introduction

The extracellular matrix (ECM) is a highly dynamic and complex structure that is involved in embryonic development, morphogenesis, tissue remodelling and repair. The major enzymes involved in the proteolytic catabolism of the ECM are the matrix metalloproteinases (MMPs). Degrading the ECM, MMPs can regulate matrix structure, cell-cell and cell-matrix interactions, growth factors availability and function of cell surface signalling systems (Murphy and Nagase, 2008). In addition, the A disintegrin and metalloproteases (ADAM) enzymes, also named "sheddases", influence cell behavior by proteolytically releasing the ectodomain of cell surface molecules such as growth factors, cytokines and cell adhesion molecules (Edwards et al., 2008).

The uncontrolled turnover of ECM components is avoided thanks to several mechanisms that keep the ECM anabolism and catabolism in equilibrium. Disruption of this balance is related to arthritis (Birkedal-Hansen et al., 1993), cardiovascular diseases (Gunja-Smith et al., 1996), cancer (Kessenbrock et al., 2010), pulmonary diseases (Dancer et al., 2011), nephritis (Tveita et al., 2008) and gut diseases (Pender and MacDonald, 2004). The proteolytic activity of matrix proteases is controlled at the expression level, the zymogen activation level and the protein inhibition level. Four endogenous tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been discovered so far.

All TIMPs are secreted, but TIMP-3 is the only family member tightly associated with the ECM. The main function of TIMP-3 is the suppression of MMPs activity. Indeed, TIMP-3 can inhibit all 23 MMPs found in humans (Brew and Nagase, 2010). The *Timp-3* KO mouse was generated in 2001 and shows spontaneous air space enlargement in the lungs (Leco et al., 2001), dilated cardiomyopathy (Fedak et al., 2004), interstitial nephritis and fibrosis (Kassiri et al., 2009), spontaneous osteoarthritis, collagen degradation and abnormal bone growth (Sahebjam et al., 2007), impaired cognitive function (Baba et al., 2009), retinal abnormalities (Janssen et al., 2008) and enhanced metabolism (Hanaoka et al., 2014).

TIMP-3 is a pleiotropic protein that shows MMPs-independent functions as well. It has been demonstrated that TIMP-3 inhibits several ADAMs including ADAM10 (Amour et al., 2000), ADAM12 (Loechel et al., 2000), ADAM28 (Mochizuki et al., 2004), ADAM33 (Zou et al., 2004). Other TIMPs can block the same enzymes but with a lower efficiency. However, an exclusive ability of

TIMP-3 is to inhibit the shedding activity of ADAM17 (TNF-α converting enzyme, TACE) (Amour et al., 1998). The interrelation between inflammation, the immune system and immune cells is mediated by cytokines, growth factors, chemokines, integrins and other molecules. Proteolysis plays an important role in regulating these molecules. TACE sheds the ectodomain of membrane anchored TNF-α, raising the levels of the soluble form of TNF-α (Black et al., 1997). Mohammed *et al*. (Mohammed et al., 2004) showed that *Timp-3* KO mice develop chronic inflamed livers due to an increase in TNF-α activity. Smookler and colleagues (Smookler et al., 2006) observed that *Timp-3* KO mice are more susceptible to LPS-induced mortality than wild-type mice, suggesting that the loss of TIMP-3 and the consequent raise in TNF-α levels lead to pathological inflammation due to an unregulated innate immune response. These papers demonstrated that TIMP-3 is a physiological negative regulator of inflammation *in vivo*. In addition, it has been reported that TIMP3 can block TACE-mediated shedding of syndecan-1 and syndecan-4 (Fitzgerald et al., 2000), L-selectin (Borland et al., 1999) and IL-6R (Hargreaves et al., 1998). Moreover, TACE is able to catalyse the cleavage of other molecules involved in inflammation such as IL-1RII (Reddy et al., 2000), IL-15Ra (Budagian et al., 2004), TNF-alphaR (Wang et al., 2003), V-CAM (Garton et al., 2003), I-CAM (Tsakadze et al., 2006) and JAM-1 (Koenen et al., 2009b).

Two papers suggested a relationship between TIMP-3 and aging. Kamei and Hollyfield (Kamei and Hollyfield, 1999) showed an increase in TIMP-3 protein levels in Bruch's membrane in patients with age-related macular degeneration and during normal aging. Macgregor and collegues (Macgregor et al., 2009) characterized the distribution of TIMP-3 in human lungs, kidneys, retinas and vascular tissues. They found that TIMP-3 protein increases with age in lung, kidney and retina and that TIMP-3 protein accumulation is an age-dependent phenomenon. The staining appears in all three tissues in early adulthood (the staining was absent in all tissues for all subjects <20 years of age) and becomes stronger among the elderly.

Aging is accompanied by a persistent, low-grade inflammation, elevated levels of circulating TNF-α (Bruunsgaard et al., 2003) and age-related diseases with an inflammatory basis. The lack or deficiency of TIMP-3 removes the inhibition towards TACE, increasing TNF-α levels and it has been related to inflammatory diseases such as vascular inflammation and diabetes (Federici et al., 2005), inflammatory bowel diseases (Monteleone et al., 2012), hepatic steatosis and adipose tissue inflammation (Menghini et al., 2009) and endotoxic shock (Smookler et al., 2006).

Given the significant contribution of TNF-α to inflammation and aging processes and the role of TIMP-3 as TACE inhibitor, *Timp-3* KO mouse might be useful as an animal model not only for inflammatory diseases but also aging, age-related pathologies and especially the relationship between aging and inflammation.

Here we demonstrated that, as they age, *Timp-3* KO mice are affected by a series of alterations in the maturation process and functionality of both NK and T lymphocytes. Our results offered new insights into TIMP-3 biological role in adaptive and innate immunity, especially its importance during the aging process.

6.2 Results

6.2.1 Proportions of total NK and mature NK cells were lower in *Timp-3* KO mice than WT mice as they age

Our investigation of how NK cell immune response varies during aging between *Timp-3* KO and WT animals started with the phenotypic analysis of splenocytes. After excluding doublets and dead lymphocytes, we identified NK cells as $CD3^{neg}NK1.1^{pos}$ cells. While no difference was found in total $CD3^{neg}$ cells (Figure 6.1A), we observed that NK cell percentage did not significantly differ at 24 weeks but decreased with age in both genotypes. However, this decline was more accentuated in the KOs leading to a significant difference compared to the WTs at older age (Figure 6.1B).

When measuring the expression of the two most used NK differentiation surface markers, we found that proportion of mature $CD11b^{pos}$ cells and CD11b MFI declined with age and resulted higher in aged WTs (Figure 6.1C). Conversely, proportion of immature $CD27^{pos}$ cells and CD27 MFI increased during aging showing a greater level in old KOs (Figure 6.1D). Expression of these molecules did not differ at young age (Figure 6.1C and Figure 6.1D).

The data above confirmed our previous results (reported in Chapter 5 of this thesis), suggesting that there is a defect in NK maturation during the aging process. This work added that the abnormality seems to be more accentuated in old *Timp-3* KOs and sets in at a time point later than 24 weeks and earlier than 50 week of age.

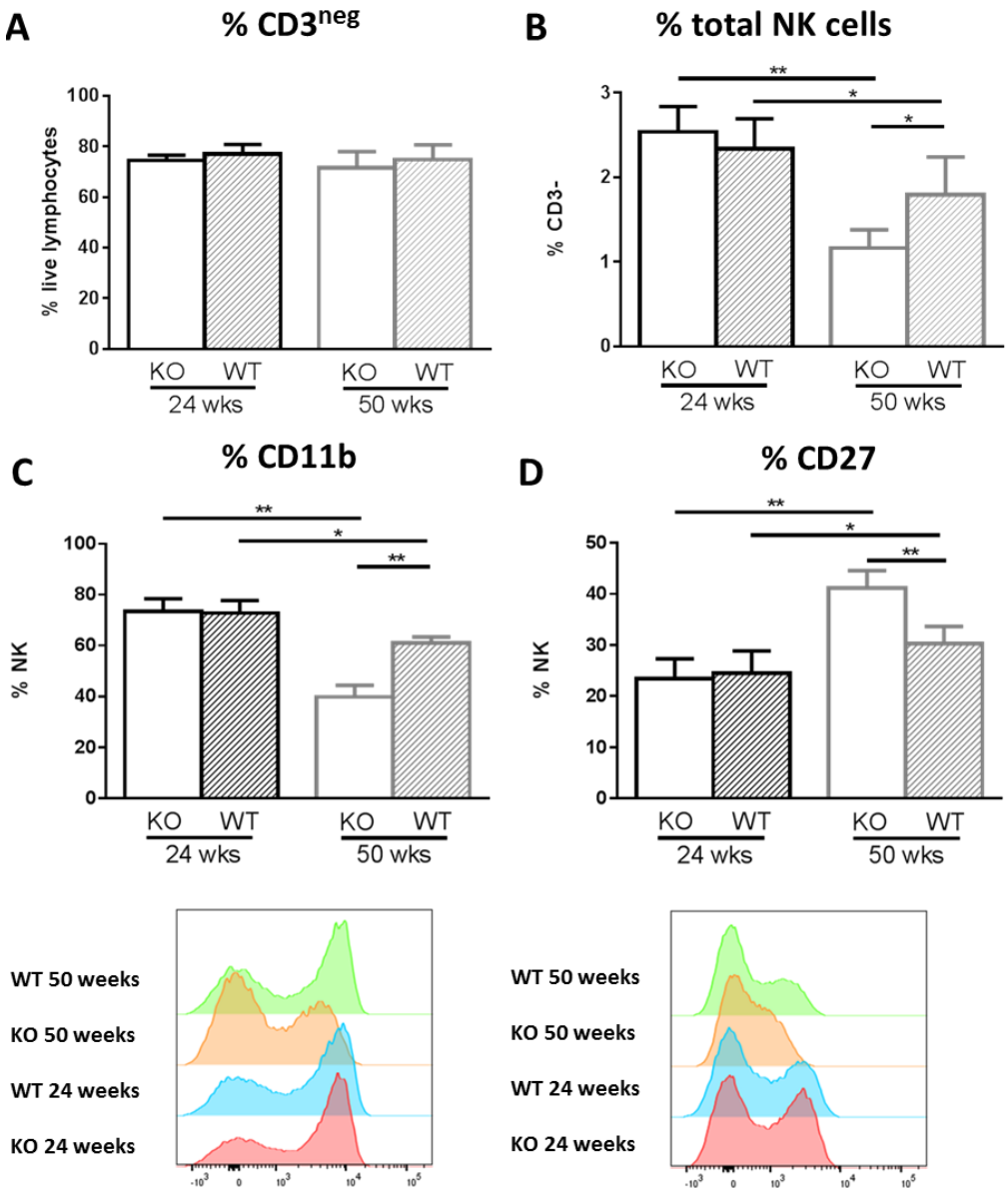


Figure 6.1 Old *Timp-3* KO mice had less total and mature spleen NK cells than WT mice at old age. (A) Percentage of $CD3^{neg}$ cells in *Timp-3* KO mice (KO) and wild type mice (WT) with aging tested by flow cytometry. (B) Percentage of total NK cells in KOs and WTs with aging tested by flow cytometry. (C) Percentage of $CD11b^{pos}$ NK cells (upper panel) and CD11b MFI (lower panel) in KOs and WTs with aging tested by flow cytometry. (D) Percentage of $CD27^{pos}$ NK cells (upper panel) and CD27 MFI (lower panel) in KOs and WTs with aging tested by flow cytometry.

6.2.2 Age-related NK immaturity was more profound in TIMP-3 KO mice

Phenotypic immaturity was further analysed investigating the expression of other surface molecules that are associated to specific stages of NK cell differentiation. The MFI and the proportion of NK cells positive for some markers of maturity, such as CD62L (Figure 6.2A), KLRG1

(Figure 6.2B) and Ly49H (Figure 6.2C), were found to be similar between genotypes at young age, decreasing with aging and significantly lower in old KOs. On the other hand, the trend was opposite for the immature status marker CD127 (Figure 6.2D), corroborating the outcome reported in section 6.2.1.

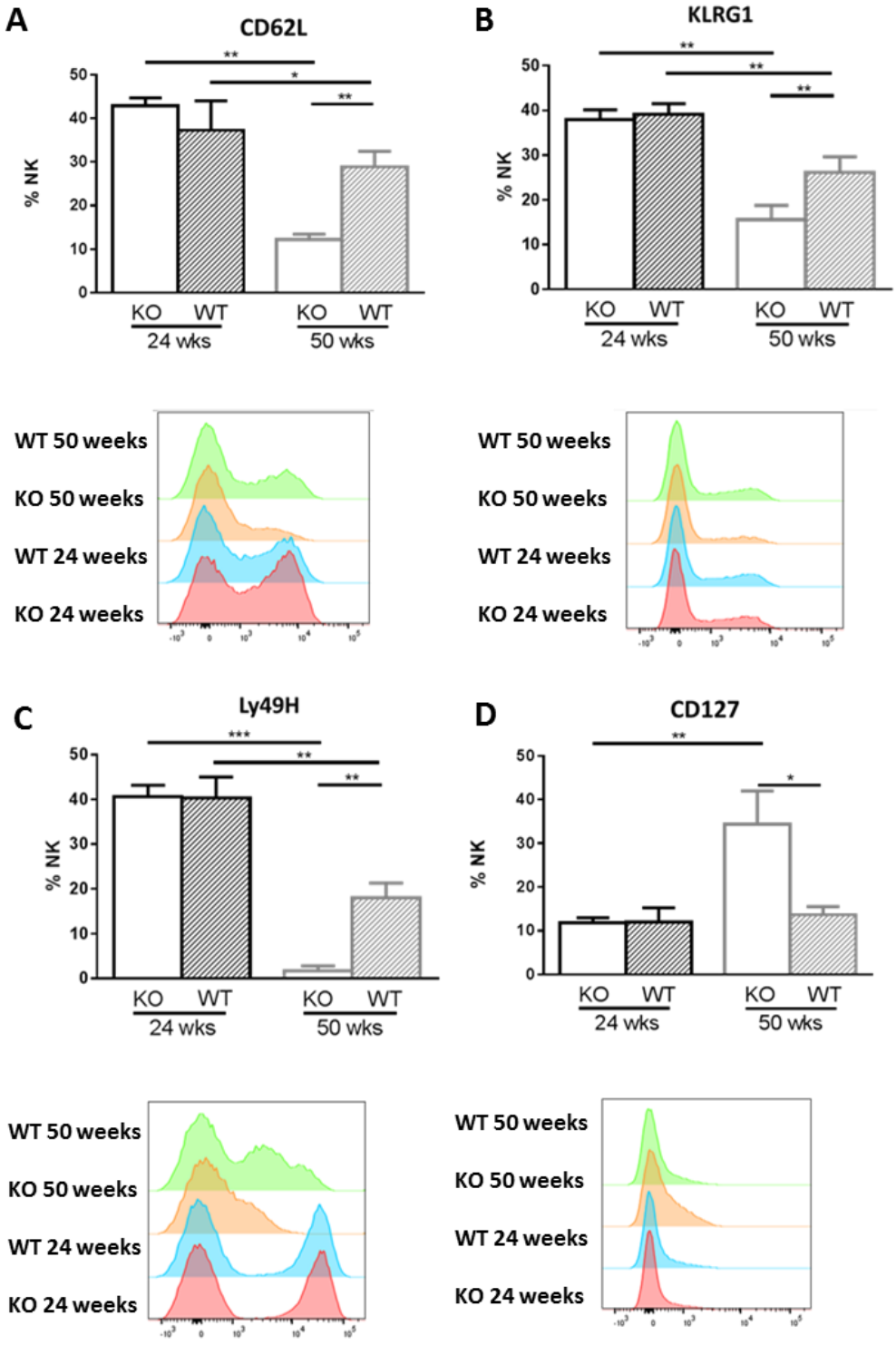


Figure 6.2 NK cells from the spleen of aged *Timp-3* KO mice showed furher signs of phentypic immaturity. Percentage of positive NK cells (upper panel) and MFI (lower panel) for (A) CD62L, (B) KLRG1, (C) Ly49H and CD127(D), tested by flow cytometry in *Timp-3* KO mice (KO) and wild type mice (WT) with aging.

6.2.3 Differentiation abnormality severely affects NK phenotype at late stages

In order to understand at what phase(s) of the differentiation process *Timp-3* KO NK cells diverge from the WT normal status, we pushed the analysis deeper dividing the total NK cell population in four stages: $CD11b^{neg}CD27^{neg}$ (double negative [DN]), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (double positive [DP]), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells, listed from the least mature to the most mature. In line with previous results, there was no difference at 24 weeks whereas, at older age, it became clear that KO mice accumulated immature (DN and CD27) NK cells (Figure 6.3A and Figure 6.3B) and lost late differentiated (CD11b) NK cells (Figure 6.3D) in their spleen at a higher percentage than the WT counterparts.

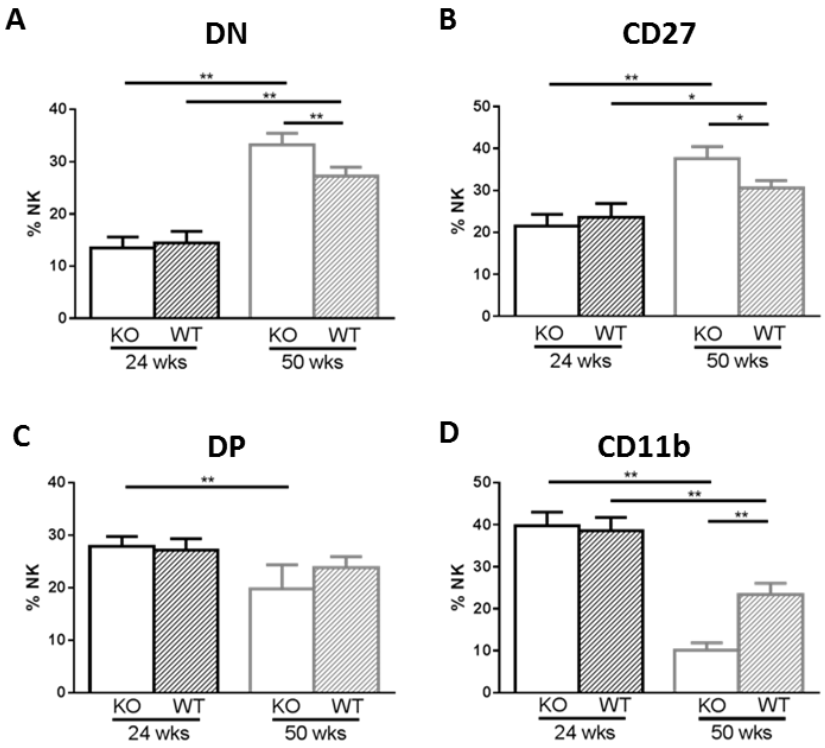


Figure 6.3 Old *Timp-3* KO mice accumulated more immature NK cells and lost more differentiated NK cells with aging than WT mice in the spleen. Proportion of (A) $CD11b^{neg}CD27^{neg}$ (DN), (B) $CD11b^{neg}CD27^{pos}$ (CD27), (C) $CD11b^{pos}CD27^{pos}$ (DP), (D) $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells in *Timp-3* KO animals (KO) and wild type animals (WT) with aging measured by flow cytometry.

Then we sought to measure the expression of maturation markers on the different subpopulations. Regarding CD62L (Figure 6.4A), CD127 (Figure 6.4B) and KLRG1 (Figure 6.4C), differences at 50 weeks of age concentrated in the intermediate DP and late CD11b phases of differentiation. Expression of Ly49H resulted dysregulated in old KOs in all maturation stages, suggesting that a

more profound abnormality affects this molecule than other markers investigated here (Figure 6.4D).

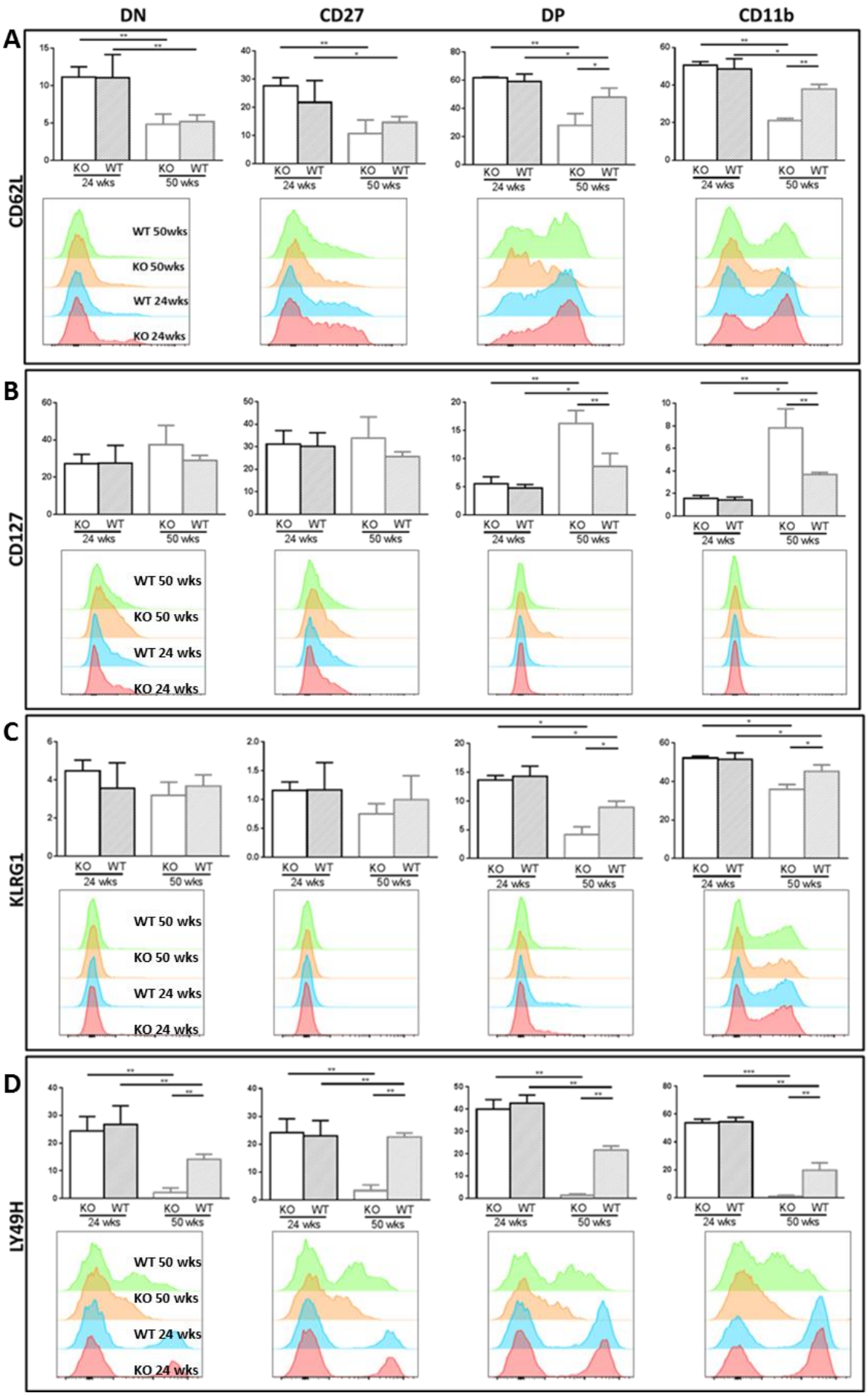

DN
CD27
DP
CD11b
A
CD62L
KO WT
24 wks
50 wks
WT 50wks
KO 50wks
WT 24wks
KO 24wks
B
CD127
WT 50 wks
KO 50 wks
WT 24 wks
KO 24 wks
C
KLRG1
D
LY49H

Figure 6.4 Defect in maturation markers were more severe in aged *Timp-3* KO spleen NK cells from late stages of maturation. Percentage of NK cells positive for (A) CD62L, (B) CD127, (C) KLRG1, (D) Ly49H and related MFI in the different maturation subsets of *Timp-3* KO mice (KO) and wild type mice (WT) during aging were measured by flow cytometry. $CD11b^{neg}CD27^{neg}$ (DN), $CD11b^{neg}CD27^{pos}$ (CD27), $CD11b^{pos}CD27^{pos}$ (DP), $CD11b^{pos}CD27^{neg}$ (CD11b) NK cells.

6.2.4 Phenotypic immaturity reflects an alteration in the functionality of NK cells from aged *Timp-3* KO mice

Having assessed that age-related altered phenotype is more prominent in *Timp-3* KO animals than WT counterparts, we investigated whether this abnormality could be accompanied by functional defects. Supernatants of PMA/ionomycin-stimulated sorted total NK cells were tested through Luminex and ELISA to measure the secretion of the cytokines IFN-γ, TNF-α, IL-6 and the cytotoxic molecule GZB. As described in Chapter 5, aging in WTs was characterized by a relevant loss in IFN-γ (Figure 6.5A) and GZB (Figure 6.5D) release while there was an upregulation of TNF-α (Figure 6.5B) and IL-6 secretion (Figure 6.5C). Additionally, we observed that functional capacity is equal in the two different genotypes at 24 weeks but age-associated alterations are more pronounced in *Timp-3* KO mice (Figure 6.5).

These data confirmed that NK cells from *Timp-3* KO mice showed more evident NK maturation defects associated to aging at both phenotypic and functional levels.

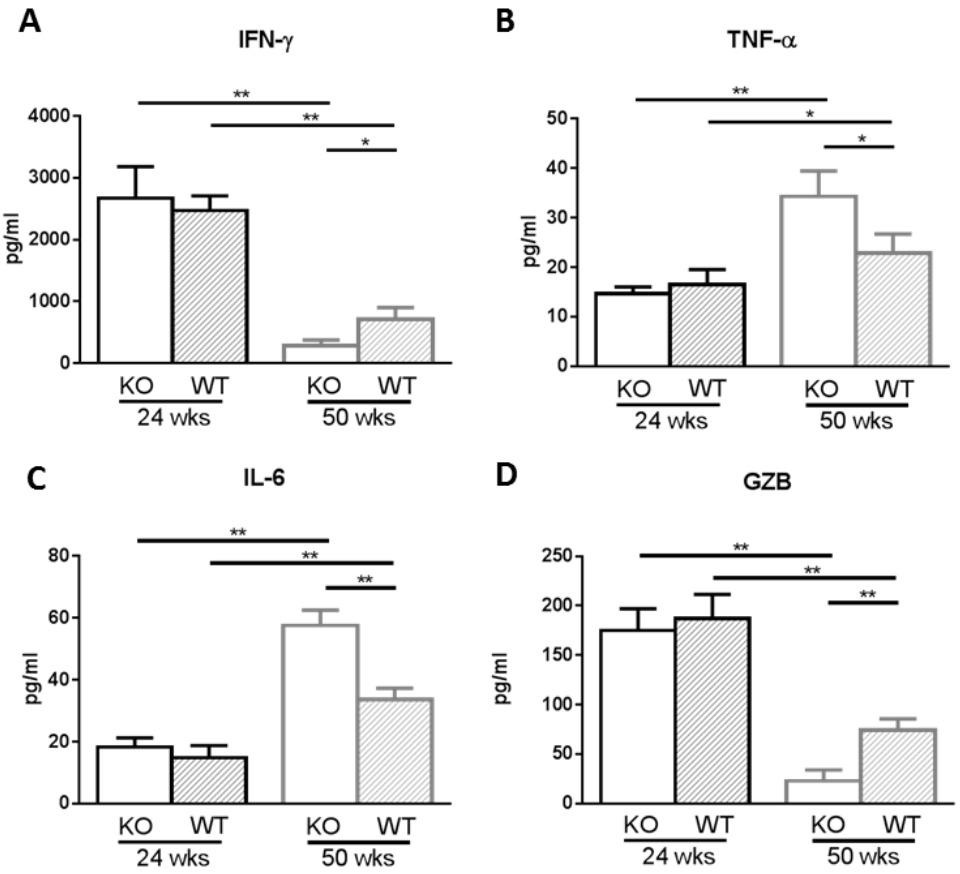


Figure 6.5 NK cells from spleen of TIMP-3 KO mice showed more evident age-related functional defects. Supernatants of PMA/ionomycin-stimulated sorted total NK cells were tested by Luminex to assess the release of cytokines IFN-γ (A), TNF-α (B), IL-6 (C) and by ELISA to measure the cytotoxic molecule GZB (D).

6.2.5 Age-associated phenotypic and functional immaturity impacts T cells more severely in *Timp-3* KO animals

In order to widen our investigation on the role on TIMP-3 in immunity, we investigated whether the adaptive branch of *Timp-3* KO mice was also affected. While no alteration resulted in the proportion of total $CD3^{pos}$ cells (Figure 6.6A) and of CD4 (Figure 6.6B) and CD8 (Figure 6.6C) T cells independently of age and genotype, both T cell subsets showed a relevant maturation deficit in *Timp-3* KO animals, as seen above with regards to the NK cell population. In CD4 (Figure 6.7A) and CD8 (Figure 6.7B) cells from WT mice, there was an increase in the percentage of cells positive for CD44 and KLRG1 with aging. Conversely, KOs did not experience any up-regulation of these molecules, leading to a significant difference between the genotypes at 50 weeks old. $CD62L^{pos}$ CD4 (Figure 6.7A) and CD8 (Figure 6.7B) T cells were present at a higher proportion in KO mice at young

age and dropped during aging in both WTs and KOs but to a lesser extent in KOs. Expression of CD127 on both T cell subsets remained stable with age and between young WTs and young KOs whereas it was greater on 50 week-old KO mice (Figure 6.7). Thus, the accumulation of mature T helper and cytotoxic cells observed in WT mice upon aging did not occur in mice lacking *Timp-3* gene.

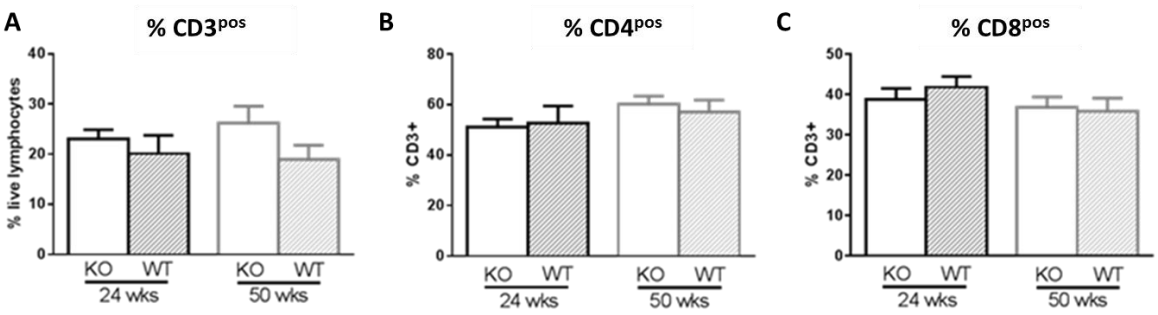


Figure 6.6 *Timp-3* KO mice did not have abnormal proportion of total T cells and main T cell subsets. Percentage of total $CD3^{pos}$ cells (A), CD4 T cells (B) and CD8 T cells (C) in *Timp-3* KO and WT mice during aging measured by flow cytometry.

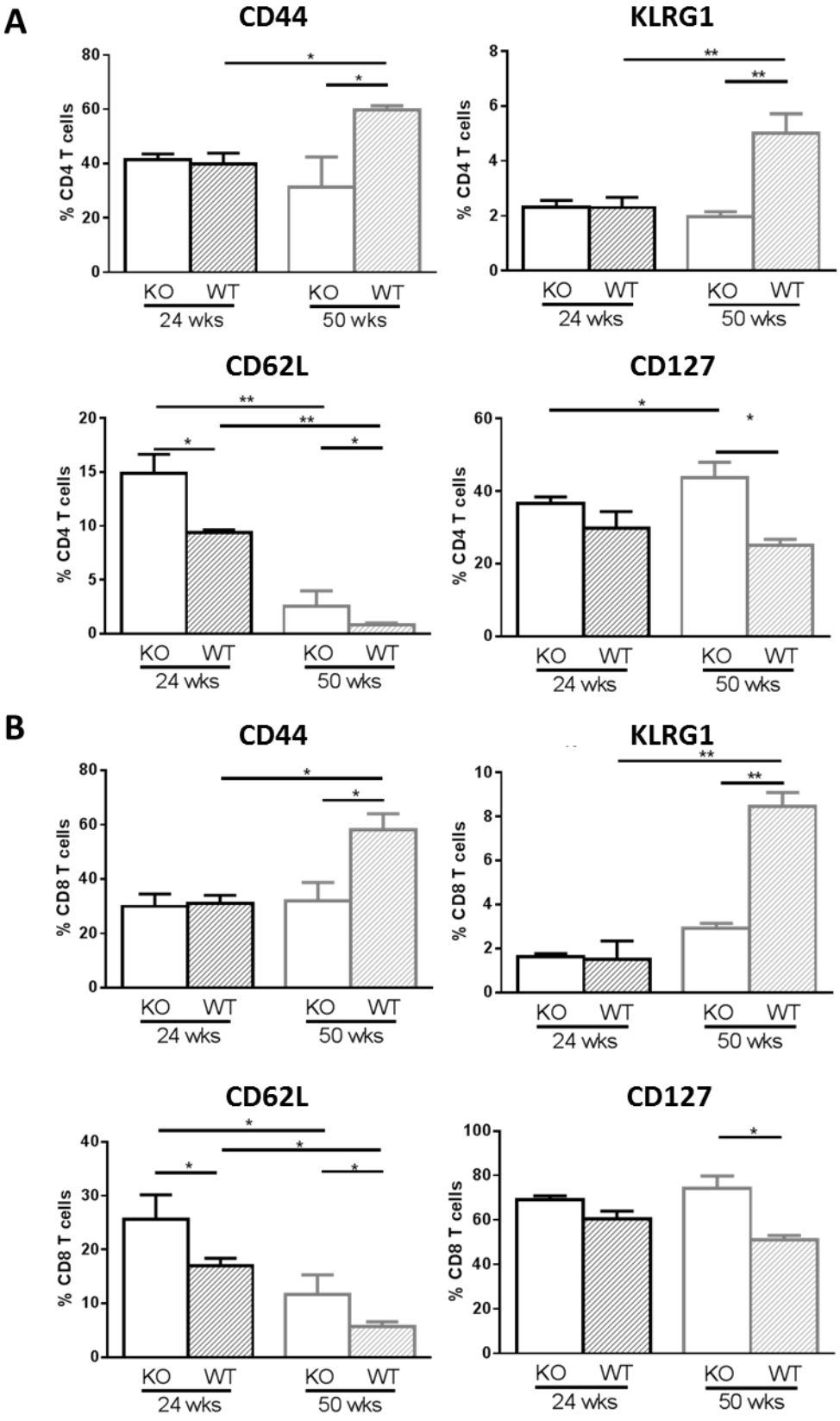


Figure 6.7 T cells from the spleen of *Timp-3* KO mice showed phenotypic immaturity. Percentage of CD4 T cells (A) and CD8 T cells (B) expressing the indicated maturation markers in *Timp-3* KO and WT mice during aging was measured by flow cytometry.

Combining the two classic markers CD44 and CD62L, T cell maturation stages were studied to understand at which point the differentiation process was altered in KO animals. We observed that *Timp-3* KO mice had a higher percentage of CD4 (Figure 6.8A) and CD8 (Figure 6.8B) naïve and central memory cells compared to their WT counterparts at 24 weeks of age. These populations from either genotypes experienced age-related decline with a less severe decrease occurring in CD4/CD8 naïve cells and CD8 central memory cells from KOs (Figure 6.8). The loss of cells in early maturation phases seen with aging was coupled with the accumulation of effector memory T lymphocytes in WT mice that was not observed in *Timp-3* KO samples. This led to the presence of less mature T cells in old *Timp-3* KO mice.

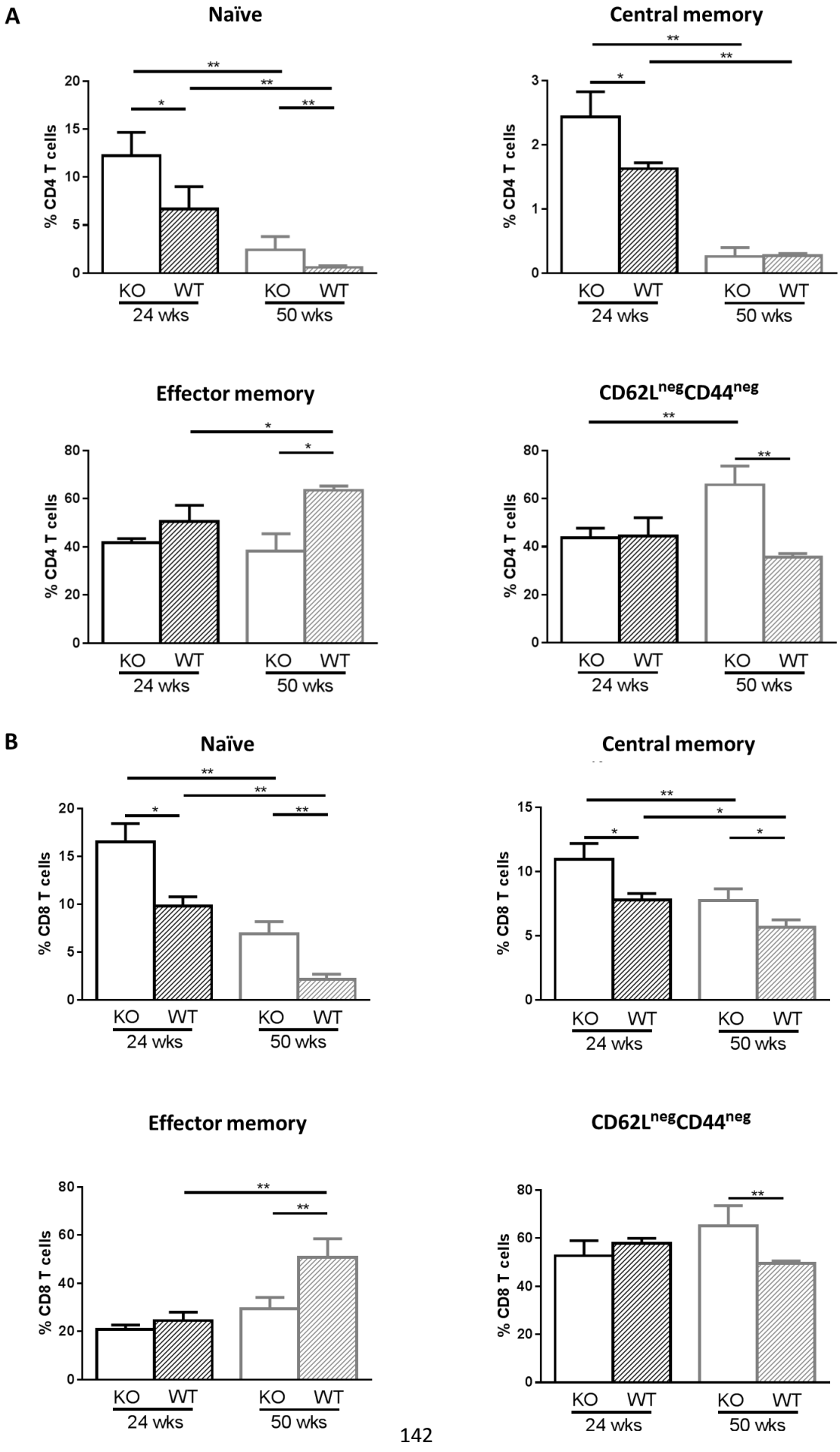
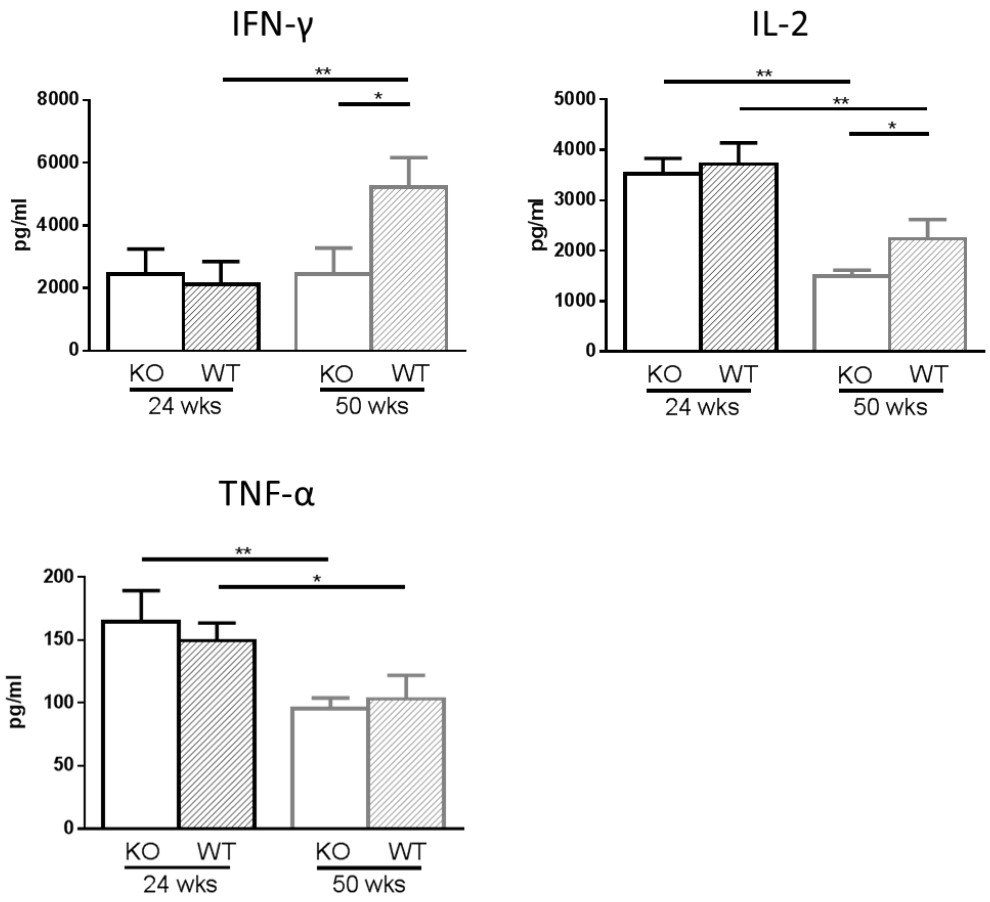

A
Naïve
Central memory
Effector memory
CD62L^negCD44^neg
% CD4 T cells
KO WT KO WT
24 wks 50 wks
B
Naïve
Central memory
Effector memory
CD62L^negCD44^neg
% CD8 T cells
KO WT KO WT
24 wks 50 wks

Figure 6.8 *Timp-3* KO animals showed a differential distribution of spleen T cell differentiation stages. Percentage of CD4 T cells (A) and CD8 T cells (B) maturation subsets in *Timp-3* KO and WT mice during aging was measured by flow cytometry. Naïve ($CD62L^{pos}CD44^{neg}$), Central memory ($CD62L^{pos}CD44^{pos}$), Effector memory ($CD62L^{neg}CD44^{pos}$).

Total CD4 and CD8 T lymphocytes were sorted and polyclonally stimulated and this allowed us to investigate the consequences of the maturation deficit reported here on the ability to secrete cytokines and cytotoxic molecules (Figure 6.9). Starting from a similar level at the younger age (24 weeks old), the functional capacity of T cells diverged from KOs and WTs at later age (50 weeks old). Release of IFN-γ and GZB from both T subsets did not increase in KOs during aging as it did in cells from WTs. Conversely, the secretion of IL-2 and TNF-α dropped in old animals of either genotypes but the decline in IL-2 resulted to be accentuated in cells from KO mice. We could conclude that *Timp-3* KO mice are characterised by an extensive maturation and functional defects that involve not only NK cells but also both T helper and cytotoxic lymphocytes.

A


IFN-γ
IL-2
TNF-α
pg/ml
KO WT
24 wks
50 wks

B

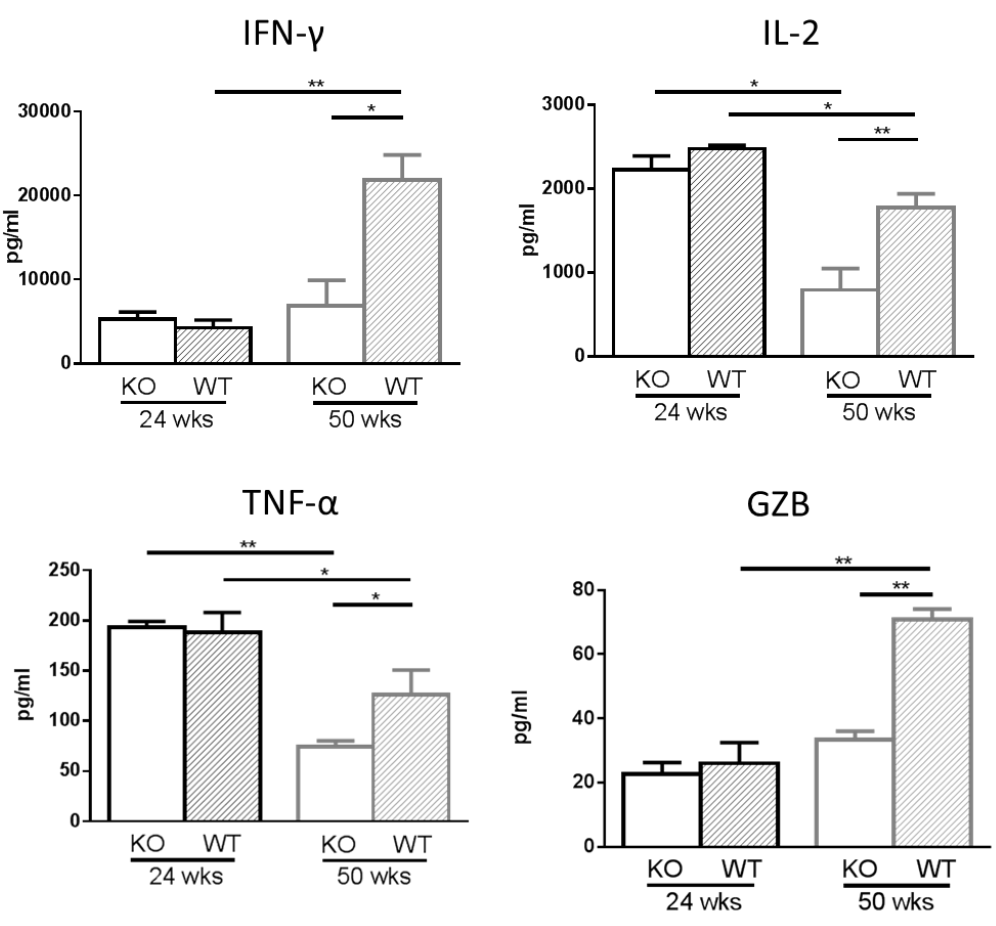

IFN-γ
IL-2
TNF-α
GZB
pg/ml
KO WT
24 wks
50 wks

Figure 6.9 Maturation deficit was accompanied by a functional alteration of spleen T cells in *Timp-3* KO animals. Secretion of the indicated molecules from CD4 T cells (A) and CD8 T cells (B) in *Timp-3* KO and WT mice during aging was measure by Luminex for IFN-γ, IL-2 and TNF-α and by ELISA for GZB.

6.3 Discussion

As discussed above, TIMP-3 has proved to be a master regulator of not only tissue structural remodelling but also inflammatory processes, given its unique ability to modulate the signalling of TNF-α and other mediators of inflammation. Therefore, researchers postulated that TIMP-3-based therapies can be developed to control systemic and local inflammation (Smookler et al., 2006). This opened up a new branch of immunological research in the topic of MMPs and TIMPs.

In our study, we profiled NK and T cells isolated from the spleens of *Timp-3* KO mice at different ages in order to reveal whether TIMP-3 has effects on how innate and adaptive lymphocytes differentiate and function during aging.

Phenotyping analyses demonstrated that maturation status of NK and T cells did not significantly differ between *Timp-3* KO and WT mice at young age. Lymphocytes from both genotypes experienced the same qualitative age-related changes. However, the kinetics of these changes was more accentuated in old KO animals, resulting in earlier NK and T cell immunosenescence. Proportions of total NK and mature NK cells were lower in old *Timp-3* KO mice than old WT mice as suggested by decreasing expression of maturity markers (CD11b, CD62L, KLRG1, Ly49H) and higher expression of molecules present on immature NK cells (CD27, CD127). Defect in maturation markers were concentrated in the late stages of maturation (DP and CD11b) except for Ly49H whose levels proved to be dysregulated at earlier phases of differentiation as well.

The accumulation of mature T helper and cytotoxic cells (marked by CD44 and KLRG1) during aging did not occur in mice lacking *Timp-3* gene as it happened in WT animals. Phenotypic immunosenescence reflected in functional alterations that were more pronounced in NK and T cells isolated from spleens of aged *Timp-3* KO mice. We could conclude that, in *Timp-3* KO mice, NK and T mmunosenescence set in at a time point later than 24 weeks and earlier than in the WT counterparts. The use of additional time points will surely help in explaining the kinetic of age-associated abnormalities in *Timp-3* KO animals. In addition, it would be interesting to understand why this series of abnormalities becomes clear at old age in *Timp-3* KO mice.

The descriptive work we have done will need to be enriched by investigation of the reasons why lack of *Timp-3* KO was associated to altered maturation and functions in innate and adaptive

lymphocytes. The proteolytic cleavage and release of transmembrane cell surface proteins has emerged as an important post-translational mechanism to regulate the function of immune cells. MMPs inhibited by TIMP3, such as TACE, MMP-9 and ADAM10, cleave growth factors or their receptors affecting haematopoietic growth factor signalling. For instance, some of these molecules are crucially involved in myeloid cell development (c-Kit/SCF) (Cruz et al., 2004; Heissig et al., 2002), in the formation of monocytes (M-CSF/c-fms) (Horiuchi et al., 2007; Rovida et al., 2001) and in the differentiation of T and NK cells (IL-2Rα, IL-15Rα, Notch) (Budagian et al., 2004; Rovida et al., 2001; Sheu et al., 2001). Any disruption of these pathways might block or skew the development, maturation and function of the related immune cell. Typically, receptor cleavage reduces signal transduction for that pathway. Indeed, the presence of sIL-15Rα and sIL-2Rα in biological fluids could affect the function of their ligands by competing for the cytokine with the cell-bound receptors and inhibiting its action. On the contrary, in the case of Notch, shedding activates the signalling pathway. TACE-mediated extracellular cleavage of Notch is coupled with its intracellular proteolysis, allowing the cytosolic fraction of Notch to translocate to the nucleus as a transcription factor (Brou et al., 2000) where it blocks hematopoietic stem cell differentiation (Duncan et al., 2005). Thus, we could speculate that the lack of TIMP-3 leads to increased cleavage of sIL-15Rα, sIL-2Rα and Notch with consequent impairment of proper NK and T cell differentiation. It has been reported that, after being transferred to aged mice, bone marrow from young mice gave rise to NK cells with maturation defects (Chiu et al., 2013). They found that aging-related functional NK cell deficiency was completely reversed by injecting soluble IL-15/IL-15Rα complexes. Chiu *et al.* demonstrated that the aged host environment is responsible for aging-related functional NK cell deficiency, suggesting that IL-15 receptor agonists may be useful tools in treating aging-related functional NK cell deficiency.

6.4 Experimental procedures

6.4.1 Mice and sample preparation

Female *Timp-3* KO mice and C57BL/6 WT mice were used at two ages: 24 weeks (young) and 50 weeks (old). Gender and age were chosen on the basis that female *Timp-3* KO mice died at 60 weeks old on average while male *Timp-3* KO mice did not show such survival abnormality. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in Singapore and the

Animal Welfare and Ethical Review Body (AWERB) in Southampton, UK. For details on spleen sample preparation, refer to Chapter 3.

6.4.2 Phenotyping

Cell phenotyping was performed by flow cytometry on 6 spleen samples from young WT mice, 6 spleen samples from young KO mice, 6 spleen samples from old WT mice and 6 spleen samples from old KO mice. For each staining, 2×10^6 splenocytes were used. Lymphocytes were gated based on FSC/SSC profile and doublets/dead cell exclusion. For NK cell analysis, T cells were excluded by CD3 expression, followed by NK cell identification on $CD3^{neg}$ lymphocytes using NK1.1 expression. T cells were tested as $CD3^{pos}$ lymphocytes and further gating on CD8 or CD4 T cells. The antibodies are listed in Table 6.1. Flow cytometry was performed on an LSR Fortessa Cell Analyzer (BD Biosciences) and automatic compensation was applied.

6.4.3 Sorting

Total NK cells ($NK1.1^{pos}$), total $CD8^{pos}$ T cells and total $CD4^{pos}$ T cells isolated from 6 spleen samples from young WT mice, 6 spleen samples from young KO mice, 6 spleen samples from old WT mice and 6 spleen samples from old KO mice were sorted. The antibodies used are listed in Table 6.1. Cell sorting was performed on a BD Influx 3 lasers (BD Biosciences). Compensation was performed using single colour controls prepared from BD Comp Beads (BD Biosciences).

Table 6.1 List of antibodies used for sorting and phenotyping

Antigen	Fluorochrome	Clone	Company
CD3	FITC	145-2C11	BioLegend
NK1.1	BV-421	PK136	BioLegend
CD27	PEC-y7	LG7F9	eBioscience
CD11b	APC-Cy7	M1/70	BioLegend
KLRG1	PE/Dazzle-594	2F1/KLRG1	BioLegend
CD4	BV-605	RM4-5	BD Biosciences
CD62L	BV-786	MEL-14	BD Biosciences
CD127	PE	SB/199	BD Biosciences
LY49H	AF-647	3D10	BioLegend
CD44	AF700	IM7	BioLegend

6.4.4 NK cell and T cell stimulation

Sorted NK, CD8 and CD4 cells were stimulated with a cocktail of phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) and ionomycin (250 ng/ml) at 0.5 million cells/ml in RPMI + 10% FBS. Supernatants were collected after 18 hours of incubation at 37 °C and stored for future use at -80°C.

6.4.5 Granzyme B ELISA

Frozen supernatants from sorted and stimulated NK, CD8 and CD4 T cells were thawed and tested with a Mouse Granzyme B ELISA Ready-Set-Go (eBioscience, Cat# 88-8022-22) according to the manufacturer's instructions. Refer to Chapter 3 for details of this assay.

6.4.6 Multiplex analyte screening

Frozen supernatants from sorted and stimulated NK, CD8 and CD4 cells were thawed and tested with a Milliplex MAP Mouse Th17 kit (Millipore, Cat# MTH17MAG-47K) according to manufacturer's instructions. Refer to Chapter 3 for details of this assay.

6.4.7 Data analysis

Flow cytometry data were analyzed using FlowJo (Treestar) and FACSDiva (BD Biosciences). Samples were compared using GraphPad Prism software (v.6.0c). Groups of young and old mice were compared by Mann-Whitney U test. The Wilcoxon matched-pairs signed rank test was used for paired testing of median values of different subsets from the same mouse. Comparisons with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered significant.

Chapter 7: Conclusion and perspective

7.1 Concluding remarks

In this thesis, we described the age-associated abnormalities occurring in humans and in two murine models, the C57BL/6 wild-type mouse and the *Timp-3* knock-out mouse. Our data showed that NK cell subset repartition and the complex multi-step process of differentiation that NK cells experience in order to become fully competent are deeply affected by aging. Alterations were found in the progressive acquisition or down-regulation of cell surface molecules, including activating and inhibitory receptors, adhesion molecules and other maturation markers. This abnormal phenotype reflected changes to cell functionality, influencing cytotoxic arsenal, cytokine production and, consequently, immunomodulatory and proliferative capacity. Aging also impacted the intricate network of transcription factors finely controlling the series of cellular and molecular events during NK cell maturation. This work deepened our knowledge of human and murine NK cell maturation and function during aging, leading to novel insights into age-related dysfunctions in innate immunity.

We analysed the phenotype and function of human NK cell subtypes using three models of persistent immune activation: aging, HCMV infection and hepato-cellular carcinoma (HCC). We showed that CD57, NKG2C and TIM-3 are hallmarks of NK cell maturation during aging and that acquisition of these markers correlated with HCMV and inflammation status of the donors. Our results demonstrated that, while cytokine production was preserved, degranulation and cytotoxic molecules secretion was altered during aging. However, mature $CD57^{pos}$ NK cells from older people were more poly-functional than those from younger controls at the single cell level. NKG2C dissected $CD57^{pos}$ NK cells in different subsets in terms of functions, gene expression and differentiation, with $NKG2C^{pos}CD57^{pos}$ NK cells as the most advanced stage of NK cell differentiation. In addition, $NKG2C^{pos}CD57^{pos}$ NK cells functionality was shown to be, at least partially, regulated by the TIM-3/Ceacam-1 pathway since the use of anti-TIM-3 antibody and the silencing of Ceacam-1 ameliorated some of their functions. To determine the relevance of these results in a pathophysiological environment, we investigated CD57-expressing NK cell functionality in patients with different stages of HCC. Disease progression was associated with tumor infiltration of exhausted and cytotoxic-deficient NK cells expressing CD57, TIM-3 and Ceacam-1. We could efficiently segregate stages of HCC progression according to co-expression of Ceacam-1 and TIM-3. This work enriched our understanding of the mechanisms underlying NK cell differentiation during persistent immune activation, possibly supporting the identification of new immunological targets

for checkpoint blockade therapies in order to rescue early innate defense in aging, chronic infections and cancer.

Moreover, we sought to expand the knowledge on how aging impacts NK cell maturation and function in murine models. Our data demonstrated that NK cells are reduced in frequency and numbers and exhibit an altered phenotype in the blood and spleens in aged mice. Investigating the expression of a variety of cell surface markers associated with the maturation process, we showed that aging is characterized by an accumulation of immature NK cells coupled with a reduction in the late differentiated subset. This phenotypic immaturity reflects a relevant functional immaturity. Our results showed that cytokine secretion, cytotoxicity and gene expression of NK cells are modulated by the aging process along a maturation pathway defined by CD11b and CD27 and, in some cases, LY49H and KLRG1. The reduced presence of NK cells in circulation and in the spleen and their altered maturation status can have severe implications on the immune responses of aged mice to viral infections and cancers.

Older *Timp-3* KO mice were affected by a series of alterations in the maturation process and functionality of both NK and T lymphocytes compared to wild-type counterparts. Indeed, NK and T cells from aged *Timp-3* KO mice experienced the same qualitative age-related changes as the lymphocytes from the age-matched wild-type counterparts; however, the kinetics of these changes was accelerated in old *Timp-3* KO animals, resulting in earlier NK and T cell immunosenescence. We could conclude that, in *Timp-3* KO mice, NK and T cell immunosenescence set in at a time point later than 24 weeks and earlier than in the age-matched WT counterparts.

TIMP-3 is a master regulator of not only tissue structural remodelling but also inflammatory processes, given its unique ability to modulate the signalling of TNF-α and other mediators of inflammation. Aging is accompanied by chronic high levels of circulating TNF-α and by age-related diseases with an inflammatory basis (Bruunsgaard et al., 2003). In case of a lack or deficiency of TIMP-3, TACE is not blocked, resulting in elevated TNF-α levels and it has been related to inflammatory diseases such as vascular inflammation and diabetes (Federici et al., 2005), inflammatory bowel diseases (Monteleone et al., 2012), hepatic steatosis and adipose tissue inflammation (Menghini et al., 2009) and endotoxic shock (Smookler et al., 2006). Additionally, it TIMP-3 levels were shown to be associated with human aging in a variety of tissues, such as Bruch's membrane (Kamei and Hollyfield, 1999), lungs, kidneys, retinas and vascular tissues (Macgregor et al., 2009). For these reasons, *Timp-3* KO mouse might be useful as an animal model not only for inflammatory diseases but also aging, age-related pathologies and especially the relationship between aging and inflammation.

In conclusion, this work deepened our understanding of human and murine NK cell differentiation and functionality during aging, leading to novel insights into age-related dysfunctions in NK cell responses and innate immunosenescence. Our findings could support the identification of new immunological targets for checkpoint blockade therapies in order to rescue early innate defense upon aging, chronic infections and cancer. Furthermore, these results offered new insights into TIMP-3 biological role in adaptive and innate immunity, especially its importance during the aging process.

7.2 Limitations of the work

This work encountered some limitations that might have influenced the interpretation of our findings. Even though recent scientific and technical advancements have greatly increased our understanding of immunity and immunosenescence, many aspects of the study design and methodology still hinder immunogerontologic investigations. There is much controversy in the topic and standardised inclusion criteria and experimental design are needed. In particular, including detailed chronic pathologies status, infectious status and gender is of crucial importance. In elderly Chinese Singaporeans, CMV prevalence is very high (99%) (Solana et al., 2012a). For this reason, we could not include an old CMV-seronegative donor group in our study, making difficult to dissect the impact of aging and CMV.

Innate immunity is diminished in unhealthy and frail old individuals. Thus, in order to describe the intrinsic effect of aging on innate lymphocytes, studies should focus more on elderly who are not affected by relevant clinical conditions and do not make use of drugs that could interfere with immunological parameters. However, it is extremely difficult, if not impossible, to build a completely healthy cohort of elderly people who are treated with medicines. Several chronic conditions were present in our study population, such as hypertension, high cholesterol and diabetes. However, subjects with a history of hospitalization in the past 6 months and high CRP levels (>3 mg/l) were excluded from the analysis.

Immunological parameters of our old cohort were compared with those of a young cohort in a cross-sectional study. A better experimental design in the immunogerontologic research field will need to include more longitudinal studies to be able to understand the clinical significance of age-associated alterations of the innate immune system as biomarkers of healthy aging and longevity.

Moreover, deeper studies on how aging mechanistically modulates the phenotype and function of NK cells are required to go beyond descriptive and non-interventional work. The precious tools available nowadays, such as proteomics, genomics and analysis of large datasets, will surely help to

identify the molecular mechanisms that contribute to the age-related abnormalities of the innate immune response.

Our animal studies were limited by restricted numbers of mice that could be studied and sample size might have influenced whether statistical significance was reached or not in some analyses. The cross-sectional nature of the animal work presented here surely represents a model much weaker than the longitudinal one. However, the length of and the types of tissues analysed in this study did not allow to follow the animals longitudinally. Additionally, few time points during the aging process were tested, hindering the ability to deduct more precise and definitive conclusions about the kinetics of the observed age-related changes. A further limitation is the lack of mechanistic explanation of our findings regarding the reasons why *Timp-3* KO mice showed altered maturation and functionality in innate and adaptive immune responses.

Finally, the main question in any research involving animal model is whether the similarities in cellular processes and age-associated disease pathogenesis between mice and humans are sufficiently robust that experimental findings from mouse models can be extrapolated to study aging in human systems. Gerontology continues to rely on models with a lifespan shorter than normal. While mice offered insights on the mechanisms governing aging and on targets for drug discovery with potential human applications, these findings must be interpreted with caution. The lack of unambiguous models where scientists can test their hypotheses is still one of the reasons why the mechanisms responsible for human aging remain largely a mystery.

7.3 Future directions

In our opinion, the key aspects of this thesis on which future work should focus are:

- How the descriptive phenotype can inform on NK cell functions.

  It has been extensively described that CD57 expression is increased on NK cells during aging and other conditions of chronic immune stimulation. As already reported in the introduction of this thesis, the CD57 antigen, also called HNK-1, LEU-7 or L2, is a terminally sulfated carbohydrate epitope that was originally reported as a marker of human natural killer cells (Abo, 1981). HNK-1 is widely conserved in animals from insects to mammals, and expression is regulated both developmentally and spatially in specific areas of the nervous system, including migrating neural crest cells, rhombomeres, the cerebellum, and myelinated Schwann cells in motor neurons. Cell surface carbohydrates function as binding sites in a variety of crucial cellular activities including recognition and adhesion to other

cells, bacteria, viruses, and toxins. HNK-1 was originally detected by immunoblot/immunohistochemical analysis of cell adhesion glycoproteins in the human nervous system, including the binding partners neural cell adhesion molecule, myelin-associated glycoprotein, 5'-nucleotidase, ICAM5, NCAM, integrins, cytotactin, TAG-1, MAG, PMP-22, contactin, L1, P0 and telencephalin, as well as on chondroitin sulfate proteoglycans and glycolipids such as sulfoglucuronylglycolipid-1 and sulfoglucuronylglycolipid-2 (Focosi, 2010). HNK-1 is now also known to act as a ligand for laminin, L-selectin, and P-selectin, as well as for the cerebellar adhesion protein amphoterin. These observations suggest that HNK-1/CD57 is associated with cell–cell and/or cell–matrix interactions. However, little attention has been paid to the precise identity of the molecules expressing the CD57 epitope on NK cells and T cells, precluding a full understanding of the relationship between CD57 expression and lymphocyte function. Although one study identified the CD57 epitope on the IL-6 receptor gp130 of resting lymphocytes (Cebo, 2002), the cells expressing CD57/gp130 were not identified and no comprehensive analysis of CD57-expressing molecules on T cells or NK cells has been reported. It would therefore be very interesting to investigate if, on immune cells, CD57 has a pivotal role in the recognition and adhesion to other cells, toxins and microbes or in some other processes such as molecular tagging/labelling and signal transduction. These studies are, however, hindered by the lack of relevant knock-out animal models, including a CD57 knock-out mouse. Yamamoto et al. (Yamamoto, 2002) have shown that mice lacking the enzyme responsible for CD57 biosynthesis (GlcAT-P gene deletion) exhibit alterations in several key higher brain functions, including spatial memory formation and synaptic plasticity, similarly to NCAM-lacking mice.

- Age-related dysfunction of $CD56^{neg}CD16^{pos}$ NK cells.

  Besides aging, this subpopulation of NK cells was also described in individuals with chronic viral infections (Cooper, 2001), including hepatitis C and HIV infection. The origins of the $CD56^{neg}CD16^{pos}$ NK cell subset and the underlying mechanisms of NK cell dysfunction are poorly understood.

  The $CD56^{neg}CD16^{pos}$ NK cells described from these varied settings have uniformly poor cytolytic ability with impaired cytokine production. For these reasons, they have been described as 'immature' (Gaddy, 1997), dysfunctional' (Mavilio, 2005), 'anergic' or 'exhausted' in the settings of HIV infection (Alter, 2005) and EBV-driven post-transplant lymphoproliferative disorder (PTLD) (Wiesmayr, 2012). However, it is possible that impaired

functional responses reflect a failure of NK cell development and/or inadequate stimulation in these diverse environments, and may therefore be potentially reversible.

In order to understand if $CD56^{neg}CD16^{pos}$ NK cells have exhausted features in aged individuals, a series of markers could be of help: exhausted phenotype (increased TIM-3, increased PD-1, increased NKG2A); exhausted transcriptional program (reduced Eomes); exhausted function (reduced cytolytic and cytokine production activity [investigation limited by small cell numbers in the $CD56^{neg}CD16^{pos}$ population]).

- The clinical significance of TIM-3 on NK cells.

  Current T cell immunotherapies aiming at overcoming immune exhaustion target PD-1 and CTLA-4, but there is an emerging interest in TIM-3. Although TIM-3 is present on T cells and other immune cell types, its highest expression has been found on NK cells more recently. However, few studies so far addressed TIM-3 role on NK cells in health and disease. Our data requires validation at a larger scale to evaluate if the profiling of intra-tumoural NK cells could be used as a reliable diagnostic or prognostic biomarker.

  Further investigations are also necessary to assess the consequences of *in vivo* TIM-3/Ceacam-1 blockade as a potential new innate and adaptive immune checkpoint inhibitor. Reversibility of exhausted NK cell functionality in the context of HCC will surely need to be tested in TILs (other than blood).

  Additionally, expansion of the HCC cohort and application of further techniques, such as histology, would surely add relevant details. For example, in situ immunohistochemistry of tumours and peritumoral tissue sections on paraffin-embedded sections from HCC biopsies will inform on the localization of tumour-infiltrating NK subsets (e.g. inside/outside blood vessels[sinusoids]). This, along with frequency and phenotype data, would help in the study of any defect in adhesion and migration processes (especially reduction in CD62L in aging) and support/validate flow cytometry analyses.

- The clinical importance of innate memory.

  Further experimental work regarding NK cell maturation, innate memory and the vast array of interacting receptors can pave the way to innovative NK cell-based adoptive immunotherapies. While NK cell immunotherapy is currently a transient therapy due to the limited life span of NK cells, the use of long-lived memory-like NK cells may offer long-term protection against cancer and infectious agents such as HCMV upon transplantations.

Strategies activating NK cell antiviral immunity could also support and improve current T cell-based vaccination protocols against many types of viruses.

- The mechanisms underlying abnormal maturation of NK cells in murine models.
  The use of additional time points will help in explaining the kinetics of age-associated abnormalities in wild-type and *Timp-3* KO mice as they age. In addition, it would be intriguing to understand why differences in NK cell phenotype and function between wild-type and *Timp-3* KO become clear only at old age. The descriptive work we have done will also need to be enriched by mechanistic investigation of the reasons why lack of *Timp-3* KO was associated to altered maturation and functionality in innate and adaptive immune responses. For instance, we could speculate that the lack of TIMP-3 leads to increased cleavage of sIL-15Rα, sIL-2Rα and Notch by TACE enzyme with consequent impairment of proper NK and T cell differentiation. It would be interesting to investigate whether treatment of old *Timp-3* KO mice with the above-mentioned growth factors is able to reverse alterations in NK cells.

While most immunogerontologic literature was limited to the investigation of adaptive immunity until recently, researchers now know that abnormalities in the innate response, such as those in the NK cell population, have severe and various effects on health of older adults. New studies are revealing that the function of NK cells stretches beyond their conventional role in anti-viral and anti-tumour immunity into new fields of research including innate memory, cross-talk with dendritic cells and macrophages, initiation of adaptive immune responses, anti-microbial immunity, resolution of inflammatory responses and the clearance of senescent cells. Indeed, along with the increase in viral infections and cancer rates, some hallmarks of aging such as the weak efficacy of vaccination, the accumulation of senescent cells and the higher incidence of fungal infections may be partially caused by the decline in NK cell function that accompanies human aging. For these reasons, devising new strategies to prevent, delay or even reverse NK cell immunosenescence might be beneficial in ameliorating many aspects of the health and quality of life in older population. Few interventions, such as exercise and dietary supplementation, have been discussed so far in order to prevent, delay or reverse age-related dysfunctions of innate immune cells and especially NK cells. It is important to keep in mind that, in boosting innate cell functionality, the exacerbation of pre-existing inflammatory conditions or overzealous inflammatory responses should be avoided. Indeed, since the inter-individual variability of the aging process is enormous, it will be crucial to identify those subjects who would be damaged or benefit most from immunomodulatory treatments. Therefore, it would be useful to discover more efficient biomarkers and implement the

existing ones (e.g. pro-inflammatory cytokines) to accurately measure the effects of aging and rejuvenating interventions.

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