I, Hesham M. Shehata Ph.D., hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Immunology.

It is entitled:
The Function and Homeostasis of Natural Killer Cells in Aging

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The Function and Homeostasis of Natural Killer Cells in Aging

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By

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Thesis Abstract

Aging is a complex process that negatively affects a wide variety of physiological functions, including the development and maintenance of robust immune responses. In fact, aging affects almost all aspects of innate and adaptive immune responses. Developing successful strategies that promote improved immune responses in the elderly against infectious pathogens and cancers that cause high mortality represents a growing public health priority. Natural killer (NK) cells play a critical role in eliminating tumor cells and viral infections, both of which occur at a high incidence in the elderly population. Aging has a considerable negative impact on the function and development of NK cells. These defects attenuate NK cell cytotoxicity to virus infected cells and tumors. Thus, studies to determine the causes of aged-related NK cell dysfunctions are critically required to address the health needs of a growing elderly population.

The precise mechanism(s) underlying the impaired maturation and function of NK cells in aging remain unidentified. This dissertation aims to fill this gap in knowledge by evaluating whether key factors known to be important in NK cell biology could be dysregulated in aging, thus impairing their functional maturation. Importantly it highlights novel avenues for research in the field geared at discovering the specific factor(s) that impair the function and terminal maturation of NK cells in aging. Herein, we provide evidence that the aged environment and in particular, the aged BM microenvironment contributes to the impaired maturation and function of NK cells. NK cells derived from both young and aged BM cells adopted an impaired maturation phenotype in an aged host i.e. were hyporesponsive to stimuli in vitro, while adopting an augmented maturation phenotype following transfer in young hosts. In our pursuit to identify the environmental factor(s) that may contribute to this phenotype, we observed a differential pattern of expression of key transcription factors including a decrease in T-bet and Eomes which
are known to regulate NK cell maturation and function. Importantly, the environment in which NK cells developed played an important role in regulating the expression of both T-bet and Eomes. One of the major factors implicated in regulating T-bet and Eomes expression and NK cell maturation is IL-15. Our data suggest that deficiencies in IL-15 production or IL-15 signaling in aging are unlikely to be the mechanisms underlying the impaired maturation of NK cells as administering a large quantity of IL-15/IL-15Rα complex to aged mice did not augment NK cell maturation in the BM, despite their capacity to expand in response to IL-15.

In addition, Foxo1, which impairs NK cell maturation and function through the downregulation of T-bet, was significantly increased in aged splenic but surprisingly, not BM NK cells. The increased Foxo1 protein levels may be due to post transcriptional modifications as Foxo1 mRNA levels were not different in any of the splenic NK cell subsets of young and aged mice. Foxo1 plays a critical role in counteracting genotoxic stress, DNA damage and is known to impair cellular proliferation. The increase in Foxo1 in aged splenic NK cells was associated with increased mitochondrial superoxide production and DNA damage as well as impaired proliferation. Additionally, the impaired NK cell maturation in aging was associated with higher mTORC1 activity, higher ATP and decreased autophagy. We hypothesize that normalizing the activity of mTORC1 and expression of Foxo1 in aged NK cells using rapamycin or proteasome activators could augment their maturation and function.

On another front, we identified for the first time a profound presence of MHC-I low cells in the lymphoid organs of aged mice. These cells have a late-apoptotic phenotype with fewer organelles and some of which appear to be necrotic cells. As MHC-I deficiency imposes NK cell hyporesponsiveness, it will be interesting to evaluate whether the presence of these cells in the aged environment contribute to the hyporesponsiveness of NK cells.
Acknowledgements

I would like to convey special thanks to my mentor, Dr. Claire Chougnet for her invaluable guidance over the last five years. During this time, Claire has not only unwaveringly offered me very practicable expertise to fulfill my scientific and literary ventures, but also offered me the opportunity to think more critically and be a better researcher. For this, I am indebted to her and I convey my deepest appreciation for her support.

I would also like to thank wholeheartedly past and present members of our team – Cesar Rueda, Courtney Jackson, Casey Wells and Maria Fields. We have all been more than just lab mates at work – but more like a big family and by and large we ensure the well-being of each member. Their invaluable encouragement over the years has not only been motivating, but has also made working together in the lab with them an extremely enjoyable experience. For these reasons, I thank each of them so much for their support and advice.

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targets could be impaired. Thus, in many ways, I view Kasper as the founder of my PhD research work. Kasper, I thank you so much for everything. I would love to continue working with you and no matter what the future holds for me, we will always have common interests in the NK cell field.

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My time in graduate school was made enjoyable in large part due to the many friends and groups that became a part of my life. I would also like to thank all my friends in the immunobiology graduate program and at Cincinnati Children’s. We have had such an enjoyable time together and I am thankful for having come to this institution and meeting them.

I would finally like to dedicate this thesis to my family – my parents and my three sisters, Wesam, Amira and Hanaa for their love and encouragement. For their unconditional love and support, I believe they have equally earned this PhD. Far as we may have been from each other,
it has felt like they were close. Words cannot express how grateful I am to them for their support throughout my education. They have cherished with me every great moment and supported me whenever I needed it and for this, I will forever be indebted to them.
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<th>Definition</th>
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<tbody>
<tr>
<td>2-NBDG</td>
<td>(2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)</td>
</tr>
<tr>
<td>2-DG</td>
<td>2- deoxyglucose</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDMs</td>
<td>Bone marrow derived macrophages</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CAR</td>
<td>CXCL-12 abundant reticular stromal cells</td>
</tr>
<tr>
<td>DAP-12</td>
<td>DNAX Activating Protein of 12KDa</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DLN</td>
<td>draining lymph node</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative NK cells</td>
</tr>
<tr>
<td>DR4</td>
<td>Death receptor 4</td>
</tr>
<tr>
<td>DR5</td>
<td>Death receptor 5</td>
</tr>
<tr>
<td>DEREG</td>
<td>Depletion of regulatory T cells</td>
</tr>
<tr>
<td>DT</td>
<td>Diptheria toxin</td>
</tr>
<tr>
<td>DTR</td>
<td>Diptheria toxin receptor</td>
</tr>
<tr>
<td>ECTV</td>
<td>Ectromelia Virus</td>
</tr>
<tr>
<td>Eomes</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Foxo</td>
<td>Forkhead box (Fox) family of transcription factors of the O subclass</td>
</tr>
<tr>
<td>Gas6</td>
<td>Growth Arrest-Specific 6</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage- colony stimulating factor</td>
</tr>
<tr>
<td>GZB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte antigen</td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>HSPCs</td>
<td>Hematopoietic stem cells and progenitor cells</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>IL-15</td>
<td>Interleukin 15</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon- gamma</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-γ inducible protein-10</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine based inhibitory motif</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>IONO</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>i.v</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IRF-2</td>
<td>Interferon regulatory factor 2</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer-cell lectin like receptor G1</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>LTβR</td>
<td>Lymphotoxin beta receptor</td>
</tr>
<tr>
<td>MCA</td>
<td>Methylcholantherene</td>
</tr>
<tr>
<td>Mc1-1</td>
<td>Myeloid cell leukemia 1</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>Mig</td>
<td>Monokine induced by interferon-γ</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Noxa</td>
<td>NADPH oxidase activator 1</td>
</tr>
<tr>
<td>NAC</td>
<td>N-Acetyl Cysteine</td>
</tr>
<tr>
<td>Ncr</td>
<td>Natural cytotoxicity receptor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>pLN</td>
<td>peripheral lymph node</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>pNK</td>
<td>NK cell precursors</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polynosinic:polycytidylic acid</td>
</tr>
<tr>
<td>Rae-1</td>
<td>Retinoic acid early inducible protein-1</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S1P5</td>
<td>Sphingosine 1-phosphate receptor 5</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol phosphatase</td>
</tr>
<tr>
<td>SHP</td>
<td>SH2-containing phosphatase</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri-carboxylic acid cycle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron micrograph</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>T_H</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>T-box</td>
<td>T-box expressed in T cells</td>
</tr>
<tr>
<td>TSC-2</td>
<td>Tuberous sclerosis complex 2</td>
</tr>
<tr>
<td>U.N.</td>
<td>United Nations</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
<tr>
<td>W.H.O</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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</table>
Chapter 1: Introduction

1.1 The world’s population is growing older

The aging of an organism is defined as an inevitable process that is both progressive and cumulative leading to the decline in function of multiple cell types and organs. These alterations lead to an inability to maintain physiological homeostasis and eventually cause death. In the past several decades, the advancement in health care, the advent of antibiotics, vaccinations and enhanced life standards have contributed dramatically to an increased lifespan across all corners of the world [1-4]. While this represents a crowning achievement it also presents a significant challenge to society.

Demographic analysis by both the United Nations (U.N.) and the World Health Organization (WHO) has shown that the proportion of the elderly (> 60 years) increased from 8% in 1950 to 10% in 2000. Worldwide, it is expected that the total number of elderly individuals will increase from the current 600 million to 2 billion by 2050 and this will account for nearly 22% of the total world population in 2050 [1,5-9]. In the United States, the number of the elderly will almost double from the current 43.1 million to 83.7 million by 2050 according to the U.N. Interestingly, for the first time in history, it is projected that the number of elderly individuals will surpass the number of young individuals in the 0-4 age group rising to about 714 million by 2020. As a consequence of this demographic revolution, pressure on health services will clearly escalate, thus highlighting the need to clearly develop strategies to promote healthy aging.
1.2 Aged individuals mount sub-standard immune responses

As the world population gets older, it is critical to understand the comorbidities associated with aging and to identify potential targets of intervention to improve the quality of life of the elderly. Importantly, despite the current improvements in vaccinations and health care, the elderly population remains highly susceptible to the development of tumors as well as to a vast array of infectious diseases and exhibit reduced vaccine efficacy [3,4,10-17]. The poorly responsive immune system of aged individuals underlies their vulnerability to efficiently control infections and their dysfunctional immune responses to vaccines.

In general, the elderly population is considered to be immunologically frail and that they mount poor immune responses to infections and vaccines. This loss of effector immune responses with age has a substantial impact on both the health and the survival of the elderly [2,18-20]. While elderly individuals are not immunodeficient, they often mount sub-standard immune responses [5,10]. Consequently, this leads to a concomitant increase in morbidity and mortality from infectious diseases in older adults [10,18,21-24]. One of the most profound consequences of immunosenescence is the increased susceptibility of the elderly to influenza. In particular, influenza vaccination is associated with an efficacy of 30-40% in the elderly compared with 70-90% in young adults [25-28]. Notably, epidemiological statistics have clearly suggested that the elderly account for more than 90% of the annual deaths from influenza [29,30]. In addition, the absolute number of cancer cases in individuals aged 65 years and older is expected to double between 2000 and 2030 [3], highlighting the need to develop strategies to alleviate the cancer burden in a growing elderly population.

Therefore, as a result of the increased burden of age-associated diseases, a significant proportion of the newly added years in life expectancy are spent in poor health. Thus, improving
our understanding of the molecular mechanisms implicated in age-related immune dysfunction is critical for a better management of the growing aging population.

In the elderly, distinct deficiencies have been characterized in virtually every component of the immune system [1,2]. Efficient immune responses to the multitude of infectious pathogens encountered over an individual’s lifetime are dependent on the coordinated interactions between the adaptive and innate immune responses. While aging is associated with sub-par immune responses from both the innate and adaptive arms of the immune system, the underlying mechanisms to the causes of age-related immune dysfunction remain to be thoroughly investigated. Studies in immunosenescence have for long focused on how aging alters T cell homeostasis and function including their development, generation, activation, \( T_H \) polarization and trafficking. However, defects in various aspects of the innate arm of the immune system are becoming increasingly evident and have recently aroused widespread interest.

We provide a case for why NK cells which eliminate viral infections and tumor cells and which regulate innate and adaptive immune responses need to be studied in aging. Improving NK cell responses could alleviate the susceptibility of the elderly to viral infections and cancer development. Next, we discuss the developmental map and the factors implicated for optimal NK cell development and functional maturation. We then highlight the impact of aging on NK cell maturation and function and the factors that may be responsible for these defects.

1.3 NK cells are critical in coordinating tumor immunosurveillance and the immune response against viral infections

Natural killer (NK) cells are bone marrow derived innate immune lymphocytes that sit at the cross-roads of both innate and adaptive immune responses and play a critical role in the control of microbial infections and malignant transformation, both of which occur at a high
incidence in the elderly [4,15,17,31]. The majority of NK cells are localized in the peripheral blood, lymph nodes, spleen and bone marrow (BM) and they represent a key mechanism in immunosurveillance.

NK cells were discovered more than 30 years ago as small lymphocytes of an undefined nature exerting spontaneous selective cytotoxic activity against the Moloney Leukemia cells, a phenomenon termed as natural cytotoxicity [32,33]. Much progress has been made in identifying the mechanisms that allow NK cells to discriminate target cells from healthy cells. NK cell activation is a complex and multi-factorial process that combines multiple activating stimuli, the loss of inhibitory signals and the enhancing role of cytokines such as IL-2 and IL-15. NK cells are crucial components of the innate immune system and form the first line of defense against pathogens as well as host cells that are stressed or have undergone malignant transformation [34,35]. In the early 1980s, NK cells were first shown to eliminate tumor cells from the blood of mice and rats and thereafter in the mid-1980s, they were shown to be able to have a spontaneous cytotoxic capacity against MHC-I low tumor cells which have been shown to downregulate their expression of MHC-I as a potential strategy to escape from CD8+ T cell mediated responses [32,36-39]. Longitudinal studies assessing the impact of low NK cell cytolytic activity have shown that this is directly associated with a substantial increase in cancer incidence and risk compared with individuals with a higher NK cytolytic potential, highlighting the important role of NK cells in modulating protection against cancer development [40]. Soon after their discovery, it became apparent that the cytotoxic capacity of NK cells was impaired in aging [41]. Thus, impaired NK cell functions could have profound consequences on the overall immune responses in the elderly as evidenced in young patients with NK cell functional deficiency. These
patients are highly susceptible to viral infections despite the presence of large numbers of NK cells [42].

1.4 The effector functions of NK cells

It has been shown that one of the functions of NK cells is to survey tissues for normal levels of MHC-I which are expressed ubiquitously. NK cells will eliminate cells that have downregulated their MHC-I expression (missing-self recognition), a process that usually occurs as a result of viral infections or malignant transformation (Figure 1) [43]. This function is determined by the integration of signals arising from the engagement of their activating and inhibitory receptors. In particular, NK cells use inhibitory receptors to gauge the absence of constitutively expressed self molecules on susceptible targets. Among inhibitory receptors are the killer immunoglobulin-like receptors (KIRs) in humans and their functional homologs, Ly49 in mice which will bind to MHC-Ia molecules, whereas other inhibitor molecules such as the CD94/NKG2A heterodimers and Ly49A will recognize non-classical MHC-Ib molecules such as Qa-1b and H2-M3 in C57BL/6J mice respectively.

These inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains which recruit intracellular tyrosine phosphatases such as SHP-1 that prevents the phosphorylation of molecules associated with an activation signal. Activating receptors on NK cells include NKP46, NKG2D as well as Ly49H, Ly49D and NK1.1. Binding of the activation receptors with their ligands initiates a signal through ZAP70/SYK when immunoreceptor tyrosine-based activation motifs (ITAM)- containing adaptor proteins such as DAP-12, FcRγ and CD3ζ are activated [44]. Upon encounter with target cells, the balance between the signals generated from these receptors will determine whether NK cell lysis and cytokine production will ensue. Thus, when an NK cell encounters a healthy cell expressing
high levels of MHC-I, the balance tilts in favor of inhibitory signals and the healthy cell is spared from lysis. In contrast, when a target cell becomes infected with a virus such as MCMV or undergoes malignant transformation, the loss of MHC-I either partially or totally will favor activating signals and this will outweigh any inhibitory signals. In this scenario, NK cells are poised to degranulate releasing the cytotoxic molecules, perforin and granzymes which leads to the elimination of the target cell (Figure 1) [45].

NK cell lysis occurs when the magnitude of inhibitory signals are reduced because the target cell lacks one or more self-MHC molecules or when the target cells express high levels of stimulatory ligands that override inhibition. Thus, NK cell cytotoxicity could deliver antiviral activity by lysing virus-infected cells at times prior to replicating virion assembly and virus spreading between cells [45].

**Figure 1.** An illustration of how the balance between activating and inhibitory receptors on NK cells governs their functions. NK cells integrate signals arising from the engagement of both activating and inhibitory receptors and the final outcome will determine whether NK cells lyse or spare their targets.
Self-MHC I engagement by NK cell inhibitory Ly49 receptors is a fundamental step in the acquisition of NK cell effector function. NK cells express an extensive repertoire of both activating and inhibitory receptors which regulate NK cell function upon binding to cognate host MHC-I molecules [46]. The interaction between inhibitory Ly49 receptors on immature NK cells with self MHC-I allows NK cells to become immunologically competent and ultimately go on to functional maturity, a process termed as licensing [47,48]. The licensing hypothesis suggests that NK cells are initially hyporesponsive and that engagement of MHC-I “licenses” NK cells to become functional. In C57BL/6J mice, licensed NK cells express one or more of the four receptors that bind appreciably to H-2^b class I molecules and non-classical MHC-I molecules, Ly49C, Ly49I, Ly49A and CD94/NKG2A. Ly49C and Ly49I recognize the MHC-I molecule H-2K^b and are expressed on about half of NK cells, thus conferring responsiveness to these NK cells [48,49].

NK cells expressing several self-MHC-I specific inhibitory receptors exhibit a higher responsiveness and cytotoxicity against target cells that down regulate MHC-I molecules, a condition that occurs during cellular stress, tumorigenesis and viral infections [50]. Experiments on a per cell basis have shown that when activation receptors are cross-linked, NK cells with a self-MHC-I specific Ly49 receptor are good producers of IFN-γ [51]. In contrast, NK cells that lack MHC-I specific inhibitory receptors are hyporesponsive and fail to eliminate cells that have down regulated MHC-I expression [51]. These findings support the existence of licensed and unlicensed tolerant NK cells. Licensed NK cells are functionally competent with regard to their activation receptor and are tolerant because they have an inhibitory receptor. Unlicensed NK cells do not have self-MHC-I specific receptors and are tolerant because they are not functionally competent [51]. Studies on human NK cells also support the concept that self-MHC-Ia specific
KIRs confer functional competence as KIR+ NK cells specific for self HLA display more robust cytokine production compared to KIR – NK cells.

When activating signals outweigh signals generated from inhibitory receptors, NK cells are poised to eliminate their targets, a process that can be accomplished through various mechanisms. One of the predominant mechanisms NK cells use to eliminate their targets is through the release of cytotoxic granules that are composed of perforin and serine proteases such as granzyme protein molecules. The release of cytotoxic granules is usually measured by the expression of the surrogate marker, CD107a on the surface of degranulating NK cells. CD107a is a vesicle transmembrane protein present in lytic granules which is exposed at the cell surface after degranulation. Through the creation of an immune synapse with its target cell, NK cells can release the contents of their cytotoxic granules, a process known as granule exocytosis. During this process, the cytotoxic granules are shuttled by microtubule-associated kinesin motors which results in the focused release of the granule contents at the target interface [45,52]. The action of perforin mirrors that of the membrane attack complex mediated by complement dependent lysis of target cells. This allows the movement of granzymes across the cell membranes of target cells and the triggering of caspase-dependent and independent target cell death. Mice deficient in perforin have a severely limited capacity to suppress syngeneic MHC-I low B16 melanoma tumor cells as well as in preventing the development of methylcholanthrene (MCA) induced sarcomas [53-57].

NK cells can also eliminate their targets through the induction of their apoptosis via tumor necrosis factor (TNF) family ligands. NK cells express at least three TNF family ligands, FASL, TNF and TNF-related apoptosis inducing ligand (TRAIL). These ligands selectively induce apoptosis in a variety of tumor cells and transformed cells but not most normal cells and
have therefore garnered a lot of interest in cancer immunotherapy. In particular, TRAIL induces apoptosis by interacting with its receptors such as DR4 and DR5. Ligation of TRAIL with its receptor ultimately leads to caspase activation and the induction of apoptosis [58]. NK cells activated by cytokines such as IL-2 or IL-15 express TRAIL and are capable of inducing apoptosis in tumor cells in vitro and in vivo [59,60]. Importantly, the neutralization of TRAIL promoted the development of the chemical carcinogen, MCA induced fibrosarcoma [60]. Additionally, many tumors do not express Fas, but NK cells can induce cancer cells to upregulate Fas expression and thereafter can kill them in a Fas-dependent manner [61]. Thus, death receptor mediated apoptosis of tumor cells by NK cells is an important strategy for NK cell mediated tumor clearance.

NK cells secrete several effector cytokines including IFN-γ, TNF and GM-CSF. IFN-γ which is the prototypical cytokine produced by NK cells is well known for its role in inhibiting viral replication and the stimulation of Th1 immune responses. Several studies suggest that IFN-γ contributes to anti-tumor immune responses [52]. One study in particular showed that IFN-γ production by NK cells was critical for promoting enhanced resistance against B16 melanoma lung metastasis [62]. Along these lines, IFN-γ attenuates tumor growth through the induction of RNase L and the double stranded specific protein kinase (PKR) both of which induce apoptosis [63,64]. IFN-γ also induces the expression of MHC-I as well as MHC-II protein expression on many cells and the higher expression of MHC-I on tumor cells renders them susceptible to CD8+ T cell mediated killing [65,66]. IFN-γ is also responsible for activating macrophages and DCs which in turn induces enhanced phagocytosis, phago-lysosome fusion and pathogen clearance. It also enhances the production of nitric oxide (NO) by activated macrophages which contributes to tumor killing [67]. In addition, IFN-γ induces the production of IFN-γ inducible protein-10 (IP-
10) as well as monokine induced by IFN-γ (Mig), both of which have strong anti-angiogenic properties and therefore leading to the attenuated growth and survival of tumor cells [68].

Additionally, NK cells can act as regulatory cells that can control the magnitude of both innate and adaptive immune responses. IFN-γ produced by NK cells is critical for Th1 polarization and the activation of DCs and macrophages. Moreover, NK cells can kill immature DCs to promote more immunogenic responses as opposed to tolerogenic responses [69-72]. NK cells can also kill activated T cells to tune down excessive immune responses and therefore can act as negative regulators of inflammation [73]. Importantly, the ability of NK cells to kill tumor cells and virus infected cells promotes cross-presentation of antigens from apoptotic NK cell targets by DCs. The uptake and cross-presentation of antigens derived from NK cell mediated cytotoxicity by DCs prompts the development of robust antigen-specific CD8+ T cell responses which could be used as a powerful strategy for vaccine development [74]. Thus, the central role played by NK cells in regulating the innate and adaptive arms of the immune system are critical to study in aging in which both innate and adaptive immune responses are impaired. These various mechanisms dictating NK cell effector functions form the epi-center of the NK cell mediated control of tumors and viral infections. One of the major mechanisms regulating the effector functions of NK cells is their development. Depending on their developmental stage, NK cells can be more or less efficient at executing their effector functions. We now discuss the various stages that occur in the life of an NK cell beginning from their commitment to the NK cell lineage until their terminal maturation.
1.5 The NK cell developmental map: from NK cell precursors to their terminal maturation and education

Development of NK cells requires two major events including: 1) commitment to the NK cell lineage, and 2) NK cell terminal maturation and acquisition of immunological competence. The first phase involves early NK cell development, which mainly constitutes NK cell precursor development from HSCs as well as progression to immature NK cells. The second phase constitutes terminal NK cell maturation, which is accompanied by the acquisition of functional competence through their education (Figure 2). Herein, the main focus is on the latter, but we provide a detailed overview of these two consecutive processes.

In both mice and humans, NK cell development begins in the fetal liver and then progresses in the BM after birth [75-77]. The first step in the development involves commitment to the NK cell lineage. NK cells develop from common lymphoid progenitors (CLPs) which possess precursor potential for T, B and NK cells [78,79]. These CLPs then develop into NK cell precursors (pNK) under the support from stromal cells in the BM. CLPs are directed towards the NK cell fate through several stages that are characterized by the differential expression of several surface markers. An earlier stage of NK cell development upstream of the pNK was recently identified in the BM as pre-pro NK cells. These pre-pro NK cells are characterized by high levels of Inhibitor of DNA binding 2 (Id2) and the IL-7Rα chain, CD127 and lack most NK cell markers except for low levels of NKG2D (Figure 2) [80]. Pre-pro NK cells can then differentiate into pNK which are typically identified as Lin− CD122+ NK1.1− DX5− NKp46− cells [35]. CD122 (IL-2Rβ) is important for permitting pNK cells to respond to IL-15 to promote NK cell development and maturation in the BM.
IL-15 is a pleiotropic cytokine that plays a central role in almost all aspects of NK cell biology including their survival, homeostatic proliferation, licensing, terminal maturation and is also a potent activator of their effector functions [81-84]. Notably, the generation of pNK is IL-15 independent and does not require other common γc dependent cytokines [85]. IL-15 has been shown to support NK cell survival in vivo as demonstrated by the observations that mature NK cells fail to survive when transferred into IL-15 deficient recipients [82,84,86-88]. In this context, IL-15 induces the expression of the pro-survival molecules Bcl-2 and Mcl-1 and at the same time down regulates the pro-apoptotic molecules Bim and Noxa to ensure NK cell survival and maintenance [88].

In addition to supporting NK cell survival, IL-15 plays an important role in regulating the NK cell maturation axis. For example, IL-15 is required for the induction of Ly49 receptors and thus plays an essential role in modulating NK cell licensing [84]. This progressive acquisition of NK cell Ly49 receptors is accompanied by terminal maturation, a process that primarily occurs in the bone marrow (BM) and plays an important role in governing NK cell function [47-49]. Along these lines, IL-15 also induces the expression of the key transcription factors T-bet and Eomes which as described later are critical for regulating key checkpoints in NK cell functional maturation. Monocytes and dendritic cells have also been described to provide maturation and survival signals to NK cells and to sustain their sequential differentiation towards terminal maturation. These factors depend on the intrinsic expression of T-bet in monocytes and on their ability to trans-present IL-15 through the IL-15Rα chain to NK cells. Equally important, Eric Vivier’s group showed that neutrophils also have an important role in ensuring the terminal maturation and maintenance of NK cells in both mice and humans as the absence of neutrophils in Genista mice, greatly impaired NK cell cytotoxicity and their IFN-γ production [89].
On another front, NK cells are primarily dependent upon an intact BM microenvironment for both early development and terminal maturation. Early experiments in mice treated with bone-seeking radioisotopes including Strontium-89 which results in the selective destruction of the BM cavity suggested that the BM microenvironment was the essential site for NK cell development. Germane to this discussion, NK cell interactions with BM stromal cells plays critical roles in regulating both early and late NK cell development and maturation. One of the key requirements for promoting the full development of mature NK cells from pNK is the physical interactions between pNK and stromal cells \textit{in vivo} as purified human or mouse HSCs have an impaired capacity to generate NK cells \textit{in vitro} without stromal cells even with the use of high concentrations of IL-15 [35,90]. In contrast, NK cell development \textit{in vitro} is significantly enhanced when pNK are cultured in the presence of a stromal cell line such as OP9 [90].

A series of early \textit{in vitro} experiments showed that in the absence of stromal cells, a mixture of soluble cytokines can act on progenitors to generate IL-15 responsive cells, but these cells failed to express the various Ly49 receptors unlike those developing in the presence of stromal cells [91]. These data suggested that stromal cells play an important role in NK cell development. Lymphotoxin (LT), a cytokine of the TNF family has been reported to be necessary for the development of NK cells \textit{in vivo}. The interactions between lymphotoxin, LTα1β2 expressing hematopoietic cells and LTβ receptor expressing stromal cells is required for the activation of stromal cells which in turn promote the expression of the IL-15 receptor on NK precursors rendering them IL-15 sensitive [92-94]. Therefore, signals via the LTβR on BM stromal cells by membrane LT is an important pathway for early and late NK cell development.

Additionally, interactions between Tyro3 receptors (Tyro3, Mer and Axl) on NK cells and their ligands, Gas6 and protein S on BM stroma appear critical for NK cell maturation [95].
Blocking the Gas6 and protein S pathway was shown to reduce NK cell T-bet expression [96]. In mice deficient for the tyro 3 receptors, the repertoire of inhibitory and activating NK cell receptors in the BM is altered and these NK cells are impaired in their capacity to kill target cells *in vitro*. Together, these findings suggest that the environment in which NK cells develop fine tunes their licensing and functional maturation. Understanding how different anatomical sites regulate NK cell development provides clues for investigating how aging may influence the early and terminal maturation of NK cells.

NK cell education is the main example demonstrating how NK cell function, development and recognition of missing-self targets can adapt to their developing environment. It is well appreciated that NK cells from MHC-I low mice display an impaired response to eliminating missing-self targets and to activation via receptor stimulation. However, when these MHC-I low NK cells are transferred into MHC-I sufficient hosts, they acquire an increased reactivity and reciprocally, WT NK cells become hyporesponsive when they are transferred into an MHC-I low host [49, 97]. These observations demonstrate that NK cells have the ability to “sense” their environment and adapt to changes in that environment.

Several transcription factors play critical roles in the development of NK cells. PU.1, Ets-1, E4BP4 and Id2 transcription factors are required for the commitment to the NK cell lineage [98]. The PU.1 transcription factor belongs to the Ets transcription factor family and is required upstream of the pre-pro NK cell stage for the development of pNK cells as PU.1 deficient mice show a marked reduction in the number of pNK cells (Fig 1). Ets-1 is also involved in the transition of pre-pro NK cells into pNK cells (Figure 2), as Ets-1 deficient mice have approximately a 50% decrease in pre-pro NK cells [99]. It is also required at later stages for promoting the optimal expression of NKp46, and Ly49 receptors.
As pNK cells develop further, they begin to express NK1.1 (stage 1), then NKp46 (stage 2), CD16 (stage 3), NKG2D and Ly49 receptors (stage 3 and 4), DX5 (stage 5), CD11b (stage 6) and ultimately KLRG1 and CD43, which characterize terminal maturation (stage 7) (Figure 2) [100]. At the later stages of NK cell development, T-bet, Eomes, GATA-3, Blimp-1, Aiolos, Foxo1 and IRF-2 are critical in regulating NK cell terminal maturation and functional competence (Figure 2). Gordon Scott’s group and Laurie Glimcher’s group both independently established that both T-bet and Eomes were essential for the optimal maturation and function of NK cells as these transcription factors also regulated the expression of perforin and granzyme B [101,102].

**Figure 2.** NK cell development occurs in 7 stages which are further categorized into phase 1 and phase 2 (Stage 4-7). The figure illustrates the pathway and the surface markers as well as the various key transcription factors that play critical roles in driving the progressive development of NK cells.
Additionally, Aiolos, GATA-3 and Blimp-1 are required for the final maturation of NK cells characterized by the diminution of CD27 and the increased expression of KLRG1 and CD43 maturation markers in mice deficient for these transcription factors. Mice with a deficiency in any one of these transcription factors have an overrepresentation of immature NK cells in their lymphoid organs [103-105].

Recently, Foxo1 and Foxo3 were identified as a negative intrinsic regulator of T-bet in NK cells, thus hampering NK cell maturation and cytotoxicity through the regulation of T-bet [106]. This regulation occurs through the recruitment of Foxo1 by its protein binding partner, SP1 to the T-bet proximal promoter region. Furthermore, there exists a dynamic correlation between Foxo1 and T-bet expression during the sequential maturation of NK cells. Foxo1 is highly expressed in immature NK cells whereas T-bet is expressed at low levels in this subset [106]. During maturation, Foxo1 expression begins to decrease and this is accompanied by a concomitant increase in T-bet expression. Thus, it can be reasoned that the decrease in Foxo1 expression relieves T-bet suppression which then promotes NK cell terminal maturation [106]. Given previous findings demonstrating that T-bet controls the expression of the NK cell cytotoxic molecules, perforin and granzyme B, it thus suggests that through its regulation of T-bet, Foxo1 is a critical regulator of NK cell cytotoxicity.

Foxo1 deficient and Foxo1 and 3 double deficient NK cells appeared to have a more mature phenotype across all lymphoid organs (spleen, pLNs and BM) and have an enhanced cytotoxic capacity against YAC-1 target cells. The proportions of CD11b+ and KLRG1+ terminally mature NK cells were significantly higher in Foxo1 deficient NK cells compared to WT controls and concomitantly there was an under representation of immature CD27+ NK cells. In fact the ratio of mature (CD27-) to immature (CD27+) NK cells almost doubles in Foxo1/3
double deficient NK cells [106]. Using the human NK cell line, NKL cells, it was reported that overexpression of Foxo1 resulted in almost a two-fold reduction in IFN-γ production post activation with IL-15. Conversely, the reduction of Foxo1 expression using short hairpin RNA, led to a significant increases in IFN-γ transcript levels post activation with IL-12. These data suggested that Foxo1 regulates both the homeostasis of NK cells (maturation and homing) and their functional competence (cytotoxicity and cytokine production).

Foxo1 has been known for its role in controlling T cell trafficking in particular by regulating the expression of CD62L which is critical for T cell homing to peripheral lymph nodes. Consistent with this, there is an overrepresentation of CD62L\(^{-}\) cells in Foxo1 deficient but not Foxo3 deficient NK cells and this was most apparent in immature NK cells. Importantly, this phenotype is more pronounced in Foxo1/3 double deficient NK cells. Taken together, these data suggest that Foxo1 and Foxo3 play a critical role in controlling NK cell homing through the regulation of CD62L expression which is required for NK cells to home to lymphoid organs [106].

**1.6 NK cell terminal maturation is associated with the acquisition of enhanced functional competence**

NK cell terminal maturation which is the last step in NK cell development is a 4-stage process that can be described on the basis of surface expression of the TNF superfamily member, CD27 and the integrin, CD11b (Figure 2). NK cell maturation starts at the double negative (DN) stage (CD27\(^{\text{low}}\) CD11b\(^{\text{low}}\)) which progressively differentiate into, immature (CD27\(^{\text{high}}\) CD11b\(^{\text{low}}\)), then transitional (CD27\(^{\text{high}}\) CD11b\(^{\text{high}}\)) and finally into mature NK cells (CD27\(^{\text{low}}\) CD11b\(^{\text{high}}\)) which display the full repertoire of Ly49 receptors and have the highest cytotoxic potential.
The acquisition of a mature phenotype enables NK cells to be fully functionally competent with higher cytotoxic and cytokine production capabilities.

Pan genomic analysis as well as the assessment of key proteins such as perforin, granzyme B, FasL, NKp46 and T-bet that are directly involved in NK cell cytotoxicity showed that mature NK cells have a higher expression of genes and protein molecules associated with NK cell mediated cytotoxicity [90,102,107,109-111]. The mature subset of NK cells also had a higher expression of genes associated with the secretory pathways such as Rab proteins which may be involved in enhancing the exocytosis of cytotoxic granules [107]. Therefore, an overrepresentation of immature NK cells reduces the overall function of NK cells at the population level [107,109,110].

1.7 NK cell terminal maturation in the BM is accompanied by their egress from the BM to the periphery

Many chemokine receptors including CCR2, CCR5, CXCR3 and CX3CR1 have been implicated in NK cell recruitment during inflammatory conditions. However, no gross abnormality in homeostatic NK cell distribution among lymphoid organs has been described in mice lacking chemokine receptors such as CXCR3, CXCR6, CCR1, CCR5 or CX3CR1. These data suggest that the aforementioned chemokine receptors do not play a critical role in NK cell trafficking under steady-state conditions. In fact, the capacity of NK cells to exit the BM parenchyma and populate the peripheral lymphoid organs is dependent on the equilibrium between CXCR4 and S1P₅ expression on NK cells [112,113] (Figure 3).

CXCL12 which is the ligand for CXCR4 is produced constitutively in the BM and functions to retain immature NK cells within the BM parenchyma. CXCL12 is highly produced by CXCL-12 abundant reticular BM stromal cells (CAR cells) compared to other non-
hematopoietic cells and the close interaction of NK cells with CXCL-12 abundant reticular BM stromal cells (CAR cells), has been shown to be critical for promoting NK cell maturation [112-114]. In contrast to CXCL-12, the lipid chemoattractant, S1P which is the ligand for S1P₅, is expressed at higher levels in the blood and lymph compared to levels within the BM parenchyma. CXCR4 is highly expressed on precursor NK cells (pNK) and immature cells [112]. However, progressive maturation is accompanied by a down regulated CXCR4 expression and an upregulation of S1P₅ [115,116] (Figure 3). This change in receptor expression relieves mature NK cells from BM retention and allows them to move to the blood through the sinusoids via the S1P-S1P₅ axis. Corroborating these findings, two other studies established that NK cells from S1P₅ deficient or mice carrying a point mutation in T-bet (Duane mutants) accumulate in the BM and have a reduced capacity to enter the blood via egress through the BM sinusoids [116,117]. Together, these studies show that S1P₅ functions to facilitate the egress of NK cells from the BM to the blood and spleen in response to S1P gradients. In sum, the early development of NK cells is associated with a high expression of CXCR4 and reduced levels of S1P₅. As NK cell progressively differentiate into more mature cells, terminal maturation is accompanied by increased levels of S1P₅ and reduced CXCR4 which allows mature NK cells to exit the BM parenchyma into the sinusoids from which these cells can home and seed the peripheral lymphoid organs (Figure 3).
1.8 NK cell terminal maturation can occur in the BM and in extra medullary peripheral sites

Although the BM serves as the primary site for NK cell development and terminal maturation, emerging evidence suggests that peripheral lymphoid organs are also capable of supporting NK cell development and terminal maturation. In this section, we discuss the data that suggests that in addition to the BM, peripheral lymphoid organs including the spleen and pLN s could support both the early development and terminal maturation of NK cells.

The adoptive transfer of purified splenic DN NK cells, immature or transitional NK cells by intravenous (i.v) injection into non-irradiated C57BL/6 mice showed that each of these subsets progressively develop into the terminally mature subset when analyzed in the spleens of
recipient mice [107]. Importantly, this provided the first line of evidence that suggested that NK cell terminal maturation could occur in peripheral lymphoid organs such as the spleen.

Along these lines, NK cell egress from the BM has in fact been shown to be dependent on their stage of terminal maturation [112,113,115-119]. In general, lymphocytes exit central and peripheral lymphoid organs through sinusoids that connect either to the blood circulation or to the efferent lymph vesicles. In discriminating the parenchymal and sinusoidal localization of BM NK cells using the anti-CD45, it was reported that sinusoidal NK cells are highly enriched for terminally mature NK cells [113]. Interestingly, a proportion of immature NK cells were capable of exiting the BM parenchyma into the BM sinusoids. These data provided another piece of evidence suggesting that immature NK cells can complete their terminal maturation program in the periphery after exiting the BM parenchyma [113].

More evidence has also emerged suggesting that pNK cells can migrate from the BM to peripheral lymphoid organs where they can undergo further maturation. In fact, in humans, Freud and colleagues identified a BM-derived CD34+ hematopoietic precursor that was present in lymph nodes and that can further differentiate into NK cells when co-cultured with activated lymph node T cells. Furthermore, based on the localization of differential NK cell developmental subsets, it has also been shown that early NK cell progenitors may migrate from the BM to the peripheral lymphoid organs after which they can progressively mature further [120].

Specifically testing where NK cells exclusively undergo their maturation may be technically challenging as a result of interference from NK cell trafficking between the periphery and the BM. However, recent data provides evidence against such a possibility as the long term tracking of transferred NK cells appeared to home to the site of their origin. Thus, transferred splenic NK cells migrated mostly to the spleen while BM NK cells migrated mostly to the BM
This preferred homing capacity may reflect the different chemokine receptors expressed on splenic and BM NK cells. Taken together, these data provide evidence that 1) peripheral lymphoid organs can support NK cell maturation and 2) transferred NK cells undergo maturation preferentially in the tissue of their origin rather than exclusively in the BM (Figure 3).

**1.9 NK cell metabolism is regulated by their maturation and activation**

As in every immune cell, homeostatic proliferation, survival and their progressive differentiation are regulated by metabolic activity which provides the biological fuel to support the generation of new daughter cells. The immune system encompasses a heterogeneous population of cells that for the most part are relatively quiescent at steady state, but are capable of rapidly responding to infection, inflammation and other perturbations. For cells to proliferate and differentiate, their metabolic profile must change from a catabolic metabolism to an anabolic metabolism because unlike in resting cells, nutrients will no longer be utilized for maintenance and homeostasis, but rather will be incorporated into biomass for new daughter cells. Thus, the link between metabolic regulation and the activation of cells in the immune system has received considerable attention particularly in developing strategies that target metabolism for immunotherapy [122]. Recently, there has been a growing interest to understand the metabolic demands of NK cells and how this regulates NK cell function and development.

ATP, which is the metabolic fuel required to drive these functional processes, can be generated from two primary processes, namely, aerobic glycolysis and mitochondrial oxidative phosphorylation. In addition to glucose, other metabolic substrates such as fatty acids, amino acid and glutamine can fuel mitochondrial oxidative phosphorylation through fatty acid oxidation and glutaminolysis respectively.
Most of the free energy released from glycolysis and the TCA cycle are channeled in the form of the reduced coenzymes, NADH and FADH$_2$. As the electrons from these coenzymes flow down the electron transport chain in the mitochondria, protons (H$^+$) are pumped from the matrix into the intermembrane space forming an electrochemical proton gradient across the mitochondrial inner membrane. As the protons flow back into the mitochondrial matrix through the ATP synthase, the energy from this electrochemical gradient drives the phosphorylation of ADP into ATP. Finally, at the end of the electron transport chain the electrons are then transferred to oxygen during oxidative phosphorylation to form H$_2$O.

Thierry Walzer’s group evaluated how IL-15 could impose both homeostatic and inflammatory effects on NK cells [123]. They began their analysis by assessing the expression of key transporters necessary for the nutrient uptake including glucose uptake in the different NK cell maturation subsets. NK cell maturation is accompanied by a decrease in the transferrin receptor (CD71) as well as the heavy chain amino acid system L transporter (CD98). In addition using the fluorescent glucose, 2-NBDG as a surrogate for glucose uptake, it was observed that as NK cells matured they became less competent at the uptake of glucose in vitro. These findings were consistent with microarray analysis depicting that mature NK cells expressed gene signatures that are characteristic of quiescent cells including autophagy as well as aerobic sugar and fatty acid catabolic pathways but had a lower expression of genes associated with cell cycle progression and ribosomal biosynthesis. Under steady state conditions as well as short term in vitro activation, NK cells primarily depend on oxidative phosphorylation as assessed using the extracellular flux assay.

Interestingly, NK cell oxidative phosphorylation is mainly powered by glucose as the inhibition of oxidative phosphorylation using oligomycin or glycolysis using 2-deoxyglucose (2-
DG) abrogated ATP levels in activated NK cells and almost completely impaired their IFN-γ production capacity [124]. In contrast inhibition of fatty acid oxidation and glutaminolysis had a minimal effect compared to the inhibition of glycolysis on oxidative phosphorylation. These data thus, suggested that NK cell bioenergetics is mainly driven by glucose through oxidative phosphorylation.

One of the central molecules that integrates various metabolic, inflammatory or stress cues is the evolutionarily conserved serine/threonine mechanistic target of rapamycin (mTOR). The mTOR kinase exists in two functionally distinct complexes: mTORC1 and mTORC2 [125-127]. mTORC1 is known to respond to amino acids, stress, oxygen and growth factors and is acutely sensitive to rapamycin. It functions to promote cell growth by inducing anabolic metabolism and by driving cell cycle progression. mTORC1 is best known for its ability to control protein synthesis mainly by directly phosphorylating the translational regulators, 4EBP1 and S6K, which in turn promote protein synthesis [125-127].

The link between the activation and development of NK cells and their metabolic regulation has received considerable attention recently. Interestingly, mTOR activity as assessed by the phosphorylation of its targets, 4EBP1 and S6K decreased with progressive maturation in splenic and BM NK cells, suggesting decreased metabolic demands with maturation, an observation consistent with the quiescent nature of mature NK cells. Importantly, the conditional deletion of mTOR in NK cells achieved by crossing mice with loxP-flanked mTOR to Ncr1- cre mice considerably impaired NK cell maturation across lymphoid organs and led to a paucity of peripheral NK cells [123]. Deletion of mTOR in NK cells also abrogated their acquisition of Ly49 receptors and the key transcription factors required for promoting NK cell maturation, T-bet and Eomes. The absence of mTOR also impaired NK cell glucose uptake and the expression
of the transferrin receptor as well as CD122 and CD132 expression, suggesting that mTOR is required for the development of NK cells and potentially for their optimal metabolic activity, although further studies will need to be conducted to explicitly demonstrate that mTOR is important for efficient oxidative phosphorylation and glycolysis in NK cells.

The next section focuses on how the terminal maturation and function of NK cells are compromised in aging and the potential factors that could be contributing to these defects. The ability to reverse the effects of aging on NK cell function could be significantly beneficial in increasing the capacity of the elderly to deter viral infections and tumors [128-130]. Studies on NK cell immunosenescence have focused on describing their impaired development, cytokine production and cytotoxic functions. However, the underlying mechanisms contributing to the impaired homeostasis and function of NK cells in aging remain largely unknown. Herein, we provide an overview of the work that has been done on the alterations in murine NK cell function and maturation in aging. Of note, we discuss how our understanding of these features could provide insight into the development of novel therapies to boost NK cell cytotoxicity in the elderly. We begin by providing an overview of the functions and the development of NK cells.

1.10 The number of mature NK cells is significantly reduced in the periphery and in the BM of aged mice

In B6 mice, it has been reported that the proportion and numbers of NK cells are reduced significantly in the blood, spleen and lungs of aged mice, whereas no differences were observed in the lymph nodes or liver [131-135]. This reduced peripheral NK cell frequencies and numbers have also been reported in other aged mouse strains including Balb/c and DBA/2 strains [136-138]. The terminal maturation of NK cells is also significantly impaired in aging with an almost two-fold increase in the proportion and number of immature NK cells (CD27high CD11blow) in
the peripheral lymphoid organs (spleen, peripheral lymph nodes and BM). Observed in all lymphoid organs in addition to the blood, the proportion of mature NK cells was decreased almost 3 fold in these organs and were almost virtually absent in the BM of aged mice [131,135,139]. Our group and others have extensively analyzed the functional maturation of aged NK cells in the periphery and BM (see chapter 2) and taken together, the results collectively support the impaired maturation of NK cells in aging.

Assessment of NK cell maturation based on other classical phenotypic markers such as CD43 and KLRG1 also revealed similar results, suggesting that aging imposes constraints on the terminal step of maturation of NK cells. Given these findings, it is highly likely that the loss of mature NK cells which constitute a larger proportion of peripheral NK cells may also explain the paucity of total NK cells in the periphery.

Although aged mice have a considerable reduction of NK cells in the periphery, intriguingly the BM has a larger proportion and number compared to young mice [131,132,135,139]. In fact, most of the BM NK cells in aged mice (70-80%) were immature compared to young mice (40-50%) [131,132,135,139].

**1.11 NK cells in aged mice and humans are less efficient at eliminating target cells *in vivo* and *in vitro***

Previous reports have shown that aged NK cells in both mice and humans exhibit impaired cytotoxicity post activation with cytokines such as IL-2, IL-12 and IL-18 or post *in vivo* activation with poly(I:C) [24,133-135,140-147]. In the context of influenza infection as well as *in vitro* cytokine stimulation, Elizabeth Gardner's group and others have shown that aged NK cells from mice infected with influenza have a four-fold reduced capacity to lyse YAC-1 target cells *in vitro* as assessed by the chromium assay [133,134]. In the same context, whole lung NK
cells from aged mice also had an attenuated degranulation (CD107a) and IFN-γ production [133]. Furthermore, peripheral splenic NK cells had a reduced capacity to eliminate missing-self, allogeneic and B16 tumor targets \textit{in vivo} [135,148]. However, these studies did not investigate the effector functions of the individual NK cell maturation subsets and whether changes in maturation of aged NK cells directly impaired their cytotoxic functions.

\subsection*{1.12 NK cells in aged mice have an attenuated homing capacity in response to viral infections.}

NK cells have also been shown to play a crucial role in mediating the resistance to Ectromelia virus infection (ECTV), the causative agent of mouse pox and which provides a model for the study of human small pox [109]. NK cells function to control the replication and spread of ECTV particularly in the draining lymph nodes (DLNs). A lower proportion of NK cells expressed granzyme B in the draining lymph nodes and there was less NK cell accrual of terminally mature NK cells in the draining lymph nodes of infected aged mice [131]. As a consequence of this failed capacity of mature NK cells to accumulate in the DLNs, ECTV spreads more rapidly to other lymphoid organs including the spleen and liver, ultimately leading to a higher mortality in challenged aged mice. Importantly, the adoptive transfer of aged or young NK cells differentially labeled with different CFSE intensities into infected young hosts suggested that terminally mature NK cells from aged mice have a defective capacity to migrate to DLNs during ECTV infection [131].

In this context, however; the authors did not account for the fact that the transferred aged NK cells initially are lower in their proportion of mature cells. To show that aged mature NK cells have defects in their homing capacity to the DLNs, a purified population of these cells and not whole NK cells could provide more insight. Analysis of chemokine receptors including
CCR2 and CCR7 as well as additional analyses through microarray did not reveal any
differences in expression of LN homing receptors in blood mature NK cells, although CXCR3, a
marker of NK cell immaturity appeared to be increased in aged mice [131,139]. Overall, the
authors concluded that the impaired migratory capacity of mature NK cells in aged mice could
be attributed to their decreased expression of the LN homing receptor, CD62L.

In the context of influenza infection, aged mice had a significantly reduced proportion
and number of lung NK cells compared to young mice after infection and lung NK cell numbers
remained consistently lower in aged mice as far as 4 days post infection [133,134]. While it is
not clear whether impaired homing was responsible for the lack of increased NK cell infiltration
in the lungs of infected aged mice, the reduced numbers of NK cells correlated positively with
increased influenza virus titers [134]. In contrast to the impaired increase of NK cells at sites of
virus infection, NK cells appeared to infiltrate the lungs similarly in young and aged mice in the
context of B16 melanoma challenge. The differences observed here suggest that the context in
which NK cells are primed may determine their homing capacity. Additionally, it may also
reflect on the defective capacity of other innate immune cells such as macrophages or dendritic
cells to release chemokines necessary for promoting NK cell recruitment in aging as shown
previously [149-151].

1.13 Aging has a characteristic chronic inflammatory environment (inflammaging).

Aging is associated with a chronic, low-grade systemic inflammation, a phenomenon
known as inflammaging. It is characterized by raised levels of pro-inflammatory cytokines such
as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF); all of which have
been shown to rise with age and are involved in the pathogenesis of most age-associated diseases
[152-155]. C-reactive protein (CRP), an acute phase protein produced by the liver in response to
IL-6, is also a useful marker of inflammaging, more commonly used in clinical practice. The underlying mechanisms responsible for the increased levels of pro-inflammatory cytokines in aging remain poorly understood. The presence of senescent cells or the inefficient clearance of dying cells by phagocytes which have an impaired phagocytic capacity in aging may contribute to inflammaging [156,157]. The lack of clearance of apoptotic and necrotic cellular debris in aging may trigger the production of pro-inflammatory cytokines initiated by nucleic acid stimulation of TLRs, causing the release of cytokines such as IL-6, TNF-α and IL-1. Additionally, the large presence of apoptotic cells also contributes to the enhanced production of the anti-inflammatory cytokine, TGF-β which is known to limit NK cell maturation and function. Thus, determining the factors promoting the increased release of these cytokines is an area of increasing interest in aging.

All in all, the goal of this dissertation is to provide insight into the mechanisms through which aging impairs the function and maturation of NK cells that play an important role in the eradication of viral infections and tumors. We reason that a better insight into the mechanisms of NK cell dysfunction that occur with age will help to delay or even reverse the detrimental effects of immunosenescence and thereby ensure a better protection of the vulnerable elderly population from disease and death imposed by viral infections and cancer.
Chapter 2: The aged nonhematopoietic environment impairs natural killer cell maturation and function

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Running title: Aging and NK cell maturation/function.

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Summary

Natural killer (NK) cells are critical in eliminating tumors and viral infections, both of which occur at a high incidence in the elderly. Previous studies showed that aged NK cells are less cytotoxic and exhibit impaired maturation compared to young NK cells. We evaluated whether extrinsic or intrinsic factors were responsible for the impaired maturation and function of NK cells in aging and whether impaired maturation correlated with functional hyporesponsiveness. We confirmed that aged mice have a significant decrease in the frequency of mature NK cells in all lymphoid organs. Impaired NK cell maturation in aged mice correlated with a reduced capacity to eliminate allogeneic and B16 tumor targets \textit{in vivo}. This could be explained by impaired degranulation, particularly by mature NK cells of aged mice. Consistent with impaired aged NK cell maturation, expression of T-bet and Eomes which regulate NK cell functional maturation were significantly decreased in aged bone marrow (BM) NK cells. Mixed BM chimeras revealed that the non-hematopoietic environment was a key determinant of NK cell maturation and T-bet and Eomes expression. In mixed BM chimeras, NK cells derived from both young or aged BM cells adopted an “aged” phenotype in an aged host i.e. were hyporesponsive to stimuli \textit{in vitro}, while adopting a “young” phenotype following transfer in young hosts. Overall, our data suggest that the aged non-hematopoietic environment is responsible for the impaired maturation and function of NK cells. Defining these non-hematopoietic factors could have important implications for improving NK cell function in the elderly.
Introduction

It is well established that aging attenuates the host’s ability to mount robust immune responses [2]. Immunosenescence is defined as age-dependent alterations that lead to an impaired function in almost every component of the immune system [158-160]. A hallmark of immunosenescence is the increased susceptibility of the elderly population to microbial infections [5,14]. In addition, the elderly are highly susceptible to the development of tumors with the incidence of cancer being particularly high in individuals aged > 60 years [4,17]. Thus, studies geared at providing novel insight into the molecular mechanisms leading to age related immune dysfunction are critically required to address the need for novel therapeutic interventions for a growing elderly population.

Whereas the function and homeostasis of aged T cells has been extensively investigated, it is becoming increasingly evident that the innate arm of the immune system is also affected in aging. Of particular importance, Natural killer (NK) cells play a critical role in coordinating tumor immunosurveillance and the immune response to viral infections [34].

The function of NK cells is determined by the integration of signals arising from the engagement of their activating and inhibitory receptors [35]. Such responses are mediated through two major effector NK cell functions, the direct cytolysis of target cells and the production of cytokines and chemokines. NK cell function is also dictated by their stage of maturation, a process that primarily occurs in the bone marrow (BM) [161,162]. During terminal maturation, NK cells become more efficient at eliminating target cells [107,109,110] and undergo a process of education (licensing) while acquiring the expression of different Ly49 receptors [100]. It has been previously reported that NK cell maturation is a 4-stage process that can be described on the basis of surface expression of the TNF superfamily member,
CD27 and the integrin, CD11b [107]. NK cell maturation starts at the double negative (DN) stage (CD27\textsuperscript{low} CD11b\textsuperscript{low}), which progressively differentiates into, immature (CD27\textsuperscript{high} CD11b\textsuperscript{low}), then transitional (CD27\textsuperscript{high} CD11b\textsuperscript{high}) and finally into mature NK cells (CD27\textsuperscript{low} CD11b\textsuperscript{high}), which display a full repertoire of Ly49Rs and the highest cytotoxic potential [100,107,108].

Previous studies showed that aged human and murine natural killer cells are less cytotoxic \textit{in vitro} and contain reduced proportions of mature NK cells [131-134,141,147,148,163]. In addition, it has been reported that the mature subset of aged NK cells have an impaired capacity to home to draining lymph nodes in the context of Ectromelia virus (ECTV) infection [131]. However, whether defective NK cell maturation in aging influences their functional competence remains poorly understood. Here, we sought to provide insight into: 1) whether extrinsic or intrinsic factors are responsible for the impaired maturation and function of NK cells in aging and 2) whether there is a correlation between the impaired maturation of NK cells and their reduced functional competence in aging. Our novel findings show that the aged non-hematopoietic environment is responsible for the impaired maturation and function of NK cells.
Results

NK cells in aged mice are less efficient at eliminating allogeneic and tumor targets in vivo.

The functional competence of aged NK cells was first determined in vivo by testing whether they could eliminate allogeneic (Balb/c splenocytes) targets. Differential CFSE labeling allowed the tracking of allogeneic (Balb/c) versus syngeneic (BL/6) targets in recipient animals. Relative allogeneic cytotoxicity (normalized to NK cell depleted recipients) was significantly decreased in aged animals (Fig. 1A). As expected, syngeneic (BL/6) targets were not eliminated by NK cells in recipient mice (data not shown).

To assess whether this functional impairment also extended to a reduced recognition of tumor cells in aged mice, we next assessed the ability of aged mice to eliminate B16 melanoma cells in vivo. B16 melanoma cells, which down regulate the expression of MHC-I [43], have been shown to be rapidly eliminated from the lung by NK cells as early as one hour post i.v challenge, thus presenting a reliable model to test the functional capacity of aged NK cells [164]. Accordingly, depletion of NK cells impaired the elimination of B16 melanoma cells in the lungs of challenged young and aged mice, as determined by both quantitative RT-PCR expression of OVA (Fig. 1B) and flow cytometric enumeration of CFSE+ cells (Fig. 1C). Importantly, 1hr post transfer, aged mice had a lung tumor burden comparable to that of anti-asialo GM1-treated mice and significantly higher than untreated young mice. Of note, there was a trend towards an increase in the number of NK cells in the lungs of challenged mice and this number was similar in both young and aged mice, which suggests NK cell homing to the lung in response to B16 melanoma challenge is not affected in aged mice (Fig. 1D).
Aged NK cells exhibit maturation defects and have a pronounced impairment in degranulation but not IFN-γ production after poly (I: C) priming or IL-2 activation.

Consistent with previous findings, the proportion of mature NK cells (defined as CD3-NKp46+ CD27^{low} CD11b^{hi}) was decreased in all lymphoid organs of aged mice (spleen, BM, LNs, lungs, liver and blood) (Fig. S1A and B and data not shown) [131-134,148,163]. We also confirmed these results using the expression of both CD43 and KLRG1, markers that are commonly used to identify terminally mature NK cells (Fig. S2). The frequency of immature stage D (CD11b^- CD43^-) was augmented while KLRG1+ NK cells were decreased in the spleen of aged mice (52.1%±1.7% in young Vs. 27.4%±6.0% in aged, p= 0.007) and BM (14.5%±0.9% in young Vs. 5.1%± 0.6% in aged, p= 0.0002).

It is well known that the mature NK cell subset has a higher expression of genes involved in NK cell effector functions, and a higher overall functionality [107,109,110]. Thus, in addition to defects at a population level (i.e. mature vs immature), it was also possible that aged NK cells are impaired in their cytotoxic capacity. To test this, we primed NK cell activity using poly(I:C) in vivo and evaluated the potential of aged NK cells to degranulate and produce IFN-γ in response to various NK cell stimuli in vitro. Aged NK cells exhibited a comparable capacity to produce IFN-γ as young NK cells (Fig. 2A). In contrast, a significant decrease in surface expression of the degranulation marker CD107a (LAMP-1), but not in its intra-cellular levels (data not shown) suggests that aged NK cells have an impaired capacity to degranulate following their in vivo priming (Fig. 2B). Similar findings were observed when NK cells were activated in vitro by IL-2 (data not shown). Importantly, this defect was particularly pronounced in the transitional and mature, but not immature subsets of NK cells that expressed very low levels of CD107a as expected (Fig. 2C and data not shown) [107,110]. Therefore, both the reduced proportion of
mature NK cells and functional defects in mature NK cells may contribute to the impaired in vivo cytotoxicity of NK cells in aging.

**The aged non-hematopoietic environment limits NK cell maturation**

To determine the underlying mechanism(s) leading to the reduced maturation of aged NK cells, we asked whether NK cell intrinsic or extrinsic defects were responsible. We constructed mixed BM chimeras in which T-cell depleted BM cells from aged (CD45.2+) and young (CD45.1+) donors were mixed at a 1:1 ratio and adoptively transferred into young (CD45.1+) or aged (CD45.2+) recipients (Fig. S3A) and compared the development of NK cells from both sources in the same environment. Intriguingly, despite aged and young NK cells being provided in the BM inoculum at a 1:1 ratio (Fig. S3B), NK cell chimerism in the BM and periphery were not proportionally established in both environments at week 2 and 6 post chimerism as assessed by the absolute number (Fig 3D and E). At week 2, there was a dominance of young NK cells in the periphery of young recipients (Fig. 3D). In contrast, in aged recipients, chimerism was established in favor of NK cells from an aged origin (Fig 3D). Surprisingly, at week 6, aged NK cells were dominant in both young and aged recipients (Fig. 3E). However, this dominance in chimerism was also observed for the whole population of cells derived form an aged origin (CD45.2+), suggesting that the dominance in chimerism was not NK cell specific (Fig. S3C and D).

The maturation status of NK cells from both origins was evaluated 2 and 6 weeks post chimerism in both a young and aged environment based on their respective congenic markers. NK cells from an aged origin had an augmented maturation that was similar to that of young donor NK cells (baseline) when they developed in a young environment at 2 weeks post chimerism in the spleen and BM (Fig. 3B and C and data not shown). In contrast, NK cells from
a young origin developing in an aged environment acquired a maturation phenotype reminiscent of that of aged donor NK cells (baseline) in both the spleen and BM (Fig. 3B and C and data not shown) 2 weeks post chimerism. While young and aged NK cells developing in an aged environment had an impaired maturation compared to those developing in a young environment at 6 weeks post chimerism, these differences were more subtle compared to those observed at 2 weeks. Interestingly, the presence of young BM cells in the BM inoculum did not augment aged NK cell maturation in sub-lethally irradiated aged recipients, and nor did the presence of aged BM cells impair the maturation of young NK cells in sub-lethally irradiated young recipients. These novel and important findings suggest that the non-hematopoietic environment in aged recipients plays a role in contributing to the impaired maturation of NK cells.

**Eomes and T-bet are significantly reduced in aged BM NK cells and are regulated by the environment in which NK cells develop**

Previous studies have shown that the transcription factors, T-bet, Eomes, GATA-3 and Blimp-1 play a critical role in directing and modulating NK cell functional maturation [101-104]. To determine if the defects in NK cell maturation and function were associated with a reduction in expression of these key transcription factors, we evaluated their intracellular expression by flow cytometry. Expression of the transcription factors, Eomes and T-bet (Fig. 4A and C) but not GATA-3 or Blimp-1 (Supplementary Table I) were significantly decreased in aged BM NK cells and their subsets. Importantly, in our mixed BM chimera studies, the development of aged NK cells in a young environment restored both Eomes and T-bet levels (Fig. 4B and D). Thus, NK cells from an aged origin maturing in a young environment, had an expression of both T-bet and Eomes, comparable to those of NK cells from a young origin, but significantly higher than that in
aged NK cells maturing in an aged environment. In contrast, development of young NK cells in an aged environment led to the down regulation of both Eomes and T-bet (Fig. 4B and D).

T-bet and Eomes have been shown to control NK cell maturation and NK cell cytotoxicity by regulating the expression of perforin and granzyme B (GZB) [101]. Consistent with decreased T-bet and Eomes, we found that the proportion of GZB+ NK cells was significantly reduced in the spleen of aged mice (28%± 7% vs. 60%± 12% in young mice, p=0.004).

One of the major factors implicated in regulating T-bet and Eomes expression and NK cell maturation is IL-15 as well as the interaction between NK cells and stromal cells [95,101,114]. Similar levels of IL-15mRNA were found in whole BM cells (4.3±0.4 in young Vs. 4.07±0.4 in aged, p=0.6) and splenocytes (5.49± 0.78 in young Vs. 8.24±1.72 in aged, p=0.22) of aged and young mice. Although the MFI of CD122 was significantly, but modestly, decreased in aged NK cells (mean 22% decrease in aged NK cells, p=0.001), the in vitro response to IL-15 was similar for aged and young NK cells (Fig. S4A) Additionally, the surface expression (MFI) of the IL-15Rα which is essential for the transpresentation of IL-15 to NK cells was similar between aged and young mice when assessed by flow cytometry in whole splenocytes, BM cells as well as splenic macrophages (F480+ CD11b+), dendritic cells (DCs= Lin- CD11c+ MHC-II+) and the different DC subsets (CD8α-CD11b-, CD8α+ CD11b- and CD8α- CD11b+) (data not shown). Importantly, administering a large quantity of IL-15/IL-15Rα complex to aged mice did not augment NK cell maturation in the BM (Fig. S4B), although the IL-15/IL-15Rα complex promoted the expansion of aged NK cells similar to that of young NK cells (data not shown). Together, the data suggest that deficiencies in IL-15 production or the
impaired capacity for IL-15 transpresentation or IL-15 signaling in aging are unlikely to be the mechanisms underlying the impaired maturation of NK cells in aging.

**Aged NK cells developing in a young environment have an enhanced functional capacity comparable to young NK cells.**

It had never been shown whether the non-hematopoietic environment in which NK cells mature dictates their functionality. Thus, we assessed the capacity of aged and young splenic NK cells developing in both aged and young hosts to degranulate and produce IFN-γ in response to robust PMA/Ionomycin and YAC-1 stimulation. Interestingly, aged NK cells developing in young hosts had improved their capacity to degranulate and produce IFN-γ, reaching levels similar to that of young NK cells developing in the same environment (Fig. 5A and B). Furthermore, this enhanced functional capacity was particularly apparent in the mature NK cell subset from an aged origin (data not shown). The aged environment had the reverse effect, in that NK cells from a young origin developing in an aged environment had an attenuated capacity to degranulate and produce IFN-γ similar to their aged counterparts, but substantially less than those developing in a young environment (Fig. 5A and B).
Discussion

Aging influences many aspects of both innate and adaptive immunity. Here, our studies provide a novel understanding of how aging influences NK cell maturation and function by revealing that the aged environment and, in particular, the non-hematopoietic compartment plays a critical role in impairing the maturation and function of NK cells in aging. This offers new insight that may provide leads on therapeutic interventions to reverse the NK cell phenotype in aging.

As demonstrated here and elsewhere [131-134], aging significantly impairs the maturation and the cytotoxic capacity of human and murine NK cells in vitro. We extended our studies to show that the cytotoxicity of murine NK cells is impaired in vivo against allogeneic and B16 melanoma tumor cells. Most studies using this later model evaluate lung tumor burden (nodules) at least 2 weeks post challenge in young mice, but this model is not useful to assess NK cell cytotoxicity in aged mice because in these mice the number of developing metastatic lung colonies is much lower than in young mice, a phenomenon attributed to impaired vascularity and tumor angiogenesis [165]. We therefore, used the model described by Grundy and colleagues in which B16 tumor cells are rapidly destroyed by NK cells in the lung (as early as 50 min post injection) with NK cell numbers peaking 20 min post challenge [164]. In the context of Ectromelia virus infection (ECTV), aged NK cells have an impaired capacity to migrate to draining lymph nodes [131]. It was thus possible that a similar phenomenon could be contributing to the reduced elimination of B16 tumor cells in the lung of aged mice. However, we found similar NK cell absolute numbers in the lung tissue of young and aged mice post B16 challenge, suggesting that the reduced elimination of B16 tumor cells in aged mice was not due to defects in NK cell homing to the lung, but suggested a functional deficit of NK cells in aging.
Consistent with the fact that the mature NK cell subset displays the full repertoire of Ly49 receptors and has the highest cytotoxic potential, the reduced frequency of this subset in the peripheral lymphoid organs of aged mice is a major contributor to the overall reduced effector functions of aged NK cells in vitro and in vivo.

At a subset level, the impaired capacity of aged NK cells to degranulate following in vivo priming with poly (I:C) had never been shown. We observed impaired degranulation mostly in the mature NK cell subset of aged mice, highlighting that not only does the reduced frequency of the mature NK cell subset contribute to the overall reduction in aged NK cell functionality, but that mature NK cells in aging are also hyporesponsive. Accordingly, reduced cytotoxicity against multiple targets and reduced GZB expression was found in aged NK cells. Mechanistically, we found that the reduced protein expression of the transcription factors, T-bet and Eomes which have been shown to regulate perforin and GZB may play an important role in reducing the cytotoxic potential of aged NK cells. Indeed, a reduced proportion of aged NK cells are GZB+ and T-bet−/− mice are highly susceptible to B16 melanoma [166]. Thus determining the factors influencing T-bet and Eomes expression may provide promising therapeutic interventions to augment the function of NK cells in aging.

IL-15 and the interaction between NK cells and stromal cells are key factors regulating T-bet and Eomes expression in NK cells [95,101,114]. IL-15 is the essential NK cell fuel that promotes both the survival and functional maturation of NK cells when it is transpresented by DCs, macrophages and stromal cells [82,87,88]. In mice lacking IL-15 or its receptors, the numbers of NK cell progenitors are relatively normal, but the maturation and cytotoxic capacity of peripheral NK cells are severely reduced compared with control animals [85]. Furthermore, the interaction of NK cells with CXCL-12 abundant reticular BM stromal cells
(CAR cells), which express high levels of IL-15, has also been shown to play an important role in promoting the maturation and function of NK cells [112,115]. Thus, defining whether a reduced expression of IL-15 was evident in aging was imperative. If IL-15 was the limiting factor in aging, then administering IL-15 would compensate for this deficiency and augment NK cell maturation in aging. However, a lack of IL-15 does not appear to be responsible for the impaired maturation of NK cells in aging as administering the IL-15/IL-15Rα complex in vivo did not augment BM NK cell maturation in aged mice. These data are in agreement with the fact that the number of IL-15+ as well as IL-15 levels increased in splenic stromal cells from middle-aged mice (10 month old) compared to young mice [167]. Furthermore, we show that IL-15 signaling or the impaired capacity for optimal IL-15 transpresentation through the IL-15Rα do not appear to be impaired in aging.

Interestingly, our findings highlight that the environment in which NK cells mature governs the expression profile of both T-bet and Eomes. Notably, the presence of young BM cells in the mixed BM chimera inoculum did not contribute to the augmented maturation and function of aged NK cells in sub-lethally irradiated aged hosts 2 weeks post chimerism. Similarly, the presence of aged BM cells in the BM inoculum did not impair the young NK cell maturation and function in sub-lethally irradiated young hosts 2 weeks post chimerism. While there were trends towards a similar pattern at 6 weeks post chimerism, the differences at 6 weeks were more subtle than those at 2 weeks. Therefore, while the aged non-hematopoietic environment may impair NK cell maturation, other factors including defects in the hematopoietic environment as well as intrinsic defects in hematopoietic stem cells (HSCs) and NK cell precursors may also contribute to the impaired NK cell maturation and function in aging. Together, these results advance our understanding of how aging influences NK cells by revealing
the contribution of the aged non-hematopoietic environment on the impaired maturation and function of NK cells. These data are in agreement with those from Chiu and colleagues [148]. However, we significantly extended these analyses and showed that the expression of T-bet and Eomes as well as the functional capacity of aged NK cells are also controlled by the non-hematopoietic environment. Our data, thus, suggest that therapeutic interventions geared at boosting NK cell cytotoxicity in aging will need to manipulate the non-hematopoietic environment in which NK cells develop. Indeed, our data highlight that aged NK cells are intrinsically capable of being functionally competent provided they undergo maturation in a “young” environment. The use of stem cell transplantation as a therapeutic intervention to reverse immune dysfunction in aging has received a growing amount of interest [168]. Thus, integrating stem cell therapy with the transfer of young non-hematopoietic cells or a way to improve the aged environment may have considerable promise in reversing the phenotype of NK cells in aging.

An intriguing finding in our analysis was the observation that in mixed BM chimeras, NK cells from an aged origin dominated the NK cell compartment in the periphery (spleen, pLNs, lung) despite the BM inoculum having a 1:1 ratio of aged and young NK cells. However, this phenomenon was not specific to NK cells as bulk CD45.2+ aged cells dominated the hematopoietic compartment in both young and aged recipients at week 6 post chimerism. While the mechanism to account for this phenomenon is yet to be defined, one explanation for these results could be an enhanced survival capacity of aged NK cells in vivo which allows them to dominate in cellular competition. Indeed, preliminary results show that aged splenic NK cells have a higher (2 fold) protein expression of the pro-survival molecule, Mcl-1, but the expression of Bcl-2 and Bim are similar between young and aged splenic NK cells (data not shown).
Similarly, aged naïve T cells have been shown to have an enhanced survival compared to young cells when transferred in the same host [169]. This suggests that cells from aged donors may have an intrinsically enhanced capacity to survive in the same environment compared to cells from young donors. An alternative explanation could be enhanced aged NK cell proliferation or skewed migration between peripheral lymphoid organs. However, NK cell proliferation as measured by the expression of the nuclear proliferation antigen, Ki-67 was similar between aged and young NK cells in our mixed BM chimera experiments (data not shown). Additionally, the dominance of NK cells from an aged origin was present not only in the spleen, but also pLNs, BM and the lung suggesting that preferential migration was unlikely to explain this phenomenon.

In conclusion, in this study, we demonstrate that the aged non-hematopoietic environment is an important contributor to the impaired maturation and function of NK cells in aging. These findings open up the possibility of therapeutic interventions geared at boosting the functional competence of NK cells against tumors and viral infections, both of which occur at a high incidence in the elderly and therefore identifying the non-hematopoietic environmental defects that exist in aging is of high significance to human health.

**Experimental Procedures**

**Mice**

C57BL/6 female mice were purchased from either Taconic Farms (Germantown, NY), The Jackson Laboratory (Bar Harbor, ME), or from the National Institutes of Aging (NIA) colony at Taconic Farms. Young mice were considered to be 2-3 months of age and aged mice were considered to be ≥ 16 months of age. All mice were acclimatized for at least 1 week before conducting any studies. Mice were housed under specific pathogen-free conditions in the Cincinnati Children’s Hospital Medical Center (CCHMC) vivarium. Congenic CD45.1
C57BL/6 mice were obtained from Jackson Laboratories. All animal protocols were reviewed and approved by CCHMC Institutional Animal Care and Use Committee.

**Cell preparations**

**Bone marrow cell preparation:** The hind legs were dissected and carefully removed from the surrounding muscle tissue. The femurs and tibia were then crushed using a mortar and pestle in 10 ml of phosphate buffered solution (PBS, Mediatech, Manassas, VA) containing 2% FBS (Invitrogen, Carlsbad, CA). The obtained solution containing the bone marrow was filtered on a 70-micron cell strainer and washed twice before red blood cell lysis with Ammonium-Chloride-Potassium (ACK) (prepared in house).

**Spleen and peripheral lymph node (pLN) cell preparation:** Spleens and pLNs (inguinal, axillary, brachial and cervical) were homogenized with a 3 ml syringe plunger through a 100-micron cell strainer using buffered saline solution (BSS) (prepared in house) and red blood cells were lysed with ACK lysis buffer (prepared in house).

**Lung cell preparation:** Lungs were isolated and digested in an enzyme mixture of 6ml of RPMI 1640 containing Liberase CI (0.5mg/ml) (Roche Diagnostics, Indianapolis, IN) and DNase I (0.5mg/ml) (Sigma-Aldrich, St. Louis, MO) for 45 minutes at 37°C. The digested lung tissue was then homogenized with a 3 ml syringe plunger through a 70-micron cell strainer and red blood cells were lysed with ACK lysis buffer (prepared in house).

**Liver cell preparation:** Single liver cell suspensions were prepared by first homogenizing the whole tissue in RPMI 1640 using a gentle MACS dissociator (Miltenyi Biotech, Auburn, CA). The resulting homogenate was then centrifuged at 2,000 rpm and the cell pellet was mixed with 33% Percoll (Sigma-Aldrich) in RPMI 1640 solution (Invitrogen). The Cell suspension was then centrifuged at 2,000 rpm for 20 minutes at room temperature. The cell pellet was
removed and washed, and red blood cells were lysed with ACK lysis buffer (prepared in house). Single cell suspensions from all tissues analyzed were then washed with RPMI 1640 (Invitrogen) containing 10% FBS and viable cells were counted via trypan blue (MP Biomedicals, Solon, OH) exclusion.

**In vivo NK cell cytotoxicity assay against allogeneic and B16 melanoma targets**

Aged and young mice were injected intravenously (i.v) with a 1:1 mixture of a low carboxyfluoresceinsuccinimidyl ester (CFSE) (Life technologies, Grand Island, NY) syngeneic C57BL/6 splenocytes (2 μM CFSE) reference population and a medium CFSE allogeneic Balb/c splenocytes (10 μM CFSE) target cell population (10⁷ total cells). Eighteen hours after injection of the CFSE-labeled cell mixture, a blood sample was taken from each recipient mouse and the presence/absence of the reference and target cell populations was determined by flow cytometry.

NK cell depletion in vivo was performed by injecting mice intraperitoneally with 20μL of anti-asialo GM1 antibody as recommended by the manufacturer (Wako Pure Chemical Industries, Richmond, VA). Administration of anti-asialo GM1 was performed twice, on day -3 and 24 hours before challenge of recipients with target cells. NK cell cytotoxicity was determined by normalizing the percentage of target cell killing in relation to NK cell depleted recipients (>90% depletion in anti-asialo GM1 treated recipient mice). Assessment of B16 melanoma lung tumor burden was performed 1 hour after i.v injection of 0.2X10⁶ (10 μM CFSE) labeled B16 melanoma cells expressing OVA using both flow cytometry (for detection of CFSE+ cells) and RT-PCR (for detection of OVA expression)[164]. The right lung was isolated and digested in a Liberase (Roche Diagnostics)/DNase (Sigma-Aldrich) enzyme mixture as described above.
Antibodies and flow cytometry

10^6 cells from single cell suspensions were suspended in 100 μL fluorescence-activated cell sorting buffer and Fc receptor was blocked with anti-mouse CD16/32 (clone 93, Biolegend). The following mAbs (purchased from eBioscience, San Diego, CA, BD, San Jose, CA, Biolegend, San Diego, CA or Cell Signaling, Technology, Beverly, MA) were used: anti-CD3 (500A2), anti-NKp46 (29A1.4), anti-CD27 (LG.3A10 or LG.7F9), anti-CD11b (M1/70), anti-T-bet (ebio4B10), anti-Eomes (DAN11MAG), anti-Blimp-1 (C14A4), anti-GATA-3 (TWAJ), anti CD45.2 (104), anti-CD45.1 (A20), anti-KLRG1 (2F1), anti-CD43 (S7), anti-CD107 (1D4B), anti-Ly49G2 (4D11), anti-Ly49I (YL1-90), anti-IFN-γ (XMG1.2), anti- granzyme B (GB11), anti-F4/80 (BM8), anti CD8α (53-6.7), anti- CD11c (N418), anti-MHC-II (M5/114.15.2), anti CD19 (6D5), anti-IL-15Rα (DNT15Ra), anti- CD122 (TM-β1). For detection of Blimp-1, secondary anti-Rabbit antibodies were used (Jackson ImmunoResearch Laboratories, West Grove, PA). Lineage (Lin) is defined using CD3, CD19 and NKp46. NK cells are defined as CD3- NKp46+ within live cells (Live-Dead fixable blue negative, Life technologies) and NK cell subsets are defined based on expression of CD27 and CD11b as previously described[107,108,170]. DN NK cells are CD27^low CD11b^low, immature NK cells are CD27^hi CD11b^low, transitional NK cells are CD27^hi CD11b^hi and mature NK cells are CD27^low CD11b^hi. Flow cytometry data were collected using an LSRII or LSR Fortessa (BD) flow cytometers and analyzed by BD FACS Diva software.

Construction of Mixed BM chimeras

C57BL/6 recipient mice were lethally irradiated first with 700 rad and then with 475 rad 3 hours later. After 24 hours, recipient mice were injected intravenously with 10^7 donor bone marrow cells containing a 1:1 mixture of young (CD45.1) and aged (CD45.2) bone marrow
donor cells that were depleted of CD3+ TCRβ+T cells using CD3ε microbead kit (Miltenyi) and biotinylated TCRβ (Biolegend) and used according to the manufacturer’s instructions. Recipient mice were then fed Doxycycline-containing chow until the time of sacrifice. Chimerism was evaluated at 2 and 6 weeks post transfer.

**NK cell degranulation assay**

Young and aged mice were treated intraperitoneally with 100µg of polyI:C. Spleens were harvested 24 hrs. later and 10⁶ splenocytes were cultured in the presence of either plate-bound anti-NK1.1 (PK136, 10µg/ml, Biolegend), IL-12(p70) (1ng/ml, PeproTech, Rocky Hill, NJ)/IL-18 (20ng/ml, MBL, Boston, MA), 0.2X10⁶ YAC-1/ B16 melanoma cells and phorbol myristate acetate (PMA) (Sigma-Aldrich) (50ng/ml)/ionomycin (Sigma-Aldrich) (750ng/ml) for 5 hours in the presence of brefeldin (Sigma-Aldrich) (2.5mg/ml) and monensin (eBioscience) (1x). Similar experiments were conducted using IL-2 (Miltenyi-biotech) (500U/ml) in the presence of the various stimuli indicated above.

**IL-15 administration in vivo**

IL-15/IL-15Rα (4.5µg) (R&D Systems, Minneapolis, MN) were mixed in vitro and the equivalent of 750ng IL-15 was injected intraperitoneally on days 0, 2 and 4 before tissue harvest on day 6.

**Quantitative gene expression**

RNA was prepared using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) and converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Quantitative gene expression analysis (quantitative PCR) was performed with Roche LightCycler 480 SYBRGreen 1 Master Mix (Roche Diagnostics) using the Roche LightCycler 480 II instrument (Roche Diagnostics).
The primers utilized were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and are as follows:

1) Ribosomal protein s14 (Rps14) which was used as a house keeping gene for analysis of OVA expression in lung tissues: 5’-TGACATCCTCAATCCGCCCCAATCT-3’ and 5’-CATCACTGCCTTGCACATCAAACT-3’; 2) OVA: 5’-GTGACTGAGCAAGAAAGCAAACCTG-3’ and 5’-TTGTCCCACTGGCAAATGGAAG-3’; 3) L19 as a house keeping gene for IL-15 analysis: 5’-CCTGAAGGTCAAAGGAATGTG-3’ and 5’-GGTCTGCCCTTGGCTGTCT-3’ and 4) IL-15: 5’-AACCTTTCTCTGGAATTG-3’ and 5’-ATGAACAGTTGGGACAAATGCGT-3’

**Statistical analyses**

Statistical analyses were performed using unpaired *t* tests or non-parametric tests when appropriate. These tests were performed using GraphPad Prism software (version 5.01).

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**Author contributions**

Hesham Shehata performed the experiments and analyzed the data. HS, KH and CAC designed the experiments and wrote the manuscript.

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Conflict of interest

The authors have no financial conflicts of interest.
References

multipotent stem cells (CB-SCs) in stem cell educator therapy: phase I/II clinical trial. 
*BMC medicine.* **11**, 160.
Aged NK cells have a reduced *in vivo* cytotoxic capacity. Aged mice, young mice and NK cell-depleted mice (anti-asialo GM1 treated) were challenged intravenously with CFSE-labeled allogeneic targets or CFSE-labeled B16 melanoma cells expressing OVA. After 18 hours, the efficiency of allogeneic target cell elimination was evaluated by flow cytometry and normalized to anti-asialo treated recipients. (A) The difference in the mean percentage cytotoxicity between young and aged NK cells. B16-OVA tumor burden in the lungs was evaluated 1 hr. post challenge by (B), RT-PCR for OVA expression and (C), flow cytometry for CFSE expression and compared to NK cell depleted mice. (D) The absolute number of NK cells (CD3- NKp46+) in the lungs of naïve (n=6) and B16- OVA challenged young, aged mice (n=11-14). Data are represented as the mean± SE of two independent experiments with n=4 mice for young and aged mice and n=2 for anti-asialo GM-1 treated mice. The *p* values (unpaired t-tests) represent the difference between aged and young mice. Means are shown as horizontal lines with each point representing one individual mouse. The *p* values between naïve and B16 challenged mice was calculated using paired t-tests.
Fig 2. Aged NK cells have an impaired capacity to degranulate particularly in the mature NK cell subset. Aged and young mice were treated intraperitoneally with 100μg of poly (I: C) and after 24 hours, 10⁶ splenocytes were stimulated with various stimuli such as plate-bound anti-NK1.1 (10μg/ml), IL-12 (1ng/ml)/IL-18 (20ng/ml), 2X10⁵ B16 melanoma cells, 2X10⁵ YAC-1 cells and PMA (50ng/ml)/Ionomycin (750ng/ml) for 5 hours before analysis of CD107a surface expression and intracellular IFN-γ within the CD3- NKp46+ population. (A) The proportion of total splenic NK cells that is IFN-γ+. CD107a degranulation was assessed in total splenic NK cells (CD3- NKp46+), (B) and mature NK cells, (C), of aged and young mice. Bar graphs show mean± SE of 8 aged and 7 young mice in two experiments. The p values represent the difference between aged and young mice (unpaired t test).
Fig 3. The aged environment plays an important role in limiting NK cell maturation *in vivo*. Analysis of splenic NK cell maturation at baseline, (A) or 2 weeks, (B) and 6 weeks, (C), post chimerism in a young or aged environment. (D) and (E), Absolute numbers of NK cells and their maturation subsets after developing in a young or aged environment at 2 weeks or 6 weeks post chimerism. Numbers in pie charts represent the mean of the proportion of each subset. Data shown are representative of at least two independent experiments with *n*=6 mice per group. The *p* values for week 2 and week 6 of the mixed BM chimera represent the difference between NK cells maturing in a young vs. aged environment while the *p* values at baseline conditions represent the differences between NK cells from young and aged mice (unpaired t tests), *P*<0.05, **P*<0.009 and ***P*<0.0005.
Fig 4. Eomes and T-bet, which regulate key checkpoints in NK cell functional maturation are significantly reduced in aged BM NK cells. (A) and (C), Eomes and T-bet intracellular expression in young and aged NK cells in the bone marrow. The mean fluorescence intensity (MFI) of Eomes, (A) and T-bet, (C), in each of the young and aged NK cell subsets in the BM. (B) and (D), The MFI of T-bet and Eomes in whole young or aged BM NK cells that developed in either a young or aged host. Data are representative from at least two independent experiments with each point representing one individual mouse. Mean MFI is shown as horizontal bars. The $p$ values represent the difference between aged and young NK cells (unpaired t test).
Fig 5. The functional capacity of aged NK cells is augmented when they mature in a young environment. 6 weeks post chimerism, splenocytes were isolated from recipients of mixed BM chimeras and cultured in the presence or absence of $2 \times 10^5$ YAC-1 cells or PMA (50ng/ml)/Ionomycin (750ng/ml) for 5 hours in addition to IL-2 (500 U/ml). Intracellular IFN-$\gamma$, (A) and CD107a degranulation, (B), were assessed 5 hours post stimulation in aged and young total NK cells, based on expression of their respective congenic markers. Bar graphs show mean$\pm$ SE of 6 aged and 6 young mice. The $p$ values represent the difference between aged and young NK cells (unpaired t test).
Supplementary Figures

Fig. S1. Aged mice have a profound enrichment in immature NK cells across all lymphoid organs with a significant accrual in the BM. A, The proportion and absolute numbers of total NK cells across lymphoid organs in young and aged mice. The differences in proportion and absolute number of NK cell maturation subsets between young and aged mice in the spleen, B and the BM, C. All statistical analysis (unpaired t test) is performed in comparison to young NK cell subsets. Numbers in pie charts represent the mean of the proportion of each subset. Data are representative from two independent experiments with n=4 mice per group. Means are shown as horizontal lines with each point representing one individual mouse. The p values represent the difference between aged and young mice (unpaired t tests), *P<0.05, **P<0.009 and ***P<0.0005.
**Fig. S2**  A, The proportions of CD11b versus CD43 from gated splenic NK cells in the spleen and BM of young and aged mice respectively. Numbers in pie charts represent the mean of the proportion of each subset. The p values represent the difference between aged (N=4) and young (N=4) NK cells (unpaired t tests), *P<0.05, **P<0.009 and ***P<0.0005.
**Fig. S3** A, Construction of mixed BM chimeras in which a 1:1 mixture of aged and young BM cells were transferred i.v into either young or aged congenic recipients. B, The fraction of NK cells derived from both young and aged origins in the BM inoculum at the time of transfer into irradiated young and aged recipients. This fraction was obtained by pooling BM cells from 6 young (CD45.1+) and 6 aged (CD45.2+) mice. The absolute numbers of total CD45.1+ (young) and CD45.2+ (aged) cells in the spleen 2 weeks (C) and 6 weeks (D) post chimerism.
Fig. S4 The response of young and aged splenic NK cells to different doses of IL-15 in vitro. 10^6 splenocytes were cultured in the presence or absence (media) of different concentrations of recombinant mouse IL-15 for 24 hours before analysis of live cells (7AAD-) on gated CD3-NKp46+ cells. B, The in vivo expansion of aged and young BM NK cells after i.p injection with the IL-15/IL-15Rα complex. C, The maturation profile of aged and young BM NK cells with and without IL-15/IL-15Rα treatment. The p values represent the differences in relation to untreated young mice (unpaired t tests), *P<0.05, **P<0.009 and ***P<0.0005.
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**Supplementary Table I** Mean MFI±SE of GATA-3 and Blimp-1 in whole splenic and BM NK cells. Data are representative from at least two independent experiments with 4 young and 4 aged mice. All p values representing the differences in GATA-3 and Blimp-1 expression between young and aged NK cells and their subsets are p>0.07.
Chapter 2.2: The BM microenvironment contributes to the impaired maturation of NK cells in aging

Data from mixed BM chimeras indicated that the aged environment was impaired in its capacity to sustain NK cell terminal maturation and function. With this information, we set out to understand which factors in the aged environment were responsible. However, a sine qua non to this question was to identify which component of the aged environment, i.e, the BM microenvironment or the periphery or both housed the defects leading to the impaired phenotype of aged NK cells given that both components can independently support NK cell development. To this end, we transferred CFSE-labeled purified splenic immature NK cells from young BoyJ mice (CD45.1) into both young (CD45.2) and aged recipients (CD45.2) and assessed the maturation of the transferred NK cells 2 weeks later (Fig 6). This time frame is synchronous with the establishment of progressive maturation of immature NK cells to terminally mature cells [107]. Intriguingly, our data demonstrated that the transferred young immature NK cells matured and proliferated equally efficiently in both young and aged environments. In contrast, when we transferred splenic aged immature NK cells, we found that they did not mature as efficiently even in a young environment (Figure 6). While this finding was surprising to us, it suggested that the aged peripheral environment can support peripheral NK cell maturation as efficiently as a young peripheral environment.

Importantly, we view these data as being complementary to our BM chimera studies. The main difference between the BM chimera and the peripheral NK transfer studies is the source of the donor cells and the tissue they home to for their maturation. In the mixed BM chimeras, transferred young BM NK cells have an impaired maturation when they develop in aged BM. However, young peripheral NK cells mature efficiently in the aged peripheral environment,
suggesting that the aged BM environment is not optimal for supporting NK cell maturation. In contrast, our data show that the aged peripheral environment can equally support NK cell maturation as a young peripheral environment. Thus, the impaired peripheral maturation of aged NK cells is not due to an impaired capacity of the peripheral environment in aged mice to support maturation. We reason that NK cells that recently emigrated from the BM may receive signals while in the BM that lead to alteration in gene circuitries regulating NK cell maturation. As a consequence, aged splenic NK cells would have a compromised peripheral maturation even in a young environment. This line of reasoning is supported by the results from our mixed BM chimeras where aged NK cells that develop in a young BM environment either at 2 or 6 weeks have an improved maturation. Thus, initial programming in the BM may influence the NK cell maturation phenotype in the periphery.

Taking these results and those from the mixed BM chimera, the data suggest that the BM environment plays a critical role in regulating NK cell maturation in aging. Additionally, peripheral NK cells may accumulate intrinsic defects that also impair their maturation. Thus, identifying defects in the aged BM microenvironment and the intrinsic defects in peripheral aged NK cells would provide insight into the mechanisms that could boost NK cell maturation and function in aging.
Figure 6. Splenic immature NK cells from aged mice have an impaired maturation even in a young peripheral environment (A) CFSE- labeled immature NK cells from the spleens of young and aged donor (N=16) mice were purified by cell sorting and 1X10^5 cells were co-transferred at a 1:1 ratio with CD8+ OT-1 cells i.v to recipient young or aged mice (N=3 or 4/group). 2 weeks post transfer, the maturation status of the transferred cells was evaluated in the spleen of recipient mice. (B) The proliferation status based on CFSE dilution was evaluated in the transferred NK cells and their subsets. Gating for CFSE low cells was based on the CFSE profile of the CD8+ T cells that were co-transferred.
Chapter 3: The impaired maturation and function of NK cells in aging is associated with a dysregulation of Foxo1 expression and mTORC1 activity

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Running title: Foxo1 and mTORC1 dysregulation in aged NK cells

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Manuscript in preparation
Abstract

The cytotoxic function of Natural killer (NK) cells against tumor cells and viral infections such as influenza are greatly impaired in aged mice and elderly humans. The impaired functions of aged NK cells are associated with an impaired terminal maturation. The underlying pathways responsible for the impaired function and maturation of aged NK cells remain unknown. Previously, we and others reported that the expression of the key transcription factors regulating NK cell functional maturation, T-bet and Eomes were significantly lower in aged NK cells. We sought to understand the intracellular pathways regulating the differential expression of T-bet and Eomes in aged NK cells. Foxo1 was recently reported to be a negative regulator of NK cell maturation and function by repressing T-bet expression. Extending those findings in the context of aged NK cell maturation and function, we observed that aged splenic immature NK cells (CD27$^{hi}$ CD11b$^{lo}$) had a significantly higher Foxo1 protein expression compared to young immature NK cells. The increased levels of Foxo1 were consistent with its role in promoting DNA repair, ROS detoxification and cell cycle arrest as aged NK cells exhibited more DNA damage, increased mitochondrial superoxide and reduced proliferation. Additionally, aged NK cells had an enhanced mTORC1 activity which correlated with higher ATP levels, but reduced mitochondrial mass and autophagy (LC3). Together, our data suggest that Foxo1 expression and mTORC1 activity are dysregulated in aging and developing strategies to normalize their profile may enhance aged NK cell functional maturation.
Introduction

NK cells are large granular cytolytic lymphocytes that belong to the innate arm of the immune system. NK cells are capable of eliminating tumors and virally infected cells as well as producing pro-inflammatory cytokines such as IFN-\( \gamma \) that shape the adaptive immune response \[34,45,74\]. It is well appreciated that aging considerably impairs the function and homeostasis of both the innate and adaptive immune systems and this is reflected on the reduced efficacy of vaccines and the increased mortality of the elderly to infectious diseases such as influenza and cancers \[1-4,6,9,15-18,158,171\]. We and others have previously reported that aging significantly impairs the terminal maturation and function of murine and human NK cells \[131-133,135,139,141,142,148\].

Importantly, the source of the defects impairing NK cell function and maturation has been identified with a consensus that the extrinsic aged environment plays an important role in limiting both the maturation and cytotoxic capacity of NK cells \[135,139,148\]. However, the precise factors and the intracellular pathways that limit aged NK cell maturation and function have not been fully elucidated. Improving our understanding of the molecular mechanisms implicated in age related NK cell dysfunctions could have important implications in developing novel therapeutic strategies to boost NK cell cytotoxicity against tumors and viral infections in the elderly, contributing to a better management of a growing aging population.

The generation of mature, educated and immunologically competent NK cells is dependent on an elaborate collaboration of various extrinsic events that are associated with intrinsic developmental regulators \[98\]. In many cases, changes in NK cell transcription factor expression profiles depend on extrinsic soluble factors released by other immune cells or physical interactions between specific receptors expressed on NK cells with their ligands.
expressed on other cells (e.g. BM stromal cells) [95,96]. T-bet and Eomes have been well characterized as key molecules that not only promote the terminal maturation and education of NK cells, but also induce the expression of the effector molecules, IFN-γ, GZB and perforin.

Our group and others previously demonstrated that the NK cell transcription factors, T-bet and Eomes were governed by the environment in which NK cells develop [135,139]. Interestingly, a recent study showed that loss of Foxo1 augments NK cell homing, maturation and function through the release of Foxo1 mediated repression of Tbx21 (T-bet) expression [106]. In this study we thus measured the levels of Foxo1 and the pathways regulated by Foxo1 including the DNA damage response, metabolism and mitochondrial superoxide production in aged NK cell development.

Another important intracellular molecule that plays a critical role in regulating NK cell maturation, education and their functions is mTORC1 [123,124,172]. Our findings reveal that mTORC1 activity is significantly higher in aged NK cells despite impaired proliferation and maturation. In addition, consistent with our previous findings that T-bet is reduced in aged NK cells, we observed increased levels of Foxo1 in splenic aged NK cells. Thus, our findings showing the increased expression of Foxo1 and mTORC1 activity are commensurate with the reduced expression of T-bet and impaired aged NK cell functional maturation. Developing strategies to normalize the expression of Foxo1 and mTOR activity in aged NK cells using ROS scavengers and rapamycin respectively could be beneficial in augmenting the maturation and function of NK cells in aging.
Results

Foxo1 but not Foxo3 is significantly increased in splenic immature aged NK cells

Foxo1 is a negative regulator of T-bet in NK cells and thus the absence of Foxo1 enhances both NK cell terminal maturation and function. We assessed Foxo expression by flow cytometry within young and aged splenic and BM NK cells. Foxo1 protein expression was significantly increased in aged splenic NK cells but not BM NK cells (Fig 1A and B). The highest difference in protein expression was seen in aged immature NK cells and this is consistent with the hypothesis that aged immature NK cells have an impaired capacity to differentiate progressively into more mature NK cells. No differences were observed in Foxo3 expression between young and aged NK cells (Fig 1C). Interestingly, differences in Foxo1 protein expression may be due to post transcriptional modifications as Foxo1 mRNA levels were not different in any of the NK cell subsets of young and aged mice (Fig 1D).

The subcellular localization of Foxo1 is an important regulator of its activity. The phosphorylation of nuclear Foxo1 leads to its translocation from the nucleus to the cytoplasm, where it becomes inactive. Conversely, dephosphorylation of cytoplasmic Foxo1 promotes its nuclear translocation where Foxo1 becomes active and can regulate gene expression. We evaluated whether the increased Foxo1 expression in aged NK cells was a reflection of increased nuclear or cytoplasmic levels. Exploiting the differential permeabilization properties of saponin which permits cytoplasmic, but not nuclear staining, our data suggest that the differences observed in Foxo1 expression in both young and aged NK cells were reflective of nuclear and not cytoplasmic expression, although aged NK cells also had higher levels of cytoplasmic Foxo1, albeit much lower than the combined levels in the nucleus and cytoplasm (Fig 2A and B) [173,174]. Using IL-15 treatment which promotes Foxo1 phosphorylation as a positive control,
we observed an increase in cytoplasmic Foxo1 expression confirming the use of this staining protocol as a reliable means to differentiate between cytoplasmic and nuclear Foxo1 localization (Fig 2A and B). Thus, in general, the increased Foxo1 expression in aged NK cells is predominantly nuclear Foxo1.

**NK cells in aged mice have a higher mitochondrial superoxide production and greater DNA damage**

The Foxo family of transcription factors is known for its role in regulating oxidative stress through the detoxification of reactive oxygen species (ROS) and the control of DNA repair pathways [175-177]. This is mainly achieved by inducing an apoptotic response to eliminate cells accumulating genotoxic stress and DNA damage. This occurs by inducing the expression of GADD45α, p130, cyclin D2, p27Kip1, Bim, Puma and FasL which are involved in cell cycle arrest, DNA repair under oxidative stress and apoptosis [178-181]. To assess if increased ROS and DNA damage in aged NK cells correlated with increased Foxo1 expression, we evaluated mitochondrial superoxide production and DNA damage using MitoSOX red and γH2AX staining respectively in aged and young splenic and BM NK cells (Fig 3). Consistent with the increased Foxo1 expression, aged total splenic NK cells as well as the immature and transitional NK cell subsets had a trend towards increased mitochondrial superoxide production as well as DNA damage (Fig 3A-C and data not shown), suggesting that increased Foxo1 levels may be part of a feedback loop to reduce mitochondrial superoxide and DNA damage. There were also trends towards higher levels of the stress molecule Rae-1 on aged splenic and BM NK cells and their subsets, suggesting that aged NK cells exhibit a phenotype associated with increased cellular stress (Fig 3D and data not shown).
Higher mTOR activity is associated with increased ATP in immature aged NK cells, but impaired NK cell proliferation.

Another important intracellular molecule regulating NK cell maturation is mTORC1. Selective deficiency of mTORC1 in NK cells impairs their maturation, expression of educating receptors as well as both T-bet and Eomes [123,172]. Thus, we assessed the activity of mTORC1 in young and aged NK cells by evaluating the phosphorylation status of 4-EBP1 and S6K (which are direct targets of the mTOR kinase) in NK cells [123,125-127]. Interestingly, we observed a higher mTORC1 activity in aged NK cells particularly in the immature subset (Fig 4A and B). Importantly, the increased phosphorylation levels of S6 and 4-EBP1 was not a consequence result of increased levels of their non-phosphorylated form in the aged NK cells (Fig 4C and D).

The mTORC1 pathway is important for promoting the de novo synthesis of proteins, nucleotides, lipids and inhibiting autophagy [182]. In addition, mTOR acts as a sensor of the metabolic environment of the cell and it functions as a master regulator of glucose metabolism [183,184]. To evaluate whether enhanced mTOR activity in aged NK cells correlates with a more metabolically active state, the intracellular levels of ATP in purified splenic NK cell subsets from young and aged mice were assessed. Consistent with a higher mTOR activity, higher intracellular levels of ATP in the immature NK cell subset were observed (Fig 5A).

Given the higher mTOR activity and increased ATP content of aged immature NK cells, we anticipated that these aged NK cells were metabolically more efficient compared to their younger counterparts. To this end, we evaluated whether aging affected mitochondrial function by measuring multiple parameters associated with mitochondrial homeostasis and function. Aged immature NK cells showed a significant decrease in mitochondrial mass (Fig 5B) and mitochondrial potential (data not shown), which is indicative of impaired mitochondrial function.
To further determine how aging influences NK cell nutrient uptake, we evaluated the capacity of aged NK cells to uptake glucose using a fluorescently labelled glucose analog, 2-NBDG. Interestingly aged NK cells had a significantly impaired capacity for glucose uptake compared to young NK cells (Fig 5D) despite similar levels of Glut 1 mRNA expression (data not shown) and similar responses to activation using IL-15 both *in vitro* and *in vivo* (Fig 2) [135,139]. Furthermore, consistent with higher mTORC1 activity, aged NK cells showed reduced autophagy (Fig 5E) [185]. Thus, our results suggest that the dysregulation in mTORC1 activity is associated with higher ATP and lower autophagy in aged NK cells.

Interestingly, despite having higher levels of ATP and mTOR activity, aged NK cells had an impaired proliferative capacity as examined by BrdU incorporation and the expression of the nuclear antigen, Ki-67 was significantly impaired (Fig 6A and B). Notably, this decreased aged NK cell proliferation was commensurate with increased Foxo1 which is known for its role in inhibiting cell cycle progression [186-190]. Thus, the reduced proliferation and reduced T-bet levels in aged NK cells in the spleen is consistent with increased Foxo1 levels.

**Discussion**

NK cell development is regulated by the integration of signals from the environment in which they develop and intracellular transcription factors which regulate the NK cell cytotoxic gene program. Extensive progress has been made in identifying the source of the defects responsible for NK cell dysregulation in aging. In particular, extrinsic defects in the aged environment play a considerable role in impairing NK cell maturation and function [135,139,148]. However, the transcriptional and molecular pathways underlying how these extrinsic defects impair NK cell homeostasis and function in aging remain poorly understood.
Foxo1 has been shown to repress T-bet through its interactions with SP1 [106]. The down regulation of T-bet through Foxo1 limits NK cell maturation and effector functions and therefore, Foxo1 deficient NK cells have a higher T-bet expression and a more enhanced NK cell maturation and cytotoxic capacity. Our group and others reported that T-bet and Eomes were markedly reduced in aged NK cells and were controlled by the environment (aged or young) in which NK cells developed [135,139]. Given these findings, we became interested in assessing whether a dysregulation of Foxo1 was associated with the impaired maturation and function of NK cells in aging.

The increase in aged splenic immature NK cell Foxo1 expression correlated well with their reduced T-bet expression [135]. Importantly, this increased Foxo1 protein expression was not a consequence of increased Foxo1 transcription as demonstrated by similar mRNA levels between purified young and aged NK cell subsets. Post transcriptional modifications such as acetylation and monoubiquitination which are known to regulate Foxo1 activity could play a role in explaining the lack of correlation between mRNA and protein levels [191,192]. Interestingly, while Foxo1 levels appeared higher in splenic aged NK cells, in the BM its expression was significantly lower compared to young NK cells. This could be attributed to differential regulation of Foxo1 expression in the different lymphoid compartments and notably, differential activity of Foxo1 may exist despite differences in expression, although this will need to be evaluated further.

To determine the potential mechanisms driving increased Foxo1 expression, we assessed some of the most obvious candidates that induce Foxo1 expression. Interestingly, mitochondrial superoxide and DNA damage were significantly increased in total aged NK cells and this was accompanied by reduced NK cell proliferation. One of the major contributors of cellular ROS is
the mitochondria from the process of oxidative phosphorylation through the electron transport chain. Indeed while aged immature NK cells had a tendency to have a subtle, but significant decrease in mitochondrial mass, they appeared to have a significantly higher concentration of intracellular ATP. Along these lines, responses to stress and DNA damage requires co-ordination between transcription, translation and cell cycle progression. In response to higher DNA damage, it is possible that Foxo1 would inhibit cell cycle progression which is evidenced by the reduced proliferation of aged NK cells.

Elevated levels of ROS induce Ataxia-telangiectasia mutated (ATM) which functions as a damage sensor and coordinates cell cycle and DNA repair. ATM can then repress mTORC1 by activating tuberous sclerosis complex (TSC2) via AMPK [185]. Furthermore, mTORC1 activity can regulate ROS production by regulating mitochondrial activity, suggesting that there is considerable cross-talk between mTOR activity and the generation of ROS [193]. The interplay between mTORC1 activity, ROS levels and cellular metabolism is tightly regulated as ROS can enhance both glycolytic flux and oxidative phosphorylation through inducing HIF-1α or NRF2 and mTORC1 regulates energy metabolism through the transcriptional control of mitochondrial genes and oxygen consumption [194,195]. Thus, it is possible that higher levels of mTORC1 activity in aged NK cells yields higher ATP levels. The increased mTORC1 activity in aged NK cells was surprising given that mTOR is important for supporting NK cell maturation and proliferation and aged NK cells had reduced maturation and proliferative capacity. The increased activity may also be partly attributed to increased total levels of S6 and 4-EBP1 as there were trends towards higher levels in aged NK cells. Thus, increased mTORC1 activity as well as increased basal levels of total S6 and 4-EBP1 may be responsible for the higher levels of pS6 and p4-EBP1. The increased mTORC1 activity may also compensate for decreased...
mitochondrial function and metabolism in aging, thus leading to increased ATP levels in immature cells through AKT activation [196]. The reduced spot area of autophagic vesicles as measured by LC3 staining was commensurate with increased mTORC1 activity and ATP levels, suggesting that autophagy could be down regulated as a consequence of higher mTORC1 and ATP availability.

We hypothesize that the increased Foxo1 expression in aged NK cells could be part of a positive feedback loop to counteract the DNA damage posed by increased levels of ROS which are known to inhibit NK cell cytotoxic function [197,198]. Extending this further, we reason that the increased levels of Foxo1 could lead to reduced levels of T-bet which yields an NK phenotype that is impaired in both function and maturation.

Given previous findings that the aged environment is responsible for the impaired maturation of NK cells, it is possible that signals from the environment such as increased metabolic stress or hypoxia could trigger genotoxic and oxidative stress which leads to increased ROS and DNA damage. Additionally other environmental signals including platelet derived growth factor (PDGF) and TNF which are known to regulate Foxo1 expression may be altered in aging and if so would reflect on these differences, although this has not been determined thus far [199,200]. The higher levels of Foxo1 may impose a greater repressive effect on T-bet expression in peripheral NK cells leading to impaired NK cell maturation and function in aging. Future studies will need to interrogate whether an intervention approach such as treating aged mice with ROS scavengers such as N-acetyl cysteine (NAC) or rapamycin to normalize mTORC1 activity could augment NK cell function and maturation. Such an approach will be essential to dissect the causal relationship between the increased Foxo1 expression and mTORC1 activity with the impaired NK cell maturation and function in aging.
Experimental Procedures

Mice
C57BL/6 female mice were purchased from either Taconic Farms (Germantown, NY), The Jackson Laboratory (Bar Harbor, ME), or from the National Institutes of Aging (NIA) colony at Taconic Farms. Young mice are 2-3 months of age and aged mice are ≥ 16 months of age. All mice were acclimatized for at least 1 week before conducting any studies. Mice were housed under specific pathogen-free conditions in the Cincinnati Children’s Hospital Medical Center (CCHMC) vivarium. All animal protocols were reviewed and approved by the CCHMC Institutional Animal Care and Use Committee.

Cell preparations

Bone marrow cell preparation: The hind legs were dissected and carefully removed from the surrounding muscle tissue. The femurs and tibia were then crushed using a mortar and pestle in 10 ml of phosphate buffered solution (PBS, Mediatech, Manassas, VA) containing 2% FBS (Invitrogen, Carlsbad, CA). The obtained solution containing the bone marrow was filtered on a 70-micron cell strainer and washed twice before red blood cell lysis with Ammonium-Chloride-Potassium (ACK) (prepared in house).

Spleen and peripheral lymph node (pLN) cell preparation: Spleens and pLNs (inguinal, axillary, brachial and cervical) were homogenized with a 3 ml syringe plunger through a 100-micron cell strainer using buffered saline solution (BSS) (prepared in house) and red blood cells were lysed with ACK lysis buffer (prepared in house).

Antibodies and flow cytometry

$10^6$ cells from single cell suspensions were suspended in 100 μL fluorescence-activated cell sorting buffer and Fc receptor was blocked with anti-mouse CD16/32 (clone 93, Biolegend).
The following mAbs (purchased from eBioscience, San Diego, CA, BD, San Jose, CA, Biolegend, San Diego, CA or Cell Signaling, Technology, Beverly, MA) were used: anti-CD3 (500A2), anti-NKp46 (29A1.4), anti-CD27 (LG.3A10 or LG.7F9), anti-CD11b (M1/70), anti-Foxo1 (C29H4), anti-Foxo3 (75D8), γH2AX(20E3), Pan Rae-1(186107), anti- phosphorylated S6 (5316), anti-phosphorylated 4-EBP1 (7547), anti- S6 (54D2), anti- 4EBP1 (53H11), anti- LC3 (D50G8), anti-Ki-67 (B56). NK cells are identified as CD3- NKp46+ cells. NK cell subsets are defined based on expression of CD27 and CD11b as previously described [107,108,170]. DN NK cells are CD27\textsuperscript{low} CD11b\textsuperscript{low}, immature NK cells are CD27\textsuperscript{hi} CD11b\textsuperscript{low}, transitional NK cells are CD27\textsuperscript{hi} CD11b\textsuperscript{hi} and mature NK cells are CD27\textsuperscript{low} CD11b\textsuperscript{hi}.

For staining mitochondria, splenocytes were incubated for 30 min at 37°C with 10 nM MitoTracker Deep Red FM (Life Technologies) and for detecting mitochondrial potential, 20 nM TMRM (tetramethyl rhodamine, methyl ester, ImmunoChemistry Technologies) after staining surface markers. ROS were measured by incubation with 5µM MitoSOX Red (Life Technologies) after staining surface markers. Glucose uptake was measured after 5 hours of IL-15 (20ng/ml) (R and D Sysytems) activation with 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose; Invitrogen). Splenocytes were resuspended in RPMI-1640 medium (Life Technologies) in the presence of 100 µM 2-NBDG and were cultured for 10 min at 37 °C, then surface markers were stained. Flow cytometry data were collected using an LSRII or LSR Fortessa (BD) flow cytometers and analyzed by BD FACS Diva software.

**Measurement of BrdU incorporation.**

Mice were given 2 intraperitoneal injections of 0.5 mg of bromodeoxyuridine (BrdU; BD Biosciences Pharmingen) during a two-hour interval as previously described [107]. 6 hours after the first injection, the mice were sacrificed and BM cells were stained for BrdU according
Measurement of intracellular ATP levels

Intracellular ATP was measured in lysed NK cell maturation subsets purified by cell sorting on the basis of CD27 and CD11b expression. Purified subsets are more than 95% pure. Levels of ATP were measured by a luminescence assay (Adenosine 5'-triphosphate (ATP) bioluminescent assay kit; Sigma-Aldrich) as recommended by the manufacturer.

Quantitative gene expression

RNA was prepared using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) and converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen). Quantitative gene expression analysis (quantitative PCR) was performed with Roche LightCycler 480 SYBRGreen 1 Master Mix (Roche Diagnostics) using the Roche LightCycler 480 II instrument (Roche Diagnostics). The primers utilized were obtained from Integrated DNA Technologies (IDT, Coralville, IA) or eurofins mwg operon (Huntsville, AL) and are as follows:

*Foxo1 Reverse primer:* 5’TGGACTGCTCCTCAGTTCCTG-3’ and *forward primer:*
5’TTCGGAATGACCTCATGGATG-3’ Foxo1 expression was normalized to the housekeeping gene, GAPDH *Reverse primer: 5’-GTTCTCTGGGTGGCAGTGAT-3’ and forward primer: 5’TGGAAATCCCATCACCATCT-3’*

Statistical analyses

Statistical analyses were performed using unpaired t tests or non-parametric tests when appropriate. These tests were performed using GraphPad Prism software (version 5.01).

Author contributions

Hesham Shehata performed the experiments and analyzed the data. HS, KH and CAC designed the experiments and wrote the manuscript.
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**Conflict of interest**

The authors have no financial conflicts of interest.
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Figure 1. Foxo1 expression is significantly increased in aged splenic NK cells. (A) The mean fluorescent intensity (MFI) of Foxo1 in splenic CD3- NKp46+ NK cells and (B) BM NK cell maturation subsets of young and aged mice. (C) The MFI of Foxo3 in splenic NK cell maturation subsets of young and aged mice. (D) The mRNA expression of Foxo1 in purified splenic NK cell maturation subsets from young and aged mice. Data are representative from at least two independent experiments. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 2. The increased Foxo1 expression in aged NK cells is mainly localized in the nucleus. (A) Foxo1 expression in gated young and aged splenic immature NK cells assessed using the ebioscience fix/perm buffer detecting both cytoplasmic and nuclear Foxo1. (B) Foxo1 expression using 0.3% saponin to detect mainly cytoplasmic Foxo1.
Figure 3. NK cells from aged mice have a higher ROS production and increased DNA damage. ROS production measured by MitoSOX Red in (A) Splenic NK cells and (B) BM NK cells from young and aged mice. (C) The MFI for γH2AX in young and aged splenic NK cells to assess the extent of DNA damage. (D) The surface expression of Rae-1 family members on young and aged splenic NK cells. Data are representative from at least two independent experiments. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 4. mTORC1 activity is significantly increased in aged NK cells and their maturation subsets. mTORC1 activity was measured by assessing the phosphorylation status of S6, (A) and 4-EBP1 (B) at baseline in splenic NK cells from young and aged mice. (C and D) shows the total levels of the non-phosphorylated forms of S6 and 4-EBP1 in young and aged splenic NK cells. Data are representative from at least two independent experiments with each point representing one individual mouse. Mean MFI is shown as horizontal bars. The p values represent the difference between aged and young NK cells (unpaired t test). *P<0.05, **P<0.009 and ***P<0.0005.
Figure 5. Aged NK cells have higher intracellular ATP, but reduced mitochondrial fitness and glucose uptake. (A) Intracellular levels of ATP in sorted splenic NK cell subsets from young and aged mice. (B) Mitochondrial mass in splenic NK cells and their subsets in young and aged mice assessed using Mitotracker Far Red dye. (C and D) show the differences in uptake of the fluorescent glucose analog, 2-NBDG in IL-15 (20ng/ml) treated splenocytes. (E) Analysis using Imaging flow cytometry quantifying the number of LC3 spots in NK cell maturation subsets. Data are representative from at least two independent experiments with each point representing one individual mouse. Mean MFI is shown as horizontal bars. The p values represent the difference between aged and young NK cells (unpaired t test). *P<0.05, **P<0.009 and ***P<0.0005.
Figure 6. Aged NK cells exhibit an impaired proliferative capacity under homeostatic conditions. (A) The percentage of BrdU positive BM NK cells and (B) the percentage of Ki-67+ BM NK cells in both young and aged mice. Data are representative from at least two independent experiments with each point representing one individual mouse. Mean MFI is shown as horizontal bars. The $p$ values represent the difference between aged and young NK cells (unpaired t test). *$P<0.05$, **$P<0.009$ and ***$P<0.0005$. 
Chapter 4: The Lymphoid organs in aged mice have a profound increase in MHC-I low apoptotic cells

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Running title: MHC-I low cells and aged NK cell responsiveness

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Abstract

Aging is associated with a considerable decline in immune function predisposing the elderly to a higher incidence and severity to a wide variety of infectious diseases and cancers. MHC-I expression governs NK cell responsiveness through a process known as licensing where the interaction of Ly49 receptors on NK cells with MHC-I expressing cells confers NK cell functional competence. Here, we evaluated the expression levels of H-2^b class I molecules on different lineages of immune cells in aged mice. While levels of MHC-I were higher in aged mice, we were intrigued to find a significantly higher frequency of MHC-I low cells in their lymphoid organs. Most of the MHC-I low cells in aged mice are late apoptotic/dying cells that are nucleated, have a higher mitochondrial superoxide production and lower mitochondrial mass and potential. Although young BMDMs did not appear to phagocytose nucleated MHC-I low cells, they were efficient at eliminating necrotic cellular debris. The lack of clearance of dying cells may contribute to the inflammatory environment characteristic in aging as well as predispose aged individuals towards autoimmune diseases and impair NK cell education which relies on MHC-I expression. Understanding the mechanisms driving the accrual of these cells could have important implications in improving the overall immune responses in aging.
Introduction

Age-related changes in the immune system contribute to the increased susceptibility of the elderly to infectious diseases, poor responses to vaccines and cancers and the development of autoimmunity [1,2,4-6,15,16]. Many studies have focused on how aging influences the functions of the adaptive immune system. However, increasing evidence suggests that different cellular components of the innate immune system including dendritic cells (DCs), macrophages, neutrophils, natural killer (NK) cells and NK T cells which are all first lines of defense against infectious pathogens and tumor cells are defective in aged mice and humans.

Previous evidence has shown that aged mice have an impaired NK cell terminal maturation which is associated with poor NK cell cytotoxicity [131-134,139]. The underlying mechanisms responsible for this phenotype remain largely unknown. It is now well appreciated that the aged environment plays an important role in regulating the maturation and function of aged NK cells [135,139,148]. However, whether the expression of MHC-I changes in an aged environment has not been evaluated thus far.

We first set out to evaluate the levels of MHC-I expression on different immune cell lineages and while we found that H-2^b^ molecules were similarly or highly expressed in aged versus young mice, we were intrigued to find a large presence of MHC-I low cells in the lymphoid organs of aged mice. Herein, for the first time our results demonstrate a novel finding of the presence of an MHC-I low cell population in aged mice which are particularly prominent in the spleen. We provide an outline describing the phenotype and morphology of these cells as late apoptotic cells that accumulate with age. The consequences for the accumulation of these cells may be profound for the aged immune system. For example, the presence of necrotic cellular debris from these cells may promote autoimmune responses and may enhance
inflammatory responses triggered by TLR recognition of nucleic acids. Furthermore, the absence of MHC-I on these cells maybe critical for influencing NK cell licensing, a process that depends on interactions with MHC-I. The development of NK cells in an environment that constitutes an MHC-I low population is known to yield hyporesponsive NK cells that have a high activation threshold and that are inefficient at eliminating missing-self targets [49,50,97].

Thus, identifying the mechanisms underlying the accrual of MHC-I low cells and how they influence the aged immune system with respect to inflammation, autoimmunity and NK cell hyporesponsiveness is critical for the development of strategies to improve the health of a growing elderly population.
Results

Lymphoid organs in aged mice have a profound increase in the proportion of MHC-I low cells.

MHC-I expression is critical for providing educating signals to developing NK cells, a process termed as licensing. In the BM, immature NK cells that express inhibitory receptors that interact with autologous self MHC-I will proceed to functional maturity. In the absence of these interactions, immature NK cells fail to attain terminal maturation and become hyporesponsive in the periphery and are inefficient at eliminating NK cell targets. Given that aged NK cells are hyporesponsive and immature we asked whether changes in MHC-I expression existed in an aged environment. We began our study by assessing the levels of expression of H-2\textsuperscript{b} class I molecules on different immune lineages in young and aged mice. The expression levels of H-2\textsuperscript{K}\textsuperscript{b} were significantly higher in splenic NK cells, T cells, B cells and CD11c\textsuperscript{+} cells in aged mice compared to young mice (Fig 1A). H-2D\textsuperscript{b} was only more highly expressed on T cells and B cells from aged mice (Fig 1B) while the expression of the non-classical MHC-I molecule, Qa-1, was not different between young and aged splenocytes (data not shown). Unexpectedly and to our surprise, we detected a larger presence of MHC-I low (lacking Ter119, H-2K\textsuperscript{b} and H-2D\textsuperscript{b}) cells in the spleen, BM, liver and peripheral lymph nodes (pLN) of aged mice (Fig 1C, D and data not shown), although no differences were observed in the lung. Overrepresentation of this population was most evident in the spleen of aged mice with a proportion that was tenfold higher than those in young mice (Fig 1D). Notably, there was a high level of heterogeneity in the proportion of MHC-I low cells amongst the aged mice. Importantly, the existence of these cells was not exclusive to C57BL/6J mice as their higher proportions were also evident in the lymphoid organs of aged Balb/c mice to a similar degree as that in aged BL/6 mice (Fig 1E and
These observations suggested that a partial deficiency in MHC-I expression may be characteristic of an aged environment.

**MHC-I low cells are nucleated and express intracellular organelles**

Qualitatively, MHC-I low cells appeared to have fewer organelles or organelles that appeared to be degrading and in some cases they appeared as necrotic cells with the release of cellular debris as evidenced visually using transmission electron micrographs (TEMs) (Fig 2A and data not shown). To further characterize this population of MHC-I low cells in aged mice, we asked whether they were nucleated as all nucleated cells except red blood cells express MHC-I [201,202]. Using imaging flow cytometry to detect positivity for DAPI staining, we found that MHC-I low cells in both young and aged mice were nucleated (Fig 2B), although they appeared to have a significantly lower DNA content as measured by acridine orange using flow cytometry (Fig 2B). In parallel, we also assessed the ratio of DAPI+ H-2+ cells to DAPI+ H-2– cells in young and aged mice. Similar to our initial analysis in Fig 1, we found a significantly reduced ratio of MHC-I sufficient cells to MHC-I low cells in the spleen of aged mice (Fig 2C). Interestingly, we also found the presence of what appeared to be necrotic cellular debris (H-2-DAPI- cells) (Fig 2C) which was consistent with the TEM data (Fig 2A: third example). These MHC-I low cells also contained similar levels of golgi apparatus (golgi), although their levels of endoplasmic reticulum (ER) and mitochondrial mass appeared slightly lower than MHC-I sufficient cells (Fig 2C and data not shown).

**MHC-I low cells are apoptotic and dying cells**

Initial immune phenotyping using a wide variety of hematopoietic and non-hematopoietic markers (CD45, CD3, CD4, CD19, CD11b, CD11c) revealed low or undetectable levels of expression in the MHC-I low population, although they expressed similar levels of non-classical
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Qa1 (data not shown). This also suggested that these cells are not only deficient for MHC-I which is how we discovered them. This further suggested that these cells could potentially be apoptotic or dying cells and as a result would shut down regular cellular machinery and therefore lose expression of surface receptors. We thus, characterized their apoptotic profile by Annexin V and 7AAD staining (Fig 3A and B). In contrast to young mice in which most of the MHC-I low cell population expressed the classic markers of apoptosis, (AnnV+ 7AAD+), only half of the MHC-I low population in aged mice had a similar phenotype. A similar data pattern was observed when these populations were assessed using propidium iodide (PI) staining (Fig 3C). However, the reduced levels of staining with PI, AnnV and 7AAD dyes may also be a consequence of changes in the plasma membrane integrity of these cells as they show a reduced intensity of the lipophilic dye, PKH26 that binds to lipids within the cell membrane (Fig 3D). **MHC-I low cells in aged mice have a higher expression of stress-induced molecules (Rae-1 family) and mitochondrial superoxide.**

In view of the above findings implicating MHC-I low cells as late apoptotic/dying cells, it was of interest to examine their mitochondrial stress through ROS production as ROS is known to induce extrinsic and/or intrinsic apoptotic signaling [203-206]. To this end, we evaluated mitochondrial superoxide production by flow cytometry using MitoSOX Red and observed that MHC-I low cells in aged mice have significantly higher levels than MHC-I low cells in young mice or MHC-I sufficient cells in either young or aged mice (Fig 4A and B). In companion experiments, we also found that MHC-I low cells from aged mice expressed significantly higher levels of Rae-1 family molecules, indicating that these cells may be under environmental stress that may lead to higher ROS (Fig 4C).
Young bone marrow-derived macrophages (BMDMs) phagocytose necrotic cellular debris from splenocytes of aged mice but not nucleated dying cells

To understand why MHC-I low cells persist in aged mice, we asked whether they were targets for phagocytosis. It is well established that the phagocytic capacity of aged DCs and macrophages is considerably impaired [207-211]. Recently, we reported that aged DCs had an impaired capacity to phagocytose apoptotic cells [156]. Given these observations, we hypothesized that an impaired capacity to clear MHC-I low apoptotic cells may contribute to their accrual in aged lymphoid organs. To this end, BMDMs from young mice were cultured with aged splenocytes either at a 1:10 or 1:5 ratios for 4 hours at 37°C or 4°C and the ratio of MHC-I sufficient: MHC- I deficient cells was tracked. Within the allotted time, MHC-I low cells from aged mice were not phagocytosed by young BMDMs (Fig 5A), which was consistent with their lower expression of “Don’t eat me signals” such as CD47 and CD31 (Fig 5C), although MHC-I low cells appeared to express high levels of phosphatidylserine which is an “eat me signal” as assessed by Annexin V staining. Additionally, we observed that young BMDMs were able to reduce the proportion of necrotic cellular debris derived from aged splenocytes (Fig 5B). The data depicted in Fig 5 shows the ratio of the percentages between MHC-I sufficient cells (H-2+ DAPI+) to MHC-I low cells (H-2- DAPI+) or MHC-I sufficient cells (H-2+ DAPI+) to necrotic cellular debris (H-2- DAPI-).

The NK cell expression of licensing receptors is dysregulated in aging

NK cells expressing inhibitory receptors for self-MHC-I are licensed NK cells and have the functional competence to kill their target cells [49-51]. Importantly, NK cells expressing more self-reactive inhibitory receptors have a greater responsive potential which indicates that licensing is a quantitative event [50,212]. Because the aged environment has been shown to be
critical in regulating the maturation and function of NK cells [135,139,148], we next sought to explore whether changes in NK cell education existed in aging. In B6 mice, NK cells expressing Ly49C, Ly49I or NKG2A (CIN+) which bind with a high affinity to H-2^b molecules have the capacity to be programmed for NK cell licensing [50,213,214]. Given that NK cells acquire Ly49 receptors during their maturation in the BM and that the most immature NK cells express the lowest levels of educating Ly49 receptors [100,108], we were prompted to evaluate how the BM NK cell licensing receptor profile was influenced with age. Aged BM NK cells showed a significant reduction in the proportion of NK cells expressing all three education receptors (CIN+ educated NK cells) (Fig 6A).

Concomitantly, there was also a significant increase in aged NK cells that do not express any of the three licensing markers (CIN). In addition, the proportion of NK cells expressing NKG2A alone was almost halved in aged mice (Fig 6B), although the NKG2A expression within the NKG2A+ cells was similar in young and aged mice (Fig 6C). Interestingly, while there was a higher percentage of Ly49C/I+ NK cells in aged mice, the expression of Ly49C/I was significantly reduced within this population (Fig 6D). These findings showed that aged NK cells have a reduced expression of key receptors that are responsible for programming NK cell education and therefore suggested that NK cell education may be impaired in aging which would be consistent with their known hyporesponsiveness to stimuli *in vitro* and *in vivo* [133-135,148].
Discussion

Here, our study reports a novel finding of a large presence of late apoptotic cells in the lymphoid organs of aged mice. This high frequency of apoptotic cells may have profound consequences on the aging immune system including driving inflammation and autoimmunity. Within the immune system, more than $10^9$ apoptotic cells need to be cleared every day. These apoptotic cells are generated in both central and peripheral lymphoid organs where efficient phagocytes such as DCs and macrophages are responsible for clearing these cells.

Failure to clear apoptotic cells or necrotic cell debris may induce inflammatory responses that are initiated by nucleic acid stimulation of toll-like receptors (TLR 3, 7, 8 and 9) leading to the production of type I IFN, TNF-α and other pro-inflammatory cytokines [215,216]. Therefore, it is possible that a high presence of apoptotic cells and necrotic cellular debris may contribute to a more inflammatory environment which is a characteristic feature in aging, a phenomenon commonly termed as “inflammaging”.

Equally important, the inefficient clearance of apoptotic bodies may lead to a constant exposure to self-antigens, potentially priming the immune system for autoimmune responses. In particular, defects in genes responsible in apoptotic cell clearance including C1q and DNase I have been shown to contribute to the development of systemic lupus erythematosus (SLE) and apoptotic bodies as well as circulating DNA are found in the sera of SLE patients [217-220]. More importantly, age is a risk for several autoimmune diseases including rheumatoid arthritis, SLE, giant cell arteritis and monoclonal gammopathies [221-224]. Auto antibodies including rheumatoid factor and anti- nuclear antibodies have also been detected in the sera of elderly individuals [225-227]. Consistent with these findings, aged mice have also been reported to have an increase in sera autoantibodies, and edema within the kidney glomeruli [210]. Together, this
signifies the importance of determining the mechanisms through which the apoptotic and necrotic cellular debris accumulate in aging.

These apoptotic cells also lacked the classical MHC class Ia molecules which is how we first identified them. Interactions between self-MHC-I and inhibitory receptors on NK cells are known to allow for terminal NK cell maturation and to license NK cells to acquire full effector functions [49,50,97,228-231]. Thus, NK cells lacking MHC-I inhibitory receptors or those developing in an MHC-I low environment would be phenotypically the most immature compared to those expressing self MHC-I specific inhibitory receptors [231].

The concept of assessing MHC-I expression in aging is critical because the MHC-I environment in which NK cells develop regulates their responsiveness towards NK cell targets. Many studies have reported that the responsiveness of NK cells can be reset depending on the steady-state MHC-environment to which the NK cells are exposed [49,50,97]. This reprogramming of NK cells ensures that NK cells become tolerant if they are transferred to a new environment with a different self [49,50,97,228]. In fact it has been shown that transferred WT NK cells into MHC-I low hosts become hypo responsive and reciprocally MHC-I low NK cells transferred into WT host acquire functional responsiveness [50,97,232,233]. Additionally, if MHC-I low cells develop together with WT cells in mixed BM chimeras for several weeks, the MHC-I low cells are tolerated [232]. However, transfer of MHC-I low cells into naïve WT hosts results in their rejection within 24 hrs. This demonstrates that the MHC-I environment is a critical determinant of the ability of NK cells to recognize and eliminate missing-self targets.

It is well appreciated that aging profoundly impairs the terminal maturation and functional competence of NK cells. In fact, there is currently a consensus that the aged environment plays a role in regulating NK cell development and function. The development of
NK cells in an aged environment which consists of a population of cells that lack MHC-I would require NK cells to be fine-tuned to have a higher reactivity threshold and thus become hyporesponsive. Many examples in the literature have characterized the behavioral hyporesponsiveness of NK cells to non-self cells. In mosaic B6 mice in which some cells express the H-2D<sup>d</sup> transgenic haplotype and others do not, NK cells do not react against cells that lack the transgene expression. Similarly, in B6 mice in which the MHC-I is replaced by the human HLA-cw3 molecule; NK cells eliminate B6 splenocytes whereas HLA expressing cells are spared as they are recognized as self and are tolerated [234]. Together, these data suggest that NK cells can be readily reprogrammed to missing-self recognition and therefore, they can adapt to an environment with a different “self”. These data also suggest that the increased presence of an MHC-I low population during NK cell development in aging may reprogram aged NK cells to become hyporesponsive and in turn this could be part of the mechanism(s) responsible for the impaired responsiveness of aged NK cells, although this will need to be investigated in the future.

Equally important, the high expression of Rae-1 on aged MHC-I low cells may also lead to persistent stimulation of aged NK cells through NKG2D and would thus reset the NK cell reactivity threshold downwards towards tumor cells that express high levels of Rae-1. As a consequence of this persistent stimulation with Rae-1 and the presence of a large population of MHC-I low cells we hypothesize that this would potentially lead to readjustments in aged NK cell responsiveness leading to reduced NK cell functional competence. Indeed, we observe a dysregulated expression of Ly49C/I and NKG2A in aged BM NK cells. This dysregulated expression is consistent with their hyporesponsive nature. Further studies will need to assess
whether NK cell education is impaired in aging by assessing the degranulation capacity of the licensed NK subsets in response to missing self targets *in vitro*.

Future studies will also need to determine why these MHC-I low cells accumulate in aging and whether they have a direct role in impairing the responsiveness of NK cells. Our preliminary data from our phagocytic assays suggested that they could not be eliminated by macrophages. These findings were interesting particularly because we found that these MHC-I low cells expressed lower levels of “Don’t eat me signals” such as CD47 and CD31 when compared to MHC-I sufficient cells. In an attempt to study how these MHC-I low cells could influence NK cell responsiveness, we tried either to purify them or label them with CFSE, PKH26 or cell trace violet, but our attempts have not been very successful thus far. In fact, visually the electron micrographs suggested that these cells were not “metabolically fit” as evidenced by their lower levels of intracellular organelles and TEM morphology.

Our future directions are to build on our preliminary data by determining whether culturing activated NK cells with aged splenocytes leads to a lower proportion of MHC-I low cells. We will also test whether the transfer of young NK cells into aged recipients leads to a reduction of aged MHC-I low cells *in vivo*. Determining the factors regulating the homeostasis of these MHC-I low cells and their role in influencing inflammaging and the propensity for autoimmune development as well as NK cell development and function in aging will be important in developing strategies to augment NK cell responsiveness in aging.
Experimental Procedures

Mice
C57BL/6 female mice were purchased from either Taconic Farms (Germantown, NY), The Jackson Laboratory (Bar Harbor, ME), or from the National Institutes of Aging (NIA) colony at Taconic Farms. Young mice are 2-3 months of age and aged mice are ≥ 16 months of age. All mice were acclimatized for at least 1 week before conducting any studies. Mice were housed under specific pathogen-free conditions in the Cincinnati Children’s Hospital Medical Center (CCHMC) vivarium. All animal protocols were reviewed and approved by CCHMC Institutional Animal Care and Use Committee.

Cell preparations

Bone marrow cell preparation: The hind legs were dissected and carefully removed from the surrounding muscle tissue. The femurs and tibia were then crushed using a mortar and pestle in 10 ml of phosphate buffered solution (PBS, Mediatech, Manassas, VA) containing 2% FBS (Invitrogen, Carlsbad, CA). The obtained solution containing the bone marrow was filtered on a 70-micron cell strainer and washed twice before red blood cell lysis with Ammonium-Chloride-Potassium (ACK) (prepared in house).

Spleen and peripheral lymph node (pLN) cell preparation: Spleens and pLNs (inguinal, axillary, brachial and cervical) were homogenized with a 3 ml syringe plunger through a 100-micron cell strainer using buffered saline solution (BSS) (prepared in house) and red blood cells were lysed with ACK lysis buffer (prepared in house).

Liver cell preparation: Single liver cell suspensions were prepared by first homogenizing the whole tissue in RPMI 1640 using a gentle MACS dissociator (Miltenyi Biotech, Auburn, CA). The resulting homogenate was then centrifuged at 2,000 rpm and the cell pellet was mixed
with 33% Percoll (Sigma-Aldrich) in RPMI 1640 solution (Invitrogen). The Cell suspension was then centrifuged at 2,000 rpm for 20 minutes at room temperature. The cell pellet was removed and washed, and red blood cells were lysed with ACK lysis buffer (prepared in house). Single cell suspensions from all tissues analyzed were then washed with RPMI 1640 (Invitrogen) containing 10% FBS and viable cells were counted via trypan blue (MP Biomedicals, Solon, OH) exclusion.

**Antibodies and flow cytometry**

10⁶ cells from single cell suspensions were suspended in 100 µL fluorescence-activated cell sorting buffer and Fc receptor was blocked with anti-mouse CD16/32 (clone 93, Biolegend). The following mAbs (purchased from eBioscience, San Diego, CA, BD, San Jose, CA, Biolegend, San Diego, CA or Cell Signaling, Technology, Beverly, MA) were used: anti-CD3 (500A2), anti-NKp46 (29A1.4), anti-CD27 (LG.3A10 or LG.7F9), anti-CD11b (M1/70), anti-CD45 (30-F11), anti-Ter119 (TER-119), anti-H-2Kᵇ (AF6-88.5), anti-H-2Dᵈ (34-2-12), anti-H-2 (M1/42), anti-H-2Dᵈ (34-2-12), anti-H-2Kᵈ (SF1-1.1), Acridine orange (Life Technologies), ER tracker blue-white DPX (Life Technologies), Wheat Germ Agglutinin (Life Technologies), Annexin V and 7AAD (BD Pharmingen), PI (Immuno Chemistry Technologies), anti-Rae-1 (186107), anti-NKG2A (16A11), anti-Ly49C/I (5E6). ROS were measured by incubation with 5µM MitoSOX Red (Life Technologies) after staining surface markers. NK cells are identified as CD3- NKp46+ cells. MHC-I low cells are additionally characterized as Ter119 negative cells to exclude red blood cells. Flow cytometry data were collected using an LSRII or LSR Fortessa (BD) flow cytometers and analyzed by BD FACS Diva software.

**Staining MHC-I for transmission electron microscopy**

Whole splenocytes from aged and young mice were stained with biotinylated anti-mouse H-
2K$^b$ D$^b$ and were subsequently stained with anti-biotin conjugated microbeads. MHC-I sufficient cells were identified as cells with a high concentration of cell membrane associated microbeads. In contrast, MHC-I low cells were cells identified as lacking cell surface associated microbeads.

**In vitro generation of BMDMs**

BM cells isolated from femurs and tibias of young mice were cultured in 100 mm petri dishes in the presence of 20 ng/ml of murine GM-CSF for 7 days. On day 7, the cultured cells were isolated and plated in 96 well culture plates at 3X10$^5$ cells per well and left in culture for another two days to promote maximum adherence. Most of the cells ($\geq 75\%$) generated were CD11b$^+$ CD11c$^+$ cells as determined by flow cytometry. On day 9, aged splenocytes were added at different ratios to the BMDM cultures as indicated in Fig 5.

**Statistical analyses**

Statistical analyses were performed using unpaired $t$ tests or non-parametric tests when appropriate. These tests were performed using GraphPad Prism software (version 5.01).

**Author contributions**

Hesham Shehata performed the experiments and analyzed the data. HS, KH and CAC designed the experiments and wrote the manuscript.

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**Conflict of interest**

The authors have no financial conflicts of interest.
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Figure 1. The lymphoid organs of aged mice exhibit a profound accrual of MHC-I low cells. (A) The mean fluorescence intensity (MFI) of H-2K^b and (B) H-2D^b in young and aged splenic NK (CD3- NKp46^+), T (CD3^+), B (CD3- CD19^+) and CD11c+ (CD3- CD11c^+) cells. (C and D) Flow cytometric analysis and enumeration of the frequency of MHC-I low (H-2K^b and H-2D^b double deficient) cells in lymphoid organs of young and aged B6 mice. (E and F) Flow cytometric analysis and enumeration of the frequency of MHC-I low (H-2K^d and H-2D^d double deficient) cells in lymphoid organs of young and aged Balb/c mice. Data are representative from at least two independent experiments. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 2. MHC-I low cells in aged mice are nucleated. (A) Transmission electron micrographs (TEMs) of MHC-I sufficient and deficient cells from aged splenocytes. (B) ImageStream-based flow cytometric analysis visually representing DAPI nuclear staining in MHC-I sufficient and MHC-I low cells within splenocytes of aged mice. (C) ImageStream-based assessment of the levels of Endoplasmic reticulum (ER) and golgi apparatus (golgi) content in MHC-I sufficient and MHC-I low splenocytes in aged mice. (D) Quantification of the amount of DNA present in MHC-I low and MHC-I sufficient cells in aged and young splenocytes using acridine orange. (E) Flow cytometric analysis depicting the ratio of nucleated MHC-I low cells to nucleated MHC-I
sufficient cells. Data are representative from at least two independent experiments. The $p$ values (unpaired t-tests) represent the difference between aged and young mice. *$P<0.05$, **$P<0.009$ and ***$P<0.0005$. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 3. MHC-I low cells are apoptotic and dying cells. (A-C) Flow cytometric analysis of the apoptotic/dying phenotype of MHC-I low and sufficient cells in young and aged splenocytes using Annexin V and 7AAD (A and B) or propidium iodide (PI) staining (C). (D) Representative staining profile of the plasma membrane lipophilic dye, PKH26 on MHC-I sufficient and MHC-I low cells in aged splenocytes. Data are representative from at least two independent experiments. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 4. MHC-I low cells from aged mice have a higher ROS production and expression of Rae-1. (A and B) Representative figure showing MitoSox Red staining in splenic MHC-I deficient and sufficient cells in young and aged mice. Antimycin (120µM) treatment was used as a positive control for inducing an increased level of ROS production. (C) Rae-1 expression on splenic MHC-I low and sufficient cells from young and aged mice. Data are representative from at least two independent experiments. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 5. Young BMDMs clear necrotic cellular debris but not nucleated apoptotic cells.

(A) Ratio of MHC-I sufficient to deficient cells analyzed using H-2 and DAPI staining (as in Fig 2C) 4 hours after co-cultures with BMDMs at the indicated ratios. (B) Ratio of MHC-I sufficient cells to “clutter” defined as the H-2 negative DAPI negative population. (C) The differential expression profile of the “Don’t eat me signals,” CD47 and CD31 on MHC-I sufficient and MHC-I low cells in aged splenocytes. Data are representative from at least two independent experiments. Means are shown as horizontal lines with each point representing one individual mouse.
Figure 6. The proportion of educated NK cells is reduced in aged mice (A) Analysis of BM NK cell education in young and aged mice based on the expression of Ly49C/I and NKG2A within the CD3- NKp46+ NK cell population. Numbers in pie charts represent the mean of the proportion of each subset. (B) The proportion of BM NK cells expressing either Ly49C/I or NKG2A. The MFI of NKG2A (C) and Ly49C/I (D) within the NKG2A+ and Ly49C/I+ BM NK cells respectively in young and aged mice. The p values (unpaired t-tests) represent the difference between aged and young mice. *P<0.05, **P<0.009 and ***P<0.0005. Means are shown as horizontal lines with each point representing one individual mouse.
Chapter 5: Summary and Discussion

NK cells are at the center of the primary immune responses against viral infections and tumor immunosurveillance. Reduced functional competence of NK cells either due to mutations affecting cytolytic functions or impaired NK cell development results in a considerable increase in susceptibility to cancer development and infectious diseases including HSV, HPV, VZV and candidiasis [42]. As the front line defense against virally infected and malignant cells, the impaired maturation and function of NK cells in aging may contribute in part to the increased incidence of tumors and viral infections reported in the elderly. Therefore, it is imperative to identify the factors that contribute to the impaired development and reduced cytotoxic potential of NK cells in aging. Identifying these factors could have important implications in the development of therapeutic strategies to augment aged NK cell functional maturation and ultimately alleviate the burden of cancer and viral infections in the elderly population. In this section of the thesis, we summarize the most important findings of our research and the potential underlying causes to the impaired homeostasis, maturation and function of aged NK cells are discussed.

5.2 The factors responsible for the impaired maturation and function of NK cells in aging

Identifying the specific factor(s) that play a role in impairing NK cell maturation and function in aging is currently of major interest in the field. In this section, we discuss the factors that do not and those that may play an important role in regulating the NK cell phenotype in aging.

a) Immature and transitional NK cells in aged mice have an impaired proliferative capacity.

It has been shown previously that immature NK cells have a higher proliferative capacity than transitional and mature cells [90,107,123]. The proliferation of aged immature and
transitional NK cells as measured by both BrdU incorporation and the expression of the nuclear proliferation antigen, Ki-67 was significantly decreased compared to their young counterparts (Chapter 3) [132,139].

This certainly indicated that the increased proportion of immature NK cells in the BM and the periphery of aged mice is not a result of their increased proliferation. This reduced proliferative potential particularly in aged BM immature NK cells suggests that these cells may not receive key signals that drive their proliferation. Overall, these results also suggest that aging is associated with an arrest of NK cell maturation at the immature phase. As a consequence of this phenomenon, immature NK cells accumulate in the BM and periphery and simultaneously fail to proliferate.

b) The aged environment plays a critical role in impairing NK cell maturation

As with many cells of the immune system, NK cell maturation and function is dependent on their interactions with other hematopoietic and non-hematopoietic cells. Thus, to determine whether the underlying mechanism(s) responsible for the reduced maturation of aged NK cells were NK cell intrinsic or extrinsic, three independent studies established mixed BM chimeras in which young and aged BM cells either depleted of NK cells or not were co-transferred into sub-lethally irradiated young and aged congenic recipients (see chapter 2) [135,139,148]. Evidence from these studies shows that immature NK cells and BM hematopoietic stem cells (HSCs) from aged mice effectively generated mature NK cells as efficiently as their young counterparts only when they developed in a young host environment. In apparent contrast to these findings, young and aged immature NK cells and HSCs had an impaired maturation that was similar to aged mice when they matured in an
aged host environment. Together, these complementary studies show that extrinsic defects in the aged environment play a critical role in impairing the maturation of NK cells (Figure 4).

c) The impaired maturation of aged NK cells is determined by the BM microenvironment and not the peripheral environment.

In an attempt to define whether the aged peripheral or BM environment housed the defects responsible for the impaired maturation of NK cells, we first interrogated whether the aged peripheral environment could support NK cell maturation. Collectively, the mixed BM chimera and peripheral adoptive transfer experiments show that the environment within the BM of aged mice plays a dominant role in limiting the maturation of NK cells (Figure 4).

![BM chimera and Peripheral NK cell transfer diagram]

**Figure 4.** The young BM environment as well as young peripheral and aged peripheral environments are capable of supporting efficient NK cell maturation. In contrast, the aged BM microenvironment has an impaired capacity to support NK cell maturation, suggesting extrinsic defects in the aged BM microenvironment. Interestingly, aged immature NK cells have an impaired maturation even in a young environment, suggesting intrinsic defects in aged peripheral immature NK cells.
d) The impaired maturation of aged NK cells is associated with their impaired cytotoxicity

It has been proposed that the impaired maturation of aged NK cells may be responsible for their impaired function particularly given that during terminal maturation NK cells become more efficient at eliminating target cells both \textit{in vitro} and \textit{in vivo} [107,109,110]. Thus an over representation of immature NK cells in aging may contribute in part to their impaired cytotoxicity. These observations prompted us to evaluate whether improved NK cell maturation in aging was associated with an improved functional responsiveness. In parallel to the mixed BM chimera studies, the assessment of aged NK cell degranulation and IFN-\(\gamma\) production 6 weeks post chimerism revealed that NK cells derived from both young and aged BM cells became hyporesponsive to stimuli \textit{in vitro} after maturing in an aged environment. In contrast, those developing in a young environment adopted normal responsiveness to stimuli \textit{in vitro} [135]. These results showed that there is a close parallel between the maturation status of NK cells and their functional responsiveness and that the aged environment also impairs NK cell functions.

e) The impaired NK cell maturation and function in aging is associated with a dysregulation of key transcription factors

Transcription factor analysis revealed that the expression of T-bet, Eomes (Chapter 2) and Aiolos (data not shown) were significantly decreased in aged BM NK cells [135,139]. In contrast, the expression of Gata-3 as well as Blimp-1 which are also critical for NK cell development appeared to be similar between young and aged NK cells [103,104,135].

One discrepancy between NK cells from T-bet deficient mice and aged mice relates to survival. In contrast to T-bet deficient NK cells which appeared to undergo increased rates of cell death, aged NK cells had a similar survival [101,132]. These findings, however, do not explain the increase in NK cell numbers within the BM of both aged and T-bet deficient mice. It has
been reported that T-bet plays an integral role in regulating NK cell trafficking from the BM through the direct regulation of S1P₅ expression. In fact, Jenne et al showed that T-bet binds to a conserved region 3' of the S1pr5 locus which regulates NK cell egress from the BM [117]. Thus, factors leading to the reduced NK cell expression of T-bet in aging may arguably lead to a reduced S1P₅ expression which could explain the accrual of NK cells in the BM of aged mice.

Therefore, identifying which extrinsic factors regulating T-bet and Eomes could provide clues as to how the aged environment impairs NK cell cytotoxicity and maturation. Alternatively, retroviral transduction of both T-bet and/or Eomes into immature NK cells could be tested to determine whether these transcription factors have a direct role in augmenting aged NK cell maturation and function.

Below, we discuss the different factors that are known to regulate T-bet and Eomes and which we have evaluated thus far. We also discuss other candidate factors which would be of interest to probe in-depth in future studies.

Several environmental components in aging could underlie the impaired maturation and function of NK cells including 1) reduced production of IL-15, the key NK cell biological fuel that is essential for their maturation and function, 2) dysregulated MHC-I expression yielding hyporesponsive NK cells, 3) Impaired BM mesenchymal stromal cell composition and 4) Defects in BM mesenchymal stromal cell expression of key ligands or cytokines required to promote NK cell maturation.
5.2 Factors regulating T-bet and Eomes in NK cells may provide insight into the extrinsic defects in the aged environment

a) Administration of exogenous IL-15/IL-15Ra complex does not augment NK cell maturation in aged mice

One critical factor that induces the expression of T-bet and Eomes is IL-15 [101,111], which has been noted to be a pivotal cytokine that regulates various aspects of NK cell homeostasis and function [81-86,235]. IL-15 specifically promotes NK cell maturation by inducing the expression of T-bet and Eomes. This pathway occurs in part due to the phosphorylation of Foxo1 leading to the release of T-bet repression from Foxo1 [106]. In light of this fact, three independent studies investigated whether a dysregulation of IL-15 in aging could be responsible. Intriguingly, the administration of IL-15/IL-15Rα complex to aged mice failed to reverse their defects in maturation, despite further enhancing NK cell maturation in young recipients [135,139]. Interestingly, the IL-15/IL-15Rα complex further decreased the frequencies of transitional and mature NK cells in aged mice [139]. Notably, this phenotype was not a consequence of a lack of response of aged NK cells to IL-15 as aged NK cells in particular; immature cells expanded profoundly post IL-15/IL-15Rα administration in the BM [135,139].

If IL-15 was the limiting factor in aging, then administering IL-15 would compensate for this deficiency and augment NK cell maturation in aging. However, a lack of IL-15 does not appear to be responsible for the impaired maturation of NK cells in aging as administering the IL-15/IL-15Rα complex in vivo did not augment BM and peripheral NK cell maturation in aged mice [135,139]. These data show that despite the normal proliferative response of aged NK cells to IL-15, maturation is not corrected. This suggests that the pathways through which IL-15 regulates NK cell proliferation are different from those governing NK cell maturation. IL-2/15Rβ
is associated with Jak1 and the γc is associated with Jak3, resulting in STAT3 and STAT5 phosphorylation, respectively, after ligation with IL-15. This pathway mainly regulates NK cell proliferation and survival. In contrast, IL-15 mediated phosphorylation of Foxo1 through PI3K/AKT plays a more important role in predominantly regulating NK cell maturation. For this reason, we have utilized both T-bet and Eomes expression as a reliable readout for how the environment regulates NK cell maturation in aging. Interestingly, T-bet and Eomes expression were not augmented when aged mice were administered a high dose of IL-15 (data not shown). This suggested that there may be a suppressive factor that imposes inhibitory signals on the upregulation of T-bet and Eomes mediated by IL-15. It will therefore be critical to determine whether in vivo IL-15 administration in aged mice leads to enhanced Foxo1 phosphorylation and how that reflects on T-bet in order to dissect the causal relationship between Foxo1 and T-bet in splenic NK cells. While this phenomenon may not be impaired in BM NK cells of aged mice, an important factor regulating T-bet in BM NK cells is the interactions between stromal cells and immature NK cells [95,96]. Therefore, identifying the factors that limit T-bet expression in aged NK cells even in the presence of high IL-15 is critically important to our understanding of how aging limits NK cell maturation.

In apparent contrast to our in vivo findings, Chiu and colleagues reported that the administration of the IL-15/IL-15Rα complex to aged mice reversed the maturation phenotype of aged NK cells [148]. This discrepancy could be attributed to the differential analysis of NK cell maturation based on CD27 and CD11b markers. Chiu et al did not explicitly assess the frequency of the different NK cell maturation subsets on the basis of CD27 and CD11b, but instead grouped the subsets as CD27+, CD90+ or CD11b+. Using this strategy, individual subsets in particular immature cells are grouped with transitional cells as one subset (CD27+) and
transitional and mature cells are grouped as another (CD11b+). Thus, the ability to establish precise conclusions may be compromised with this form of analysis. Additionally, Chiu et al reported that treatment of aged mice with IL-15/IL-15Rα complex enhanced their in vivo cytotoxicity against missing-self targets and therefore while IL-15 may not enhance NK cell maturation in aging, it could potentially be used as a therapeutic strategy to boost NK cell functionality in the elderly [148].

Consistent with the lack of augmented maturation post IL-15/IL-15Rα treatment, aged mice did not acquire enhanced resistance to infection with mouse pox as their rate of survival was comparable to untreated aged mice [139]. This lack of protection shows that despite an expansion of immature cells, the presence of mature NK cells is critical for optimal resistance to viral spread and mortality [109,131]. Overall, IL-15/IL-15Rα complex treatment does not augment NK cell maturation nor provide enhanced resistance to ECTV infection in aged mice, but maybe used as a strategy to boost aged NK cell cytotoxicity in vivo.

On another front, these experiments certainly do not address precisely whether there is a reduced production of IL-15 in aging and thus, the use of IL-15 reporter mice in the future will be invaluable in answering this question thoroughly. Nevertheless, Cui and colleagues recently showed that that the number of IL-15+ as well as IL-15 levels increased in splenic stromal cells from middle-aged mice (10 month old) compared to young mice and complementary studies by our group found similar levels of IL-15mRNA in whole BM cells of aged and young mice [135,167]. Taken together, these data suggest that the production of IL-15 by stromal cells in aging is unlikely to be compromised (Figure 1). Importantly, Marçais and colleagues recently reported that mTOR activity was mainly under the control of IL-15 and that IL-15 was the only cytokine to establish a considerable increase in mTORC1 activity as assessed by the
phosphorylation status of S6 which is a direct downstream target of mTORC1. These data suggested that assessment of mTORC1 activity could be used as a surrogate for IL-15 bioavailability in vivo. Interestingly, analysis of the phosphorylation status of S6 and 4-EBP1 demonstrated a higher mTORC1 activity in aged NK cells compared to young NK cells. This suggested that IL-15 bioavailability and NK cell responsiveness to IL-15 were unlikely to be dysregulated in aging.

While the aged environment and in particular the non-hematopoietic compartment has thus far been implicated in playing a role in impairing NK cell function and maturation, it is important that researchers in the field do not rule out other potential hematopoietic factors as these may also affect NK cells either directly or indirectly. One example of this as suggested by Chiu et al is the possibility for increased competition for IL-15 bioavailability in vivo which may arise from the clonal expansion of CD8+ T cells and regulatory T cells (Treg), both of which are dependent on IL-15 for their maintenance and homeostasis [148,236,237]. Aged Treg have been shown to have a higher expression of CD122 and thus could potentially outcompete NK cells for IL-15. In addition, the accrual of Treg in aging [236,238,239] could impair NK cell development and function as other groups have reported that TGF-β, IL-10 and Treg can limit NK cell maturation and function respectively [90,240-243]. However, preliminary results from our laboratory shows that neither the depletion of CD8+ T cells (using a CD8β depleting antibody) nor Treg depletion (using the DEREG model) augmented the maturation status of NK cells in aged mice. Moreover, the in vivo blockade of IL-10 signaling also had no effect on NK cell maturation in treated aged mice [148]. Future studies will need to evaluate whether these factors may hamper NK cell function in the clearance of tumor cells or virus infection, but thus far there is no strong indication that these factors influence the maturation of NK cells in aging. All in all,
our data and those from other groups suggest that impaired IL-15 production or signaling are unlikely to be the major mechanisms driving the impaired maturation and function of NK cells in aging and that increasing in vivo levels of IL-15 does not override the defects responsible for the impaired maturation of aged NK cells.

b) Dysregulation of Foxo1 and mTORC1 in aged peripheral NK cells may contribute to their impaired maturation and function

The finding of the significant increase in the expression of a negative regulator of T-bet, Foxo1, in aged splenic NK cells prompted us to assess the factors that could be responsible for increased Foxo1. Foxo1 has a well-known role in counteracting ROS and DNA damage. The increased levels of mitochondrial superoxide and DNA damage in aged NK cells correlated with increased Foxo1 levels. Higher Foxo1 in aged NK cells may be part of a feedback loop geared at reducing DNA damage and mitochondrial superoxide levels. We hypothesize that the increased Foxo1 results in decreased levels of T-bet and this in turn may be one of the contributors leading to impaired maturation and function of NK cells in aging and that this may be part of the intrinsic defects in aged splenic NK cells. Importantly, the reduced expression of T-bet and CD62L in aged NK cells are commensurate with increased Foxo1 levels.

The increased activity of mTORC1 correlated well with reduced autophagy and increased mitochondrial superoxide. With respect to the latter, mTORC1 has been shown to regulate mitochondrial function and oxidative phosphorylation and thus may also influence the rate of mitochondrial superoxide production [193,195]. Thus we envision a pathway in which high levels of mTORC1 could lead to higher production of mitochondrial superoxide which leads to increased DNA damage. This in turn increases Foxo1 which leads to increased repression of T-bet. On another front, it is virtually possible that the increase in mTORC1 and mitochondrial
superoxide are directly unrelated and could be triggered by independent events. Therefore, interventions geared at normalizing the expression of Foxo1 and mTORC1 activity through the reduction of ROS using scavengers such as NAC and rapamycin may be important for augmenting NK cell maturation and function. In these studies, the expression of ROS, Foxo1, T-bet, DNA damage and mTORC1 activity will need to be evaluated. Such studies will provide mechanistic evidence on whether the dysregulation in mTORC1 and Foxo1 has a role to play in augmenting aged NK cell maturation and function.

In parallel, assessing whether changes in the serum as well as splenic mRNA levels of PDGF and TNF that are known to regulate Foxo1 expression may provide insight on how the aged environment impairs NK cell maturation and as to why splenic aged NK cells have a higher Foxo1 expression. Equally important, the differential expression of Foxo1 in aged splenic and BM NK cells may reflect that several differential mechanisms and not a single mechanism are responsible for the impaired function and maturation of aged NK cells.

c) The development of aged NK cells in a partially deficient MHC-I environment may contribute to their hyporesponsiveness

In B6 mice, licensed NK cells express one or more of the three receptors that bind appreciably to H-2$^b$ class I molecules, Ly49C, Ly49I and CD94/NKG2A. Preliminary results from our group and that published by others shows that the frequency of NK cells expressing these inhibitory receptors is significantly lower in aged BM and peripheral NK cells [139]. A dysregulation in NK cell education receptors or the levels of MHC-I expression may influence NK cell licensing through Ly49-MHC-I interactions. Consequently, this could reduce the optimal NK cell reactivity threshold in aging leading to the generation of NK cells that are hyporesponsive against targets and stimuli both in vitro and in vivo (Figure 3).
The acquisition of NK cell tolerance over repeated exposure to MHC-I low cells or when developing in their presence has received widespread interest. In fact, recent studies underline the plasticity of NK cells by demonstrating that NK cells can become hyporeactive when they develop with MHC-I low cells or if they are transferred into an MHC-I low host. The resetting of NK cell reactivity is associated with a tolerant phenotype where these NK cells become hyporesponsive to missing-self targets and to activating receptor cross-linking with plate-bound antibodies in vitro. We unexpectedly discovered a population of MHC-I low cells in the lymphoid organs of aged mice. While the proportion of these cells was variable in aged mice, on average they constituted about 10-20% of splenocytes. However, this proportion is heterogeneous among aged mice, albeit being significantly elevated compared to that observed in young mice. Importantly, we have evidence of a direct correlation between the presence of MHC-I low cells and the impaired NK cell maturation in aging. Generally, aged mice with a higher proportion of MHC-I low cells have a more severely impaired NK cell maturation. This is further supported by evidence that there is an intermediately impaired NK cell maturation in middle-aged mice which also have a reduced proportion of MHC-I low cells compared to aged mice.

Given that NK cells are highly plastic to the MHC-I environment in which they develop, we hypothesized that a partial deficiency in MHC-I expression in the aged environment may contribute to the impaired function of NK cells. The existence of these cells in an aged environment may impose a tolerogenic phenotype on NK cells configuring them for a hyporesponsive immature phenotype. Our hypothesis is consistent with data from our mixed BM chimeras where we found that the higher proportion of MHC-I low cells present in aged mice
was commensurate with a more pronounced impairment in NK cell maturation at week 2 than at week 6 where the proportion of MHC-I low cells was not as profound as that at 2 weeks.

We reasoned that the elimination of these cells could have a considerable impact on aged NK cell development and therefore, we set out to learn more about the phenotype of these cells. Our data shows that MHC-I low cells are apoptotic/dying cells, but the mechanism behind their existence or lack of clearance remain unknown. The data further suggests that these MHC-I low cells contain reduced DNA content as well as reduced levels of organelles such as ER and mitochondria. These data were consistent with the apoptotic phenotype of these cells which depicts a reduction in the general cellular fitness of these cells.

Given the well-established relationship between NK cell responsiveness and MHC-I, the potential role these cells play in aging raises profound interest in the field as developing strategies to siphon these cells could have important implications in augmenting the function of NK cells in aging. Indeed, in collaboration with Dr. Edith Janssen, we showed that the ability to phagocytose apoptotic cells by DCs decreases significantly in aging both in terms of the total amount and size of the particles taken up [156]. Recognition and clearance of apoptotic cells by phagocytes plays pivotal roles in the development, maintenance and resolution of inflammation. Impaired phagocytosis of apoptotic cells in aging ultimately could lead to their accrual. To test this hypothesis, we cultured bone marrow-derived macrophages (BMDMs) with aged splenocytes and observed that there was no significant difference in the frequency of MHC-I low cells in the absence or presence of BMDMs. Surprisingly, the young BMDMs were able to phagocytose necrotic cellular debris from aged splenocytes and this clean-up process would be important for reducing circulating nuclear antigens that have the potential to cause inflammation and autoimmunity.
Another interesting approach to rid the MHC-I low cells is to induce a break in tolerance to the MHC-I low population. As reported by Sun and Lanier, NK cell tolerance to missing-self targets can be broken in the presence of a high inflammatory stimulus e.g. MCMV [233]. Observations from those studies suggested that NK cell tolerance to missing-self is actively maintained but is not permanent and NK cells can be “reset” to readily eliminate missing-self targets in their developing environment using a physiological stimulus such as a viral infection. Taking this further, our future experiments aim to determine whether a break in tolerance to MHC-I low cells would lead to their elimination in vivo.

Importantly, it has been well established that lymphocytes undergoing FasL-induced apoptosis release the anti-inflammatory cytokine TGF-β. Additionally, macrophages which have ingested apoptotic cells also produce TGF-β. TGF-β has been shown to impose constraints on NK cell maturation through the downregulation of T-bet, Eomes, IRF-2 and GATA-3 [90]. Therefore, it is possible that with the high frequency of apoptotic cells in aging may in turn lead to an augmented TGF-β production.

Taking this knowledge together with the inability of IL-15 to augment NK cell maturation in aging, it remains possible that the presence of a suppressive environmental factor in the aged environment such as TGF-β may prevent the optimal response to aged NK cells to IL-15, thus reflecting the lack of maturation in the presence of a high dose of IL-15. In future experiments, we will evaluate whether depleting TGF-β in vivo plays a role in limiting NK cell terminal maturation in aging.

Overall, our work shows that the aged nonhematopoietic BM environment is responsible for the impaired terminal maturation and function of NK cells in aging (Figure 5). This impaired maturation and function is accompanied by a decrease in the expression of positive regulators of
NK cell maturation such as T-bet, Eomes and Aiolos and a concomitant increase in negative regulators such as Foxo1 (Figure 5). We hypothesize that an increased Foxo1 expression may be a consequence of increased ROS and DNA damage which was detected in aged NK cells. This increased levels of genotoxic and oxidative stress may lead to increased Foxo1 which downregulates T-bet, hence impairing NK cell functional maturation. Interestingly, this impaired maturation is accompanied by increased levels of mTORC1 activity which may lead to higher mitochondrial activity, increased ATP levels and mitochondrial superoxide production as well as reduced levels of autophagy (Figure 5).

Our novel finding of the profound presence of MHC-I low cells in the lymphoid organs of aged mice may also contribute to the hyporesponsiveness and impaired education of aged NK cells. The persistence of these cells could impose aged NK cells to reprogram their functional reactivity downwards and would promote tolerance to missing self by raising their threshold of reactivity. Taken together, we hypothesize that the collective effects of all these various factors impose a phenotype that reflects on the impaired maturation and function of NK cells in aging.

**Figure 5.** A summary of the defects in NK cells in aging and how the aged environment may contribute to the impaired maturation, education and function of NK cells through regulating multiple mechanisms such as different transcription factors like T-bet and Eomes.
Chapter 6: Future Directions

Chapters 3 and 4 raise a number of interesting questions and areas of future research:

**Determine directly whether the BM microenvironment in aged mice does not support NK cell maturation.** We will adoptively transfer CFSE-labeled congenic immature BM NK cells from young and aged donors into the femurs of young and aged recipients. This will allow us to assess the maturation of the transferred cells in the BM and the cells that exit the BM into the periphery. We expect that the aged BM will yield impaired NK cell maturation compared to the young BM.

We are also interested in determining whether the decreased T-bet expression in aged NK cells lead to their accumulation in the BM due to reduced S1P₅ expression. As outlined in chapter 1, the paucity of peripheral NK cells (spleen) in aged mice is accompanied by an accrual of NK cells especially immature cells in the BM. Given that T-bet directly regulates the expression of S1P₅, the chemotactic receptor that plays an essential role in promoting the egress of NK cells from the BM, we hypothesize that the reduced aged BM NK cell T-bet expression may translate to a decrease in S1P₅ expression. To this end, we will evaluate the expression of S1P₅ in purified immature BM NK cells from young and aged mice. We expect to find a reduced S1P₅ expression in immature BM NK cells from aged mice, consistent with a reduced T-bet expression. In addition, we will also evaluate whether the immature aged/young NK cells in the femoral transfer experiments described above have a reduced capacity to exit the BM and how this correlates to T-bet expression in NK cell developing in either a young or aged BM microenvironment.
Are aged BM stromal cells able to support optimal NK cell development?

HSCs are generally in close proximity with a highly organized 3-dimensional structure formed by stromal cells, called the BM niche. The important contribution of the BM stromal cells to providing a supportive BM niche has been well documented and is supported by the use of BM stromal cell lines that support HSC differentiation in vitro. The stromal cell compartment of the BM is composed of cells of various lineages, including osteoblasts, adipocytes, chondrocytes, endothelial cells and fibroblasts all of which derive from mesenchymal stem cells [244-249]. Importantly the specific role of osteoblasts in supporting the HSC niche in particular, lymphopoiesis has been noted [250-253].

Interactions between NK cell progenitors and BM stromal cells in vivo and in vitro are critical for the generation of mature NK cells. Interestingly, it was shown that aged NK cells and all their constitutive maturation subsets have a significantly lower expression of the integrin, α2β1 (CD49b CD29), the receptor for type I collagen and a higher expression of the α2β1 (CD49a CD29) which forms the receptor for type IV collagen [139]. Importantly, both type I and type IV collagen are produced by BM stromal cells. This altered receptor expression could impair immature NK cell adhesion to BM stromal cells, thus limiting their capacity to progressively achieve terminal maturation. Related to these findings, recent data illustrated that aged HSCs and progenitor cells (HSPCs) have a reduced capacity to form favorable adhesive interactions with BM stromal cells [254,255]. It is possible that the dysregulated collagen receptors on immature aged NK cells promote less favorable interactions with stromal cells, ultimately impairing terminal maturation.

Germane to this discussion, interactions between Tyro3 receptors (Tyro3, Mer and Axl) on NK cells and their ligands, Gas6 and protein S on BM stroma appear critical for NK cell
maturation [95]. Additionally, blocking the Gas6 and protein S pathway was shown to reduce NK cell T-bet expression [96]. Whether a reduction in the expression of Gas6 and protein S in aged BM stromal cells contributes to the reduced maturation of NK cells in aging remains unexplored (Figure 2). Preliminary data from our lab suggests that the stromal cell composition and the mRNA expression of Gas6 and protein S is similar between young and aged BM stromal cells. However, whether the protein expression is similar in distinct stromal cell subsets such as CAR cells that are known to closely interact with NK cells remains to be demonstrated. Furthermore, stromal cells are known to express LTβR and upon ligation with LT, they become activated and primed to support NK cell maturation [92-94]. Assessing whether differential expression of LT and LTβR exist in aged mice will also be critical in demonstrating whether this pathway may contribute to the NK cell defects in aging.

To study whether stromal cells from the BM of aged can efficiently support NK cell terminal maturation and at the same time to avoid NK cell trafficking between the BM and the periphery, we will develop a BM stromal cell culture system in vitro to model NK cell maturation inside the BM without the caveat of trafficking between tissues as illustrated previously [94]. Adding BM purified immature NK cells isolated from young and aged mice to young or aged BM stromal cell cultures and subsequently monitoring their progressive maturation over time will us to determine whether aged BM stromal cells are defective in their capacity to support NK cell maturation.

**Does the increased Foxo1 and mTORC1 activity directly contribute to impaired NK cell maturation in aging?**

We showed in chapter 3 that the impaired maturation of aged NK cells was associated with an increased expression of Foxo1 and mTORC1 activity. This correlated with increased
mitochondrial superoxide production, DNA damage, reduced T-bet and increased ATP levels. However, the direct causal relationships between these molecules and whether they are responsible for influencing the maturation of NK cells in aging has not been determined yet. Administering the mTORC1 inhibitor, rapamycin and the ROS scavengers such as NAC to aged mice will allow us to interrogate whether these pathways are related and whether they have a role in regulating maturation of NK cells in aging.

Additionally, in chapter 2, our mixed BM chimera studies showed that T-bet and Eomes expression was commensurate with NK cell maturation and were regulated by the environment (young or aged) in which NK cells developed. To determine whether the aged environment also regulates both Foxo1 and mTORC1, we will construct mixed BM chimeras from young and aged donors and determine whether the aged environment modulates the expression of these molecules and whether this is associated with improved NK cell maturation.

Is the development of NK cells in an environment that is partially deficient in MHC-I contributing to their hyporesponsiveness in aging?

Chapter 4 described for the first time the novel presence of a high frequency of MHC-I low cells that are apoptotic in aged lymphoid organs. The presence of these cells has several implications for the immune system in general and on NK cells in particular. In our analysis, we detected the presence of necrotic cellular debris which appeared to be present in aged splenocytes and also to be part of the MHC-I low cell population. The larger presence of the necrotic cellular debris may suggest inefficiency in the phagocytic clean-up process. Indeed young BMMDMs were able to phagocytose the necrotic debris which may promote inflammation and autoimmunity as a consequence of continuous exposure to nuclear DNA. With respect to the effect of a lack of MHC-I on NK cells, we will adoptively transfer young NK cells into aged
mice to assess whether MHC-I low cells can be eliminated as missing self targets and whether this augments aged NK cell functional responsiveness.

We are also interested in determining whether MHC-I low cells also exist at a higher frequency in elderly humans and non-human primates. Our preliminary studies show that a higher frequency of MHC-I low cells and necrotic cellular debris are present in the spleen of aged macaques. We aim to extend these observations to elderly humans. In pursuit of this goal, we are currently testing to evaluate whether we can identify MHC-I low cells in the peripheral blood of aged mice. If detected, this would provide the basis for evaluating the presence of these cells in the blood of elderly individuals. We are also currently set up to acquire spleen samples from young and aged macaques from the California National Primate Center (CNPRC). This will allow us to test whether MHC-I low cells are present in the tissues of aged non-human primates.

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Appendix 1: Loss of Phagocytic and Antigen Cross-Presenting Capacity in Aging Dendritic Cells Is Associated with Mitochondrial Dysfunction.

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Loss of Phagocytic and Antigen Cross-Presenting Capacity in Aging Dendritic Cells Is Associated with Mitochondrial Dysfunction

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Impaired functionality of dendritic cells (DCs) significantly contributes to decreased adaptive immune responses in aged hosts. The expression of MHC-peptide on the DC surface is the critical first step in T cell priming, but few studies have addressed the effect of aging on Ag acquisition, processing, and presentation by DCs. In this study, we show that aged murine DCs were less efficient in the cross-presentation of cell-associated Ag and subsequently in the cross-priming of CD8+ T cells than were their young counterparts. The decreased cross-presentation was associated with a reduction in the frequency of CD8α DCs and merocytic (CD8α−CD11b+) DCs that could endocytose cell-associated Ag, as well as the number and the size of the endocytosed particles in the DC that did internalize cell-associated materials. Mechanistically, phagocytic capacity has been associated with mitochondrial activity and membrane potential (ΔΨm). Aged DCs exhibited profound signs of mitochondrial dysfunction, illustrated by lower ΔΨm, reduced ATP turnover and coupling efficiency, decreased baseline oxidative phosphorylation, and greater proton leak and reactive oxygen species (ROS) production. Mimicking the aged metabolic phenotype in young DCs by pharmacologic manipulation indicated that the reductions in ΔΨm and ATP impeded the phagocytic capacity whereas ROS interfered with a later step in the cross-presentation process. Conversely, in vitro scavenging of ROS partially restored cross-presentation by aged DCs. Taken together, these data suggest that improvement of aged DC functionality might be feasible in the elderly by targeting metabolic dysfunction or its downstream sequelae, thereby opening new avenues for enhancing vaccine efficiency in this population.

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production (27, 28). Exploring how the mitochondrial function is affected, as well as its relationship with decreased DC functionality in aging, is thus of particular interest.

To address these important and unresolved questions, we used a well-established model of cross-presentation (the presentation of exogenous Ag on MHC class I [MHC I]) to determine the capacity of the aged DCs to cross-present cell-associated Ags in vitro and in vivo. We also studied whether age-related changes in mitochondrial functions are linked with DC impaired functionality.

**Materials and Methods**

**Mice, cells, and peptides**

Young (2–3 mo old) and aged (16–20 mo old) C57BL/6J mice were obtained from the National Institute on Aging aging colony. Mice expressing OVA under the actin promoter (actmOVA) were crossed to the K<sup>b</sup>-<sup>−/−</sup> background in our facility. Mice were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

DCs were isolated from spleens of naïve mice as described before (29). Briefly, DCs were first enriched by negative selection using anti-hematopoietic fluorochrome-conjugated antibodies and biotinylated Ab to TCR, CD19, IgM, IgD, and NKp46, followed by positive selection with CD11c beads (Miltenyi Biotec). Enriched DCs were either used directly or further subselected by flow cytometry based on their expression of CD11c, CD11b, and CD8α by using CD3, CD19, IgM, IgD, and NKp-46 as lineage/dump markers (MoFlo, Beckman Coulter). Overall DC purity after flow cytometric sorting was ≥98% and after bead isolation was ≥95%.

Cellular Acidification Rate and/or OCR were analyzed in response to 10 mU/ml of antimycin A (all from BD Pharmingen, San Diego, CA) according to the manufacturer’s directions. Capacity for secondary expansion in vitro was determined by staining the splenocytes on irradiated MEC.B7.SigOVA cells for 6 d and dividing the absolute number of endogenous Ag-specific CD8<sup>+</sup> T cells at the beginning of the culture by the absolute number of endogenous Ag-specific CD8<sup>+</sup> T cells at the end of the culture as described before (35, 36).

**Statistical analysis**

Unless stated otherwise, the data are expressed as means ± SEM and evaluated using an ANOVA followed by a Dunnett test. A p value <0.05 was considered statistically significant.

**Results**

**Increased DC frequency but poorer T cell priming capacity in aged mice**

To assess the effect of aging on the capacity of DCs to cross-prime cell-associated Ags, we first analyzed the DC composition in young and aged C57BL/6J mice. Aged mice contained higher frequencies as well as absolute numbers of total DCs (CD11<sup>c</sup> MHC II<sup>+</sup> DCs), purified young or aged DCs (5 × 10<sup>5</sup>) were i.v. transferred into young and aged wild-type BL/6 recipients. Seven days after the DC transfer, endogenous Ag-specific CD8<sup>+</sup> T cell responses were assessed by intracellular cytokine production after a 5 h stimulation with OVA<sub>257–264</sub> peptide (cognate) or gp33–41 (control) in the presence of brefeldin A. Surface staining and intracellular cytokine staining for IFN-γ, IL-2, and TNF-α were performed using a CytoTox/Cytoperm kit (BD Pharmingen, San Diego, CA, CA) according to the manufacturer’s directions. Capacity for secondary expansion in vitro was determined by stimulating the splenocytes on irradiated MEC.B7.SigOVA cells for 6 d and dividing the absolute number of endogenous Ag-specific CD8<sup>+</sup> T cells at the beginning of the culture by the absolute number of endogenous Ag-specific CD8<sup>+</sup> T cells at the end of the culture as described before (35, 36).

**Metabolism parameters**

To determine mitochondrial mass, ΦmAm, and ROS production, enriched and sorted DCs were stained with CD11c, CD11b, CD8α, and combinations of Mitotracker Green, Mitotracker Deep Red, JC1, and H2D-CFDA (Invitrogen). Cellular ATP was determined using an ATP bioluminescent assay kit (Sigma-Aldrich).

For real-time analysis of oxidative phosphorylation, DCs were analyzed using an XF-24 extracellular flux analyzer (Seahorse Bioscience) (34). Purified DCs (5 × 10<sup>5</sup>) were plated in XF running buffer (XF media, 10 mM dextrose, 2 mM l-glutamine, 1 mM sodium pyruvate) per the manufacturer’s instructions to obtain real-time measurements of the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). Where indicated, extra-cellular acidification rate and/or OCR were analyzed in response to 10 μM oligomycin, 4 μM FCCP, and 1 μM rotenone plus 10 μM antimycin A (all Sigma-Aldrich).

**In vitro cross-presentation by DCs**

Flow cytometry–sorted DC subtypes or total DCs were incubated with irradiated actmOVA-K<sup>b</sup>−/− cells (1500 rad/3 × 10<sup>5</sup> cells/well) together with 1 × 10<sup>7</sup> OVA<sub>257–264</sub>-specific B3Z hybridoma cells in 96-well U-bottom plates in the presence or absence of NAC (0.5 mM). In parallel experiments DCs were incubated with irradiated actmOVA-K<sup>b</sup>−/− in the absence or presence of FCCP (1 nM) or oligomycin (50 nM) for 4 h, after which the DCs were repurified and cultured with B3Z cells in the absence or presence of NAC (0.5 mM). B3Z activation was determined 24 h later by a chlorophenol red–β-galactosidase conversion assay (30). OVA<sub>257–264</sub>-pulsed DCs were used as positive controls.
We next tested whether aging affected the DC potential for cross-priming in vivo. As mcDCs were the most dysregulated in number in old animals, we focused on this subset. mcDCs from young and aged mice were purified and cultured with irradiated actmOV A-Kb2/2 cells, repurified, and transferred into young and aged wild-type BL/6 recipients. Transfer of young mcDCs induced an ∼4-fold higher frequency and absolute number of endogenous OV A257–264-specific CD8+ T cells than transfer of aged mcDCs into young hosts (Fig. 2A–C). Moreover, young endogenous OV A257–264-specific CD8+ T cells induced by young mcDCs underwent significant expansion upon secondary encounter with Ag in vitro (Fig. 2D). In contrast, young endogenous OV A257–264-specific CD8+ T cells induced by aged mcDCs failed to undergo secondary expansion (Fig. 2D). Similar results were obtained when aged mice received young or aged mcDCs. Whereas overall priming in aged mice was significantly lower than in young mice, transfer of young mcDCs into aged mice induced significantly more endogenous OV A257–264-specific CD8+ T cells than did transfer of aged mcDCs into young hosts (Fig. 2A–C). Moreover, young endogenous OV A257–264-specific CD8+ T cells induced by young mcDCs underwent significant expansion upon secondary encounter with Ag in vitro (Fig. 2D). In contrast, young endogenous OV A257–264-specific CD8+ T cells induced by aged mcDCs failed to undergo secondary expansion (Fig. 2D). Similar results were obtained when aged mice received young or aged mcDCs. Whereas overall priming in aged mice was significantly lower than in young mice, transfer of young mcDCs into aged mice induced significantly more endogenous OV A257–264-specific CD8+ T cells than did transfer of aged mcDCs (Fig. 2A–C). Importantly, aged endogenous CD8+ T cells primed by young mcDCs retained some of their potential for secondary expansion, whereas aged endogenous OV A257–264-specific CD8+ T cells primed by aged mcDCs contracted upon secondary encounter with Ag (Fig. 2D). Taken together, these data reveal an intrinsic defect in the capacity of aged DCs to prime CD8+ T cells to cell-associated Ags.

**Altered endocytosis of cell-associated Ags in aged DCs**

To identify the underlying mechanisms for this decreased cross-priming, we first determined whether aging affected uptake of cell-associated Ags. Young and aged DCs were cultured with CellTrace Violet–labeled irradiated splenocytes, and the endocytic process for each DC population was analyzed using classic and imaging flow cytometry (29). Aged CD8α DCs and mcDCs were less efficient at endocytosing irradiated cells than were their young counterparts, as determined by flow cytometry (Fig. 3A–C). Detailed analysis by imaging flow cytometry showed that aging also reduced the number and the size of the endocytosed particles in the DC (Fig. 3B–D). Indeed, most (75%) endocytosing young CD8α DCs and mcDCs contained more than two particles, with ∼45% of the DCs containing more than four particles (Fig. 3C). In contrast, less than half of the aged CD8α DCs and mcDCs contained more than two particles, and 15% contained more than four particles. Additionally, the size of endocytosed particles in the young DCs was significantly larger than in the aging DCs (Fig. 3D). As we had shown before, CD11b DCs were poorly endocytic (Fig. 3A–C), but again, the size of the endocytosed particles by these aged CD11b DCs was significantly smaller than those in young CD11b DCs (Fig. 3D).
Aging alters DC metabolism

Endocytic capacity has recently been associated with mitochondrial activity and $\Delta \psi_{\text{m}}$ in bone marrow–derived murine macrophages and fibroblasts (24). Because aging has been associated with impaired mitochondrial function and decreased $\Delta \psi_{\text{m}}$ (25, 27, 28, 38–40), we explored whether decreased mitochondrial function could be the mechanism underlying the decreased endocytic capacity in aged DCs.

We first used Seahorse FX24 analyses to assess mitochondrial function and cellular respiration in purified young and aged DCs. Young DCs showed higher baseline OCRs than did aged DCs (Fig. 4A, 4C). Basal respiration is strongly controlled by ATP turnover and partly by substrate oxidation and proton leak. To dissect which of these three processes was altered in aged DCs, we sequentially added the ATP synthase inhibitor oligomycin to assess ATP turnover and proton leak (“b” and “c,” respectively in Fig. 4B), then the uncoupler FCCP to assess maximal respiration (“d” in Fig. 4B), and finally antimycin/rotenone to assess the non-mitochondrial OCR rate (“e” in Fig. 4B). The nonmitochondrial OCR rate was comparable between young and aged DCs (Fig. 4A, 4C). For young DCs, 134.8 ± 17.2; aged, 124.1 ± 14.7) and these values were subtracted from all other values to strictly assess mitochondrial functions.

Addition of oligomycin resulted in a significantly greater reduction of OCR in young DCs compared with aged DCs (“b” values in Fig. 4C), indicating decreased ATP turnover in aged DCs. Moreover, the coupling efficiency, that is, the fraction of basal mitochondrial oxygen consumption used for ATP synthesis (“b/a” values in Fig. 4C), was significantly higher in young DCs. Additionally, aged DCs showed a greater oligomycin-insensitive respiration (proton leak, “c” in Fig. 4C) than did young DCs. The decreased ATP turnover and lower coupling efficiency suggested a deficiency in the ATP synthesis machinery of the aged DCs.

Using flow cytometry, we next assessed the mitochondrial mass and $\Delta \psi_{\text{m}}$ in the different subsets of young and aged DCs. Mitotracker Green staining intensity was similar between young and aged DCs, for all subsets, suggesting comparable total mitochondrial mass in the young and aged DCs (Fig. 4D). We next stained DCs with JC-1, a lipophilic cationic dye that can selectively enter into mitochondria and reversibly change color as the $\Delta \psi_{\text{m}}$ increases. As shown in Fig. 4D, all subsets of aged DCs showed significantly decreased $\Delta \psi_{\text{m}}$. Additionally, all subsets of aged DCs had increased ROS levels (Fig. 4D), further suggesting mitochondrial dysfunction.

Genetic or chemical reduction of $\Delta \psi_{\text{m}}$ and/or ATP levels was recently shown to accelerate trafficking of phagocytosed materials to low pH organelles in the LR73 cell line (24). Accelerated acidification in DCs would significantly reduce the amount of Ag available for MHC I loading and could therefore reduce cross-presentation. To determine whether the decrease in endocytosed material in aged DCs resulted from accelerated degradation of the fluorescently labeled cellular material, we assessed the endosomal acidification rate in young and aged DC subsets using beads that were dual-labeled with a pH-sensitive and pH-resistant dye (Supplemental Fig. 2) (33). Although there were large differences in endosomal acidification rates between DC subsets, no differences between young and aged DCs were found, suggesting that increased degradation was not the dominant process reducing the Ag availability in aging DCs.

Decreases in mitochondrial functionality reduce cross-presentation by young DCs

We next tested whether creating an “aged” phenotype in young DCs by reducing ATP, $\Delta \psi_{\text{m}}$, or increasing ROS were sufficient to affect their phagocytic and T cell priming capacity. Young DCs were incubated with CellTrace Violet–labeled irradiated actinOVA-Kb+/- cells in the absence or presence of combinations of chemicals that affect distinct aspects of mitochondrial function. We used low concentrations of FCCP (reduces $\Delta \psi_{\text{m}}$, increases ROS, little effect on ATP) or oligomycin (little effect on $\Delta \psi_{\text{m}}$, increases ROS, inhibition of ATP). As shown in Supplemental Fig. 2, these drugs had the expected effect in the young DCs. They were used alone or in combination with NAC (scavenges ROS, no effect on $\Delta \psi_{\text{m}}$ or ATP; Supplemental Fig. 3). Endocytosis (Fig. 5A) and cell viability (Supplemental Fig. 3) was assessed 4 h later. Both FCCP and oligomycin used alone significantly reduced the phagocytic capacity of CD8α+ DCs and mcDCs (filled bars). NAC alone during the endocytic period did not alter the DC phagocytic activity nor did it alter the inhibitory effects of FCCP and oligomycin on this phagocytic capacity (open bars versus filled bars). Taken together, these data indicate that $\Delta \psi_{\text{m}}$ and ATP, but not ROS, are important for the endocytic process (Fig. 5A).
To determine the effect of the same drugs on the cross-presentation of young DCs, young DCs were incubated with irradiated actmOV A-K\textsuperscript{b2}/ cells in the presence of FCCP or oligomycin, followed by sorting and coculture with the B3Z cells in the absence or presence of NAC. As expected owing to their inhibitory effects on endocytosis, FCCP- and oligomycin-treated young DCs showed poorer capacity to activate B3Z cells than did untreated DCs. Interestingly, addition of NAC to the FCCP and oligomycin-treated DCs partly restored their ability to activate B3Z cells (Fig. 5B). These data suggest that in conditions where ROS generation is augmented, such as in aged DCs, DC cross-presentation is impaired, although not through impaired endocytosis.

Scavenging of ROS partially restores cross-presentation by aged DCs

We next tested whether ROS scavenging could improve the poor cross-presenting capacity of aged DCs. Addition of NAC to cultures of young DCs did not significantly improve endocytosis, DC survival, or the B3Z response to irradiated actmOV A-K\textsuperscript{b2}/ cells (not shown). Similarly, NAC did not enhance endocytosis or survival in aged DCs (Fig. 6A). However, addition of NAC to aged DCs significantly improved the B3Z response (Fig. 6B). As B3Z cells are relatively resistant to oxidative stress (not shown), these data imply that the inhibitory effect of ROS on cross-presentation resulted from a direct effect on the DCs.

Discussion

Aging has been shown to reduce DC functionality, but the underlying mechanisms are poorly understood. In this study, we show that aging affected DC numbers as well as DC subpopulation composition. Moreover, aging significantly reduced the capacity of DCs to phagocytose and cross-present cell-associated Ags, and this impairment was associated with decreased ATP production, decreased $\Delta\psi_{m}$, and increased ROS production. Our data further indicate that decreased...
ATP and Δψm conferred the defect in phagocytic capacity whereas ROS impaired DCs at a later phase of the cross-presentation process. Although various groups have studied DCs in aging, there is little consensus about the impact of aging on DC numbers, composition, and function. Moreover, it is currently unclear whether the observed changes result from intrinsic defects in the DCs or their precursors, extrinsic factors associated with the aged environment, or a combination of both. Several studies reported normal DC numbers and subset composition in lymphoid tissues from young and aged mice, whereas others suggested that aging decreases CD8α+DCs and increases CD8α-DCs (4, 14, 15). Our data are in partial agreement with these latter studies, as we found the total number and frequency of splenic DCs to be increased, predominantly due to increased CD8α-CD11b+DCs. These discrepancies likely arise from the study of different background strains, as well as differences in staining and analysis strategies (3). The increase of the CD8α-CD11b-DCs is of high interest, as this population has significant phenotypic and functional overlap with mouse CD8α DCs in young animals, as well as having a human equivalent (BDCA3+DCs) that is the most potent at priming T cells to cell-associated Ags (41–45). Consequently, CD8α DCs and mcDCs are very potent inducers of antitumor responses, but they can also play a role in autoimmunity, as both processes are driven by cross-presentation of cell-associated self-Ags (29, 32, 46, 47). However, despite their functional importance, nothing is currently known how aging affects their functionality, and particularly how they cross-present cell-associated Ags.

**FIGURE 4.** Reduced mitochondrial functionality in aged DCs. Young (open circles/bars) and aged (filled circles/bars) DCs were probed for their mitochondrial function. (A) DCs were seeded into Seahorse Bioscience plates and OCRs were determined under basal conditions followed by the sequential addition of oligomycin, FCCP, and antimycin/rottenone. A representative experiment (of three) is shown. Data are expressed as mean ± SEM with n = 2 (Y) or 3 (O). *p < 0.05. (B) Scheme outlining the approach to identify parameters for the calculation of the relative contribution of nonrespiratory chain oxygen consumption, ATP-linked oxygen consumption, proton leak, and coupling efficiency. (C) Decreased baseline OCR, ATP-linked OCR and coupling efficiency, and increased proton leak in added DCs. (D) Flow cytometric analysis of mitochondrial mass, Δψm, and ROS production at steady-state in young and aged DCs. A representative experiment (of three) is shown. Data are expressed as mean ± SEM with n = 3. *p < 0.05. MFI, mean fluorescence intensity; O, aged; Y, young.
Cross-presentation of cell-associated Ags is a coordinated process, starting with the recognition of cell-associated materials and their subsequent internalization into endosomes that eventually fuse with acidic lysosomes where the materials are degraded. To facilitate cross-presentation, DCs need to actively delay endosomal acidification to allow the transport of the endocytosed Ags from endosomal vesicles into the cytosol, where they are processed by the proteasome and loaded on MHC I molecules in the endoplasmic reticulum (48).

Our data indicate that aging affects multiple aspects of the cross-presentation process. Indeed, we found that aging significantly reduced the endocytic capacity of CD8 DCs and mcDCs, resulting in a reduced frequency of phagocytosing DCs as well as a reduction in the number and size of the endocytosed particles. Importantly, our data suggest that reduced endocytosis resulted from the disruption of the active internalization process and not from defective binding/recognition, as tethering of cellular materials was similar in aged and young DCs. Age-associated attenuation of phagocytosis has been reported for a variety of cell types, including primary and derived macrophages without detectable effects on total cellular ATP production. How changes in ∆ψ\text{in}\text{Dc} affect phagocytic capacity remains unclear, but some studies suggest that changes in ∆ψ\text{in}\text{Dc} may alter mitochondrial Ca\text{2+} accumulation and mobilization, thereby affecting phagocytosis, which is a Ca\text{2+}-sensitive process (51–53).

Besides a role for ATP, we also found a relation between lower ∆ψ\text{in}\text{Dc} and reductions in phagocytic capacity. Park et al. (24) showed that ∆ψ\text{in}\text{Dc} increased after uptake of apoptotic cells in bone marrow–derived macrophages without detectable effects on total cellular ATP production. How changes in ∆ψ\text{in}\text{Dc} affect phagocytic capacity remains unclear, but some studies suggest that changes in ∆ψ\text{in}\text{Dc} may alter mitochondrial Ca\text{2+} accumulation and mobilization, thereby affecting phagocytosis, which is a Ca\text{2+}-sensitive process (51–53).

Whereas this is an intriguing hypothesis, especially in light of the accumulation of Ca\text{2+}, more research is needed to causally link these different phenomena.

Another important finding of our studies is that aged DCs expressed significantly higher levels of ROS than did young DCs, and that in vitro ROS scavenging significantly improved cross-presentation by aged DCs. ROS has an important role as a secondary messenger in several signaling pathways, but when the production exceeds its deactivation it leads to oxidative damage to proteins, lipids, and nucleic acids (28, 33, 55, 56). How ROS affect phagocytic capacity in aged DCs remains unclear, but some studies suggest that changes in ∆ψ\text{in}\text{Dc} may alter mitochondrial Ca\text{2+} accumulation and mobilization, thereby affecting phagocytosis, which is a Ca\text{2+}-sensitive process (51–53).

Whereas this is an intriguing hypothesis, especially in light of the Ca\text{2+} dysregulated homeostasis in aged cells (54) and the possible suppression of ∆ψ\text{in}\text{Dc} by high levels of Ca\text{2+}, more research is needed to causally link these different phenomena.
likely that overproduction of ROS in aged DCs directly damages molecules involved in the cross-presentation pathway. Further studies aimed at the dissection of the molecular processes that drive mitochondrial dysfunction, as well as their differential impact on the process of cross-presentation, are thus warranted. Furthermore, the ability of ROS scavengers such as NAC to restore the cross presentation ability in aged mice will need to be rigorously tested in an in vivo setting of cross priming to test whether this avenue could be targeted to increase immune responses in aged individuals.

Taken together, the present study shows that different components associated with age-related mitochondrial dysfunction, that is, reduced ATP synthesis, reduced ΔΨm, and increased ROS production, have specific deleterious effects on the cross-presenting capacity of aging DCs. Cross-presentation is only the first step in the cross-priming of cells to cell-associated Ags. Importantly, high levels of MHC-peptide can lower the requirement of costimulation by lowering the threshold for T cell activation (57). Given that aging has been reported to negatively affect expression of costimulatory molecules and proinflammatory cytokines (3, 4, 7, 14, 58), lowering the threshold for T cell activation by increasing MHC-I peptide levels is of clear interest. Although more research is needed to dissect how aging affects DC–T cell communication, our data thus suggests that improvement of DC functionality might be feasible in the elderly by targeting metabolic dysfunction, or its downstream sequelae, thereby opening new avenues for enhancing vaccine efficiency in this fragile population.

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Disclosures

The authors have no financial conflicts of interest.

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**Supplemental figure 1.** **Surface marker expression on young and aged DCs.** Spleens for young and aged mice were stained for lineage, CD11c, MHCII, CD8, CD11b, and indicated markers or appropriate isotype controls and analyzed by flow cytometry. **A.** Mean fluorescent intensity of indicated marker (black bars) and appropriate isotype controls (white bar). Data are expressed as mean ± s.e.m. with n=4-5. **B.** Representative intensity data for Kb and isotype control staining in indicated splenic DC subpopulations of young and aged mice.
Supplemental figure 2. Comparable endosomal acidification rate in young and aged DCs.
Young (white circles) and aged (black circles) DCs were cultured with dually-labeled beads (pH sensitive FITC and pH insensitive FluoProbes 647) and analyzed at different time points by flow cytometry, using a gating FCS/SSC selective for cells containing one bead. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined and compared with a standard curve (ranging from pH 5.5 to 8). Data are expressed as mean ± s.e.m. with n=3.
Supplemental figure 3. Effect of FCCP and Oligomycin on viability and mitochondrial function.
Young DCs were cultured for 4 hrs in the presence of vehicle, FCCP, or oligomycin after which intracellular ATP levels, mitochondrial membrane potential (Δψ_m), ROS production, and viability were determined. One (out of 3) experiments is shown. Data are expressed as mean ± s.e.m. with n=3.* p<0.05.
Appendix 2: *Toxoplasma gondii*- derived profilin triggers human toll-like receptor 5-dependent cytokine production.

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Toxoplasma gondii-Derived Profilin Triggers Human Toll-Like Receptor 5-Dependent Cytokine Production

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Key Words
Toxoplasma gondii · Monocytes · Humans · Pathogen-associated molecular patterns · Pattern recognition receptors · Toll-like receptor

Abstract
Up to a third of the world’s population is infected with Toxoplasma gondii. Natural infection in humans can be life threatening during pregnancy and in immunocompromised individuals. Toll-like receptor (TLR) 11 is the mouse innate sensor that recognizes T. gondii profilin; however, in humans the TLR11 gene leads to transcription of no functional protein. Herein, by using a multiple sequence alignment phylogenetic analysis program between human and mouse species, we found that human TLR5 seems to be the evolutionarily closest member of the TLR gene family to mouse tlr11. We therefore asked whether human TLR5 could mediate IL-6, IL-8 and IL-12p70 production in response to the T. gondii profilin. We found that this was the case both in human cell lines as well as peripheral blood monocytes. Moreover, TLR5 neutralization and gene silencing mediated specific ablation of cytokine production after profilin exposure. Finally, peripheral blood monocytes carrying the TLR5 R392X mutation failed to produce cytokines in response to stimulation with profilin.

Introduction
Microbial recognition by the innate immune system is mediated by a multitude of cellular and endosomal membrane-bound as well as intracellular receptors. Toxoplasma gondii-derived pathogen-associated molecular patterns (PAMPs), namely cyclophilin-18 and profilin, have been shown to be recognized by receptors present in macrophages and dendritic cells, triggering cell activation and production of proinflammatory cytokines, including IL-1β, IL-6 and IL-12. While cyclophilin-18 is recognized by both mouse and human CCR5 [1, 2], profilin has been shown to mediate powerful cytokine production from mouse dendritic cells via activation of Toll-like receptor (TLR) 11 [3]. In fact, TLR11, which was previously found to mediate recognition of uropathogenic bacteria, has been identified as a major component and is essential for the development of the protective immune response in infected mice through the induction of massive IL-12
production by dendritic cells. IL-12-mediated induction of type 1 immunity is crucial for containing parasite replication and mediating long-term immunity to infection. However, due to the presence of several stop codons, transcription of the human TLR11 gene does not produce a functional protein [4]. Yet, as we show here, human cells are responsive to T. gondii profilin. Therefore, we asked whether there could be a functional ortholog for mouse TLR11 that is responsible for recognition of T. gondii profilin in humans. To do so, we performed evolutionary genetic taxa comparisons. We found that TLR11 is, perhaps, the most ancient TLR family member and that the subsequent members of this family of genes were derived from successive gene duplications. Both human and mouse TLR5 seemed to be evolutionarily the oldest relatives of mouse TLR11. This result led us to hypothesize that human TLR5 could have conserved (or rescued) mouse TLR11 biological function and mediate T. gondii profilin recognition. To test this hypothesis, we systematically examined whether human cell lines as well as peripheral blood monocytes expressed functional TLR5, followed by examining their cytokine response to T. gondii profilin in the absence of TLR5 through loss-of-function approaches [antibody (Ab)-mediated neutralization and siRNA gene silencing]. Our results show conclusively that T. gondii profilin induces a TLR5-dependent proinflammatory response by human monocytes.

**Materials and Methods**

Reagents and Cells

IgA anti-human (hu)TLR5, recombinant flagellin and recombinant T. gondii profilin were purchased from Invivogen, and proteinase K was purchased from Roche. Human embryonic kidney (HEK) 293 cells were purchased from ATCC (CRL-1573.3) and grown in 10% FCS RPMI medium. Peripheral CD14+ blood monocytes were purified from whole blood of healthy donors using Ficoll density gradient and a highly specific monocyte isolate kit (CD14+ antibody magnetic labeled beads, Miltenyi). Proteinase K digestion of flagellin and profilin was performed as described previously [5, 6]. Briefly, proteinase K-agarose was reconstituted in endotoxin-free water to 10 mg/ml, incubated at 4°C for 2 h and washed 5 times with endotoxin-free water. Digestion buffer was prepared by supplementing PBS with 2.7 mM KCl, 1.5 mM K2PO4, 137 mM NaCl and 8.1 mM Na2PO4. Subsequently, 100 μg of flagellin or profilin were incubated in digestion buffer with proteinase K-agarose slurry on a shaking platform for 3 h at 37°C, followed by centrifugation and harvesting of supernatants. Both the cell lines and human peripheral blood monocytes were cultured overnight with native or proteinase K-predigested PAMPs, with or without anti-huTLR5 Ab. Culture supernatants were harvested and stored at −40°C until assayed for cytokine production.

**Evolutionary Relationships of Taxa**

The evolutionary history was inferred using the neighbor-joining method [7]. The evolutionary distances were computed using the Poisson correction method [8] and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 102 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [9, 10] and with ClustalW-Phylgeny [11].

**Human Cytokine Measurements**

Human IL-6, IL-8, IL-12p40 and IL-12p70 levels were evaluated in culture supernatants using ELISA Duo-Set kits from R&D.

**TLR5 Flow Cytometry Analysis**

HEK293 cells and human peripheral blood monocytes were incubated with mouse R-phycoerythin (PE)-labeled anti-huTLR5 mAb (clone 85B152.5, Enzo Life Sciences) or isotype mouse IgG2a-PE control Ab in FACS buffer (surface staining) or Perm-Wash solution (surface and intracellular staining; BD) for 30 min. Cells were then washed in FACS buffer, resuspended and acquired for flow cytometry analysis. Data were analyzed using FlowJo software.

**siRNA TLR5 Gene Silencing**

Control (sc-37007) and TLR5-specific (sc-40253) siRNA oligos were obtained from Santa Cruz Biotechnology. Gene silencing was performed using a transfection kit from Amaxa, following their specific instructions. Briefly, highly enriched peripheral blood CD14+ monocytes were transfected with control and TLR5-specific siRNAs using a nuclease device and transfection reagent (Amaxa) in media. Afterwards, cells were placed in a 24-well plate with prewarmed transfection media and incubated for 24 h. Green fluorescent protein-labeled empty vector control was used to determine the transfection efficiency by flow cytometry. To verify the TLR5 gene silencing, we analyzed TLR5 expression in transfected monocytes by flow cytometry using mouse R-PE-labeled anti-huTLR5 (Enzo Life Sciences). In order to test the functional ablation of TLR5 expression, transfected monocytes that showed decreased TLR5 protein levels were stimulated with flagellin and/or profilin (1 μg/ml) for 24 h, and supernatants were harvested and assayed for cytokine production by ELISA.

**TLR5 (R392X) Genotyping**

Genomic DNA samples (25 μg) from 35 peripheral blood monocytes were isolated and screened for TLR5 (R392X, rs5744168). Genotyping was carried out by allelic discrimination real-time PCR using the following primers: WT TLR5 T5–10, forward, 5’-ATGGGAGACCACCTGGACCTTCTCC-3’; reverse, 5’-GGAGATGGTTGCTACAGTTTGCAACGG-3’. PCR primers for TLR5 (R392X) were T5–10, forward, and T5–31, reverse, 5’-GGATCCAGGTCCTGTAATTCTTCAGGG-3’ [12]. End-point analysis was performed by high-resolution melting curve analysis using LightCycler 480 software (Roche).

**In vitro huTLR5 Ectodomain Binding Assay**

An in vitro huTLR5 ectodomain binding assay was performed as indicated by the manufacturer’s instructions, as follows. Flagellin and profilin (Invivogen; 100 ng/ml in PBS) were incubated overnight in 96-well ELISA plates. Wells were washed 3 times with PBS.
and incubated with titration curves of huTLR5-Fc (Invivogen; 100–3.125 ng/ml) with PBS alone or with flagellin or profilin (100 ng/ml). After a 2-hour incubation, wells were washed 5 times with PBS and incubated with anti-human IgG1-horseradish peroxidase conjugates for 1 h. Wells were developed with TMB substrate, and optical density was measured at 405 nm. Nonlinear regression curves were plotted, normalized and analyzed using Prism software.

**Statistical Analysis**

Student’s t test was performed to determine statistical significance of differences (p < 0.05) between control and treated groups using the GraphPad software.

**Results**

**Human TLR5 and Mouse tlr11 and tlr12 Are Part of an Ancient Cluster within the TLR Phylogenetic Tree**

Human innate immune system cells can recognize the presence of *T. gondii* parasites and produce proinflammatory cytokines, including IL-12 [13–15]. *T. gondii* profilin was shown to be a major component of innate recognition by mouse innate cells via activation of TLR11 [3]. The human *TLR11* gene is not translated due to the presence of a stop codon within its coding region [4], and to date, there are no homologs of mouse *tlr12*. We hypothesized that *T. gondii* profilin activates human cells through interaction with another TLR present in human cells. The approach to select which candidates to test was to examine the comparative evolutionary background of the TLR gene family between humans and mice. Figure 1 shows a phylogenetic tree comparing the amino acid sequences for TLRs 1–13 using the neighbor-joining method. Interestingly, the data indicate that mouse TLR11 is the most ancient member of this family, with all subsequent clusters derived from gene duplications and amino acid substitutions. In this regard, the oldest event gave origin to a cluster with mouse TLR12 and with human and mouse TLR5. Later, clusters containing TLRs 1, 2, 3, 4, 6 and 10 and, more recently, another cluster containing TLRs 7, 8 and 9 were derived. Based on these observations, we hypothesized that human TLR5 could potentially perform the microbial recognition executed by mouse TLR11. Although this method is limited with regards to interpretations that indicate complete evolutionary estimation, for the question posed in this article, we consider that it fulfilled its potential as a general sequence comparison analysis of gene family evolution between the two species based on amino acid sequences. We therefore raised the hypothesis that human TLR5 is involved in innate recognition and induction of cytokine production by *T. gondii*-derived profilin.

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**Fig. 1.** Evolutionary relationship comparison of the TLR gene family between human and mouse. The evolutionary history was inferred by the neighbor-joining method using a MEGA5 cladogram tree (a) or a ClustalW2-Phylogeny radial tree (b). The optimal tree with the sum of the branch length equal to 7.94970641 is shown. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 20 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 102 positions in the final dataset.
HEK293 Cells Are TLR5+ and Respond to Both Flagellin and Profilin in a TLR5-Dependent Manner

Next, we focused on investigating the potential involvement of human TLR5 in the recognition of *T. gondii* profilin. We adopted a widely known approach using the HEK293 cell line transfected with the respective TLRs. However, to our surprise, we noticed that in the presence of both *T. gondii* profilin and the prototypical TLR5 ligand, flagellin, there was significant IL-8 production from nontransfected cells, independent of the presence of TLR5-containing plasmid. At this point, we followed up on testing whether HEK293 cells expressed detectable amounts of human TLR5. As shown in figure 2a, we found significant levels of TLR5 in HEK293 cells. On the other hand, THP-1 cells did not express detectable levels of TLR5 above isotype control Ab staining. These results suggest that the profilin-triggered IL-8 response in HEK293 cells could be derived from activation of this receptor.

In fact, figure 2b shows that both flagellin and profilin triggered a dose-dependent IL-8 production from HEK293 cells but not THP-1 cells (fig. 2b). Upon transfection with human but not mouse TLR5, HEK293 cells produced extremely high levels of IL-8 in response to flagellin (fig. 2c) and profilin (fig. 2d). Such a potent yet nonphysiological response overshadows the endogenous TLR5-triggered cytokine production. Moreover, mAb-mediated neutralization of human TLR5 inhibited IL-8 production by HEK293 cells in response to flagellin and profilin but not lipopolysaccharide (LPS) stimulation (fig. 2e–g). Therefore, these data clearly indicate that TLR5 expressed in HEK293 cells triggers IL-8 production in response to both flagellin and *T. gondii*-derived profilin.

*Human Peripheral Blood-Derived CD14+ Monocytes Produce Proinflammatory Cytokines in Response to Flagellin and Profilin in a TLR5-Dependent Manner*

To further establish the role of TLR5 in mediating cytokine induction by human monocytes, we inhibited TLR5 gene expression by transfection with siRNA-coding plasmids. Figure 4a shows the effect of TLR5 siRNA transfection versus control siRNA transfection on the cell membrane TLR5 expression levels as determined by flow cytometry. Figure 4b and c show that while control siRNA-transfected cells presented production of IL-6 and IL-12p70 in response to all microbial stimulants, there was a significant reduction in cytokine production by cells transfected with TLR5 siRNA after stimulation with both flagellin and profilin. Taken together, these results indicate that TLR5 is a required component of the human monocyte response to *T. gondii*-derived profilin.

*TLR5 Gene Silencing Inhibits the Response of Human Monocytes to Flagellin and Profilin*

To further establish the role of TLR5 in mediating cytokine induction by human monocytes, we inhibited TLR5 gene expression by transfection with siRNA-coding plasmids. Figure 4a shows the effect of TLR5 siRNA transfection versus control siRNA transfection on the cell membrane TLR5 expression levels as determined by flow cytometry. Figure 4b and c show that while control siRNA-transfected cells presented production of IL-6 and IL-12p70 in response to all microbial stimulants, there was a significant reduction in cytokine production by cells transfected with TLR5 siRNA after stimulation with both flagellin and profilin. Taken together, these results indicate that TLR5 is a required component of the human monocyte response to *T. gondii*-derived profilin.

*TLR5 (R392X) Peripheral Blood Monocytes Are Unresponsive to *T. gondii* Profilin Stimulation and Hyporesponsive to *Tachyzoite Exposure in vitro*

Human polymorphisms of the TLR5 gene had been described previously to be relevant in several infectious diseases and chronic inflammatory diseases, including Legionnaires’ disease [12], Crohn’s disease [16], cystic fibrosis [17] and obesity [18]. In particular, the mutation R392X, which leads to the insertion of a stop codon at the position 392, leads to complete loss of TLR5 protein expression. R392X is a highly frequent (up to 10%) mutation among Caucasians of European background [12]. Notably, TLR5 (R392X) cells were shown be unresponsive to flagellin stimulation [12]. Here, we aimed to establish a more physiological model to further dissect the function of TLR5 in mediating monocyte cytokine responses to *T. gondii* profilin. To do so, we determined TLR5 expression in purified CD14+-monocytes. Figure 5a shows a histogram overlay profile from monocytes...
Fig. 2. Endogenously expressed human TLR5 in HEK293 cells mediates IL-8 responses to flagellin and *T. gondii*-derived profilin. 

**a** HEK293 or THP-1 cells were suspended in FACS buffer with PE-labeled anti-huTLR5 antibody. Cells were then washed and acquired for flow cytometry. Data shown are histogram overlays of samples stained with isotype antibody control (IgG2a-PE) and anti-TLR5.

**b–d** Subsequently, cells were stimulated with several concentrations of recombinant flagellin C (**b**), recombinant *T. gondii* profilin (**c**) or LPS (**d**) for 24 h. 

**e–g** HEK293 cells were plated and incubated in the presence of medium alone or with anti-TLR5 mAb, as indicated, followed by stimulation with flagellin (**e**), profilin (**f**) or LPS (**g**) for 24 h. Supernatants were harvested and assayed for the presence of IL-8 by ELISA. Regression curves and $r^2$ and median inhibitory concentration (IC$_{50}$) values for anti-TLR5 mAb-mediated inhibition of IL-8 responses to flagellin, profilin or LPS are shown. Data shown are representative of at least 3 independent experiments.
Fig. 3. *T. gondii*-derived profilin triggers a TLR5-sensitive human peripheral blood-derived monocyte proinflammatory cytokine production. a Peripheral blood monocytes were purified and stained for intact (solid line) and permeabilized cells (dotted line). CD14-MACS bead-purified peripheral blood monocytes were suspended in FACS buffer with or without PermWash buffer followed by incubation with PE-conjugated isotype control IgG2a (gray histogram) or anti-TLR5 mAb. b–g Cells were plated and incubated with medium alone, flagellin (b, c), profilin (d, e) or LPS (f, g) in PBS (PAMP), PAMP predigested with proteinase K (PAMP + proteinase K) or anti-huTLR5 mAb (PAMP + anti-TLR5; 1–0.1 μg/ml). After incubation for 24 h, supernatants were harvested and assayed for IL-6 (b, d, f) or IL-12p70 (c, e, g) by ELISA. Data shown are representative of 3 independently performed experiments.

Fig. 4. siRNA-mediated silencing of human TLR5 inhibits profilin- and flagellin-mediated proinflammatory cytokine production by human peripheral blood-derived monocytes. Peripheral blood-derived monocytes were isolated and electroporated in the presence of medium alone, control siRNA or human TLR5 siRNA oligos. Cells were then stained for TLR5 as described in figure 3. a Transfected cells were gated and TLR5 expression was analyzed. A histogram overlay of isotype control-stained cells (gray histogram), control siRNA-transfected cells (dotted line) or TLR5 siRNA-transfected cells (solid line) is shown. b, c Cells were plated and incubated in the presence of medium alone, flagellin, profilin or LPS for 24 h. Culture supernatants were then harvested and assayed for IL-6 (b) and IL-12p70 (c) by ELISA. Data shown are representative of 3 independently performed experiments.
that expressed low and high levels of TLR5. Figure 5b shows the mean fluorescence intensity of such samples and the low/high profiles of TLR5 expression within CD14+ cells. We then confirmed by real-time genotyping that the cells that showed low levels of TLR5 staining also showed high detection using primers containing the R392X mutation (online suppl. fig. 1, www.karger.com/doi/10.1159/000362367). We then examined their cytokine profile in response to LPS, flagellin or profilin (all at 1 μg/ml) for 24 h. Supernatants were harvested and assayed for IL-6 (c) and IL-12p40 (d) by ELISA. e, f Peripheral blood monocytes from donors CTDC C14 and CTDC C42 were incubated in the presence of live T. gondii Rh strain tachyzoites (m.o.i. ranging from 1,000 to 0.0001); 24 h later supernatants were harvested and assayed for IL-6 (e) and IL-12p40 (f). g HEK293 cells were plated and incubated in the presence of isotype control Ab or anti-huTLR5 mAb and live tachyzoites as indicated in e and f. Culture supernatants were harvested and assayed for IL-8 by ELISA. Data shown are representative of triplicate samples from 2 independent experiments. * p < 0.05 between donors or between isotype control and anti-huTLR5 mAb treatment as determined by t test.

Fig. 5. TLR5 (R392X) mutation abolishes monocyte cytokine production after T. gondii profilin stimulation. Peripheral blood CD14+ monocytes were purified and stained as described in figure 3. a Histogram overlays of isotype control versus TLR5 staining of CD14+ monocytes obtained from two donors (CTDC C42, gray histogram; CTDC C14, black histogram). Empty histograms represent isotype control Ab staining (black line for CTDC C14 and gray line for CTDC C42). b Mean fluorescence intensity (MFI) of the samples. c, d Cell suspensions from the same donors were then cultured in the presence of medium alone, LPS, flagellin or profilin (all at 1 μg/ml) for 24 h. Supernatants were harvested and assayed for IL-6 (c) and IL-12p40 (d) by ELISA. e, f Peripheral blood monocytes from donors CTDC C14 and CTDC C42 were incubated in the presence of live T. gondii Rh strain tachyzoites (m.o.i. ranging from 1,000 to 0.0001); 24 h later supernatants were harvested and assayed for IL-6 (e) and IL-12p40 (f). g HEK293 cells were plated and incubated in the presence of isotype control Ab or anti-huTLR5 mAb and live tachyzoites as indicated in e and f. Culture supernatants were harvested and assayed for IL-8 by ELISA. Data shown are representative of triplicate samples from 2 independent experiments. * p < 0.05 between donors or between isotype control and anti-huTLR5 mAb treatment as determined by t test.

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Flagellin and profilin bind to the ectodomain of human TLR5 in vitro. Flagellin or profilin (1 μg/ml) were immobilized on ELISA plates. Wells were then incubated with increasing concentrations of huTLR5-Fc fusion protein (ranging from 1.5 to 200 μg/ml) in the presence of 1 μg/ml BSA, profilin or flagellin for 2 h. Wells were washed 3 times with PBS-Tween 0.5%, followed by incubation with anti-human IgG-horseradish peroxidase conjugates. HuTLR5-Fc binding was determined colorimetrically using TMB substrate in an ELISA plate reader. Data were then normalized to a percentage of maximum values and nonlinear regression curve fit using Prism. Data shown are means of triplicate samples from 1 of 2 independent experiments. mOD = ■■■■.

Discussion

Some studies have shown an overlap of TLR5 and TLR11 in the mouse system, with TLR5-dependent responses to previously assigned TLR11 ligands [19–21] and vice versa [22]. This set of overlapping activity might be rooted in the selective pressure for recognition of PAMPs from pathogens well adapted to their hosts. Our functional clustering of the TLR gene family from humans and mice suggests an older relationship between TLR11 (supposedly the oldest TLR in both species) and TLR5 – the first gene theoretically product of an ancient trl11 gene duplication event. Despite the evolutionary distance, our results suggest that function and microbial ligand affinity is conserved between human TLR5 and mouse TLR11. Interestingly, overlap with regard to mouse TLR5 and TLR11 ligand specificity has been reported previously; however, a thorough comparative study of TLR5 and TLR11 ligands in mouse cells has not yet been conducted to date.

Previous literature relied vastly on the HEK293 transfection system to test ligand specificity with a great degree of reproducibility. Nevertheless, our study raises a central issue for the correct interpretation of these results. We have shown here strong evidence supporting the endogenous expression of TLR5 in nontransfected HEK293 cells. Past analysis of TLR/PAMP interaction in transfected HEK293 cells was certainly made under very high gene expression levels (more than 1,000 times higher than baseline) by comparing untreated versus stimulated cells (usually represented as fold increase over control). However, this method introduces a bias in the interpretation of the results due to the fact that TLR activation by PAMP in nontransfected or mock-transfected cells is proportionally increased. However, the magnitude of the TLR activation signal is too high in transfected cells as for the signal levels observed in nontransfected cells to be appreciated after ligand exposure. Another potential complicating factor with this method of analysis is that the signals coming from subtle affinity changes between receptor and PAMPs are minimized by the extremely high activation threshold over baseline. In fact, the commercial source for the use of HEK293 cells in a TLR/NOD...
reporter assay alerts to the endogenous baseline levels of TLR3, TLR5 and NOD1 in these cells (Invivogen, catalogue No. 293-LacZ). Moreover, several previous reports indicated increased endogenous TLR5 expression in HEK293 cells [23–25]. Therefore, our results are consistent with several lines of published data.

Human cells show an obvious response to T. gondii profilin that is independent of any cognate signal (i.e. CD40L, IFN-γ), an observation that highlights the innate character of this interaction. However, it is not clear that profilin is the only PAMP from this protozoan to trigger a human innate cytokine response in vivo. The mouse model suggests a very complex scenario, where several receptor/ligand pairs play a relevant role early after infection in vivo. As such, TLR11 is required for profilin-triggered cytokine production [3], while TLR9 has been shown to mediate some response [26]. However, both TLR11- and TLR9-deficient mice show resistance to acute infection, while MyD88-deficient mice quickly succumb to infection [27]. Moreover, we and others have shown the activation of CCR5-dependent cytokine dendritic cell responses by exposure to cyclophilin-18 from T. gondii [1, 28]. CCR5-deficient mice also showed high mortality upon infection concomitant with lower type 1 cytokine production [1].

More recently, a series of studies have shown that the TLR11-mediated response to T. gondii is compounded by coactivation of TLR12, as well as TLR7/TLR9 triggering by parasite RNA/DNA [29]. In the absence of all these pathways combined, mice show a susceptibility phenotype that resembles T. gondii-infected MyD88-deficient hosts [29]. Such a complex response can be further supported by the observations using UNC93B1-deficient mice, in which the activation of TLRs 3, 7 and 9 by RNA/DNA is abolished [30]. Taking all these observations together with the fact that humans have a truncated non- functional TLR11 gene and no homolog for mouse tlr12, we propose here that TLR5 ‘fills in’ for the absent human TLR11. Further interactions resulting from recognition of parasite RNA and DNA in the context of profilin-initiated responses remain to be further characterized. Our experiments were performed using recombinant profilin to focus on a specific ligand/receptor interaction, although crude parasite lysates (STAg) can trigger monocyte cytokine production (J.A., personal observations). Furthermore, proteinase K digestion of recombinant profilin completely abolished cytokine induction by this molecule, thus suggesting that potential nucleotide, polysaccharide or other nonpeptide contamination is unlikely.

The relative contribution of TLR5 to the protection against toxoplasmosis in humans, especially within populations in which there is high frequency of the TLR5 R392X mutant, remains to be fully investigated. Finally, the biological implications of the studies presented here open a new venue for PAMP-based vaccine adjuvants. Vaccine research using the mouse system has not accounted for the potential role of TLR5/profilin interaction seen in human cells, as we showed here. The use of profilins as vaccine adjuvants has been proposed previously [31]. Our results clearly identify that the receptor/ligand interaction involved in profilin recognition in humans is therefore highly relevant for the future development of PAMP-based vaccine adjuvants as well as other clinical applications.

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Disclosure Statement

The authors declare no conflict of interest.

References


Appendix 3: Homeostasis and function of regulatory T cells in aging.

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A hallmark of aging is the progressive deterioration of immune function. Age-related immune suppression increases susceptibility to infectious diseases and cancer, significant causes of morbidity and mortality in the elderly. In particular, age-related T cell dysfunction is a major contributor to ‘immune-senescence’. Recently, it has become clear that the frequency of regulatory T cells (Treg) significantly increases in aged mice and humans. As Treg control the intensity of T cell responses, their accrual probably contributes to age-related immune dysfunction. This review will focus on mechanisms underlying Treg homeostasis and function in aging.

Defective T cell responses are a critical component of immune suppression in the elderly. Indeed, defects in TCR signal transduction affect effector T cell expansion and function, clonally expanded memory T cells impinge upon the breadth of the memory compartment, and aged hosts produce less naive T cells available for de novo responses. Recent work has found that regulatory T cells (Treg), a subset of CD4+ T cells, are substantially increased in aged mice and humans [1++2,3,4*,5,6,7**,8,9]. In addition to their role in peripheral tolerance, Treg also play critical roles in controlling both acute and persistent infections (viral, bacterial, parasitic, and fungal) (reviewed in [10]). Thus, Treg may contribute substantially to inefficient T cell responses in aging.

Increased frequency of regulatory T cells in aged hosts
In young mice and humans, roughly 3–15% of the CD4+ T cells expresses forkhead box protein 3 (FoxP3), a transcription factor that is both necessary and sufficient for Treg development [11]. Using FoxP3 as a marker, several groups have shown an increased frequency of Treg in the lymphoid organs of aged mice, but not in the circulating blood or the thymus (Table 1). This accumulation is progressive, already visible in middle-aged mice [2*].

Owing to the limited access of tissues, the picture is not as clear in humans (Table 1). Several studies have assessed Treg (defined as CD4+FoxP3+ or CD4+CD25+) frequency in human blood, and age-associated differences, when found, were minimal. Other studies did not observe increased Treg frequency when Treg were defined as CD25+CD45RA− [12] or CD25+NCD127lo [13]. The only study assessing the effect of aging on the percentage of Treg in human tissues also showed a substantial accumulation in the skin [7**], strongly suggesting that Treg accrual also occurs in elderly humans. Moreover, these data emphasize that Treg frequency in the blood does not accurately represent their accumulation in the tissues.

Treg are comprised of two major populations: a so-called natural Treg population (nTreg) that is produced in the thymus and a second population that is induced extrathy mically in the peripheral lymphoid tissues (iTreg) [14]. The gut-associated lymphoid tissue (GALT) provides a unique environment poised for generation of iTreg. Components of the GALT that promote iTreg development are TGFβ and CD103+, retinoic acid-producing dendritic cells (DC) (reviewed in [15]). It remains unclear whether the Treg that accumulate in aging are comprised of nTreg, iTreg, or a combination of both. We have found that the majority of Treg in aged mice express high levels of the transcription factor Helios (unpublished data), described to be a specific marker of nTreg [16], although recent data have questioned its stringency as an nTreg-specific marker [17]. Furthermore, we found that aged T cells (either naïve or memory) were less prone to conversion in vitro [2*]. Thus, these data suggest that aged Treg may be derived from nTreg, although further work is required to rule out a contribution of increased Treg conversion in aging.

Increased Treg may also be the result of clonal expansion, as described for CD8 cells [18]. Recent work has suggested that chronic viral infection can activate endogenous superantigens that drive expansion of VB5+ Treg in C57BL/6 mice [19]. In elderly cytomegalovirus (CMV) seropositive patients, Treg and non-Treg were also enriched for VB2+ cells [20]. However, Treg
Table 1

| CD4+ FoxP3+ Treg proportion and absolute numbers in aged versus young hosts |
|-----------------------------------------------|-----------------|-----------------
| **Human**                                    | **Frequency**   | **Absolute number** |
| Peripheral blood                             | - Increased [2**,6] | - Not increased [13,47] |
|                                                | - Not increased [1**,3] | - Not increased [8] |
| Skin                                          | - Increased [7**] | - N/A |
| **Mouse**                                    | **Frequency**   | **Absolute number** |
| Peripheral blood                             | - Increased [9] | - Not increased [3] |
|                                                | - Not increased [1**,2] | - Not increased [3] |
| LN                                            | - Increased [1**,2*,3,4] | - Increased in undescribed LNs [4*], mediastinal LNs [3], mLNs [2*] |
| Thymus                                       | - Not increased [2*,3] | - Decreased [2*,3] |

*Defined and determined the Treg frequency as the percentage of CD25+ TCD4+ cells, but also confirmed the expression of FOXP3 in CD4+ CD25+ Treg.

N/A = not assessed.

from aged mice have a Vβ profile similar to young mice, suggesting Treg accrual can occur independently of superantigen or chronic infection (unpublished data). The Treg TCR repertoire in young mice and humans has been shown to be as diverse as non-Treg [21,22], but similar studies have not yet been done in aged Treg. These will be important because changes in repertoire could impact the overall responsiveness and suppressive ability of aged Treg.

Cytokines and maintenance of Treg

Development of nTreg requires common γc signaling as loss of CD132 results in the loss of Treg during thymic development [23–27]. IL-2Rβ-deficient mice also have reduced thymic Treg and while their loss of Treg is more profound than observed in IL-2-deficient mice, it is not as severe as that observed in γc-deficient mice, suggesting that IL-2, IL-15 and perhaps other γc signaling cytokines promote Treg development [23,28–30]. Consistently, loss of Stat5 prevents Treg development while overexpression of a constitutively active Stat5 restores Treg development in IL-2Rβ-deficient mice [23]. Maintenance of Treg beyond the thymus is probably also governed by γc signaling [27]. Interestingly, Treg do not appear to require CD4 or class II MHC for their development or peripheral survival as ample numbers of Treg are observed in CD4− or class II MHC-deficient mice [31].

Given the disturbed cytokine environment in aging, mechanisms governing young Treg homeostasis may not be entirely the same as those operating to maintain aged Treg. Indeed, we and others have found that a substantial fraction of Treg in aged mice have significantly decreased expression of CD25 [1**,2*,32*], although these CD25lo Treg reside predominantly in the spleen and not the lymph nodes (LN) [1**]. Substantial decline in IL-2 with age has been described, mainly based on *ex vivo* assays, in which T cell production of IL-2 was shown to be deficient in aged animals [33,34]. To our knowledge, no study has measured IL-2 levels *in vivo* in aged animals, perhaps because bio-active IL-2 is bound to heparan-sulfate [35]. Nonetheless, it is likely that factors other than IL-2 contribute to the survival of CD25lo aged Treg. We found that aged Treg express increased levels of IL-7Rα and IL-2Rβ, and while we showed that neutralization of IL-7 in aged mice did not reduce Treg [2*], we cannot rule out a potentially redundant role of IL-15. Thus, the use of neutralizing antibodies to all three cytokines (or the γc) will be needed to critically evaluate the roles of γc cytokines in Treg survival in aged mice.

Expression of the pro-apoptotic molecule Bim is progressively downregulated in aging and contributes to Treg accrual

Our data point towards increased peripheral survival as the main mechanism involved in Treg accrual in aging, as Treg in aged mice survive significantly better than Treg from young mice, while *in vivo* Treg proliferation was similar in young and aged mice, and thymic Treg was decreased in aged mice (Figure 1A) [2*].

A link between γc cytokines and cell survival is their ability to manipulate expression and function of Bcl-2 family members (reviewed in [36*]). γc cytokines promote cell survival by increasing expression of Bcl-2 [37] and by decreasing expression of the pro-apoptotic molecule Bim [38]. Mechanistically, we found that Treg have a progressive and significant decrease in their
Increased survival contributes to accrual of functional Treg with age. Treg frequency progressively increases with age, although overall thymic output declines dramatically with age, due to thymic involution while output of Treg from the thymus is not enriched as mice age. By contrast, the progressive downregulation of Bim contributes to increased Treg survival and accrual with age. Peripheral proliferation, as assessed by in vivo BrdU incorporation, showed no difference in Treg proliferation between aged and young mice [2*]. While in vitro conversion of naive or memory T cells to Treg was not different between young and old mice [2*], the contribution of peripheral conversion of naive or effector T cells to Treg accrual in vivo remains unclear. Treg from aged mice and humans express similar or higher levels of multiple inhibitory molecules, and may act directly on naive/effector T cells or on dendritic cells (DC) to restrain T cell responses. Treg depletion in vivo significantly enhances protective memory T cells in aged mice.

expression of Bim with age. Further, genetic loss of Bim resulted in a rapid accrual of Treg (Treg frequency is similar between 6-month old Bim-deficient and 24 month-old WT mice) [2*]. Thus, this normally decreased expression of Bim within Treg provides them with a survival advantage that promotes their accumulation.

There are multiple, non-mutually exclusive explanations for decreased expression of Bim in aged Treg. First, the accumulated Treg in aged mice may be Treg that were produced as part of an effector response, so-called ‘memory’ Treg. We have found that effector memory T cells (which bear a similar phenotype to Treg) have lower levels of Bim compared to central memory T cells [39]. Second, levels of the anti-apoptotic molecules Bcl-2 and Mcl-1 were also decreased in aged Treg [2*]. We recently found that the level of Bcl-2 ‘determines’ the level of Bim that CD8+ T cells can tolerate and survive [39]. Thus, the loss of Bim by Treg may be a selective event driven by a primary loss of an anti-apoptotic family member. Third, we found that exogenous administration of IL-2 promoted the accrual of Treg with low levels of Bim [2*], suggesting that yc cytokines control the accrual of long-lived Treg. Fourth, it is possible that epigenetic mechanisms lead to methylation of the Bim promoter that decreases Bim expression in aged Treg, as such mechanism is involved in survival of Epstein-Barr virus (EBV)-infected B cells [40]. Together, our data suggest that the accrual of Treg is due to the progressive down modulation of Bim, but further studies are required to determine the underlying mechanism(s).

Expression of markers associated with Treg-mediated suppression
FoxP3 is not only critical for Treg development, but also for Treg function [41]. Treg control T cell responses by targeting effector T cells (Teff) and DC [42], using both contact dependent (CTLA-4, CD39, etc.) and contact independent (IL-10, TGF-β, etc.) mechanisms. FoxP3 expression per cell is maintained in aged Treg (Table 2), suggesting that Treg function is maintained with age. Indeed, most studies have found that several markers associated with contact-dependent suppression were preserved in aged Treg (Table 2). Few studies have examined levels of contact independent mediators in aging, but we found that Treg IL-10 production is preserved in aged mice [1**]. Paradoxically, increased levels of IL-10 and TGF-β have been correlated with healthy human aging [43], although the source of these cytokines (Treg vs. non-Treg) is unclear. Overall, it appears that

Table 2

<table>
<thead>
<tr>
<th>Treg-associated markers</th>
<th>Percentage positive (%)</th>
<th>MFI</th>
<th>Absolute number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3</td>
<td>++</td>
<td>=</td>
<td>++</td>
<td>[1**,2*,3,5]</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>=</td>
<td>=</td>
<td>N/A</td>
<td>[1**]</td>
</tr>
<tr>
<td>GITR</td>
<td>=</td>
<td>=</td>
<td>N/A</td>
<td>[1**]</td>
</tr>
<tr>
<td>PD-1</td>
<td>+</td>
<td>N/A</td>
<td>++</td>
<td>[1**,8,50]</td>
</tr>
<tr>
<td>ICOS</td>
<td>N/A</td>
<td>N/A</td>
<td>++</td>
<td>[50]</td>
</tr>
<tr>
<td>LAG-3</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
<td>[50]</td>
</tr>
<tr>
<td>CD103</td>
<td>++</td>
<td>N/A</td>
<td>N/A</td>
<td>[1**]</td>
</tr>
</tbody>
</table>

*: Similar value between aged and young mice.
+: 1–2 folds increase in aged mice.
++: >2 folds increase in aged mice.
N/A: not analyzed.
MFI: Mean Fluorescence Intensity.
aged Treg express as much, if not more, suppressive markers as young Treg, with the exception of CD25 (at least in the spleen).

Regardless of the mechanism Treg use to suppress, their ability to traffic to or be retained within sites of inflammation is also important. The integrin CD103 may facilitate Treg homing to nonlymphoid tissues. Interestingly, the percentage of splenic CD103+ Treg increases with age [1**]. Treg in the skin uniformly express CD103, although they may acquire CD103 expression only after arrival [44]. Nevertheless, similar to its proposed role in retaining T cells in the intestinal epithelium, CD103 could increase Treg retention in the skin, especially in humans [7**], and this could be an underlying mechanism for increased incidence of skin cancers and infections with aging [45].

**Functionality of aged Treg**

In general, *in vitro* studies assessing the capacity of human peripheral blood Treg have reported similar, or increased suppressive capacity of aged Treg versus young Treg. *In vitro* CD8 effector function (i.e. cytotoxicity, perforin expression, and IFN-γ production) was similarly decreased by CD25+ Treg from young and elderly subjects. On the contrary, CD4 effector function (IL-2 or IFN-γ production) was more suppressed by aged Treg than young Treg, but their effect on proliferation was the same [20,46,47]. The same degree of suppression of human Treg on non-Treg activated in the presence of antigen-presenting cells (APCs) was found irrespective of the source of APCs (autologous [20,48] or allogeneic young APCs [6]).

In mice, aged and young CD25+ Treg similarly suppressed APC-driven activation of non-Treg *in vitro* [3,9,32]. By contrast, when Treg were purified from the LN on the basis of FoxP3 expression, not CD25, aged Treg reduced the proliferation of CD4+ non-Treg roughly 3-fold better than their young counterparts [1**]. The differences observed in these studies can be attributed to technical factors (Treg separation by microbead versus fluorescence-activated cell sorting), the markers used to define Treg (CD25 versus FoxP3 expression), or the source of Treg (LN Treg express more cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and CD25 than splenic or blood Treg). Importantly, the suppressive function that aged Treg exert on young effector cells may not reflect the suppressive potential of aged Treg on aged effector cells.

Despite the significance of the question, few studies have so far investigated the *in vivo* functionality of aged Treg. One study showed an age-dependent defective tumor clearance, which correlated with Treg elevation [4*]. Importantly, CD25-depletion led to tumor clearance, suggesting that aged Treg limit anti-tumor immunity [4*]. Similarly, using the *Leishmania major* infection model, which is exquisitely sensitive to the Treg/Teff ratio [49], we showed that depletion of CD25+ T cells reduced lesion size in aged infected mice [1**]. Transfer of aged CD25+ Treg also controlled delayed type hypersensitivity responses albeit less than their young counterparts [9]. Combined, these studies showed that depletion of CD25+ Treg could significantly enhance protective memory T cell responses. Although the suppressor mechanism(s) used by Treg remain unclear (Figure 1), one group showed that CD25-depletion increased co-stimulatory molecule expression on myeloid DC, suggesting that Treg may suppress by limiting APC function [3]. Importantly, to our knowledge, *in vivo* depletion of FoxP3+ Treg in aged mice, instead of depletion of CD25+ Treg, has not yet been done. These studies are technically feasible since the development of FoxP3-DTR mice, and will be crucial to our understanding of Treg functionality in aged mice.

**Summary and future directions**

Age-related immune suppression is a growing problem commensurate with the dramatic increase in the aged human population. Novel therapies to boost or reverse immune aging are needed to offset the resulting economic and health burden. Increased Treg proportion in aged mice and humans probably contributes to age-related immune-suppression. However, several tantalizing questions remain unanswered: what maintains elevated numbers of Treg in aged hosts? How do aged Treg suppress immunity? Can partial Treg depletion be employed to enhance vaccine or anti-tumoral responses and/or elimination of chronic infection? Experimental answers to these questions will hopefully lead to new therapeutic strategies to enhance the quality (and quantity) of life for the world’s aged population.

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**References and recommended reading**

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


This was the first paper to show that the accumulation of Treg in aged mice could affect reactivation of latent infection. Notably, the data demonstrate that effector T cells, once relieved of Treg suppression could regain functionality. Further, aged Treg were as, if not more, suppressive than young Treg.

This paper demonstrates that Treg accrual is not due to altered thymic output or peripheral expansion, but rather to altered survival due to the decreased expression of the pro-apoptotic molecule Bim.


This was the first paper to show that Treg were functional in aged animals as their removal promoted tumor regression.


This was the first paper to show a significant accrual of CD25hi Treg in aged mice and that aged Treg (both CD25hi and CD25lo) were fully functional in vitro.


34. Haynes L, Eaton SM, Swain SL: The defects in effector generation associated with aging can be reversed by addition of IL-2 but not other related gamma(c)-receptor binding cytokines. Vaccine 2000, 18:1649-1653.


This paper showed that the level of Bcl-2 within effector T cells dictated the amount of Bim that the cells could tolerate and survive. Thus, Bcl-2 levels determine whether effector T cells can enter the memory compartment.


