

**NATURAL KILLER CELL RESPONSES TO INFLUENZA
VIRUS INFECTION IN AGED MICE**

By

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ABSTRACT

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Influenza is a public health concern, especially for the elderly. While influenza vaccination is efficacious in the young, it offers only limited protection in the elderly. Thus, it becomes imperative to understand age-related changes in the primary response to influenza infection. Studies presented in this dissertation identified potential age-related defects in natural killer (NK) cell function during influenza infection. We showed that NK cells from aged mice were reduced and had impaired function and altered phenotype in lungs during influenza infection. Aged NK cells demonstrated decreased IFN- γ production but not degranulation after influenza infection. However, after *ex vivo* activation with YAC-1 cells, aged NK cells demonstrated both reduced IFN- γ production and degranulation. IFN- γ was also reduced in aged NK cells after activation with anti-NKp46 and soluble cytokines. IFN- β , and IL-12p40 mRNA expression was not significantly different from that observed in adult mice. Analysis of NK cell subsets indicated that aged mice had more immature and less terminally mature NK cells that were available to respond during the early days of the infection. Thus, we investigated the age-related defects in NK cell tissue and subset distribution, development and function in resting uninfected mice. Our findings indicate that aged mice have reduced NK cells in most peripheral tissues but have increased NK cells in the bone marrow. Analysis of NK cell subsets based on CD11b and CD27 expression revealed that the reduction of NK cells in the periphery was

attributed to a specific reduction of the most mature, CD11b⁺ CD27⁻ NK cells and the accumulation of NK cells in the bone marrow was attributed to a specific increase of the immature, CD11b⁻ CD27⁺ NK cells. A detailed analysis of the *stages* of NK cell development in the bone marrow revealed that NK cells go normally through the *stages I-IV* of development, but they accumulated in the *stage IV* and could not further mature. We related this defect with the reduced levels of proliferating NK cells in the bone marrow of aged mice. We further showed that CD11b⁺ CD27⁻ NK cells exhibit reduced survival in the aged peripheral tissues. Finally, with an extensive phenotypic characterization of young and aged NK cells we have shown that impaired NK cell function is not related with their altered Ly49 receptor repertoire, but rather with their maturation status. Indeed, aged NK cells that had acquired the appropriate Ly49 receptors were less responsive, and were composed of more immature NK cells. Overall we provide data that support for an age-related defective development of NK cells.

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I dedicate this work to my family for all their limitless trust and love.
I dedicate this work to my mother Trigona, because my absence from home has deeply impacted her and to the memory of my father Dimitri, who left us during my studies and I never had the chance to say goodbye.
I dedicate this dissertation to my sister Oly, and my brother Ari for all the years I have not been around to support them in their difficult times and to share their happy moments.
I wish this dissertation would be an inspiration for my sweet nephew Lefteri.

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PREFACE

Influenza infections and secondary complications are the 4th leading cause of death among elderly people in the USA. It is suggested that immunosenescence comes with aging and contributes to the impaired ability of the elderly to respond effectively to influenza virus infection. It is known that the adaptive immune responses to influenza infection are reduced in the elderly and vaccination provides only partial protection. However, little is known about how aging affects the innate immune responses to influenza virus. Among the innate immune cells, natural killer (NK) cells exert antiviral activities early during the course of the infection and before the initiation of the adaptive immunity. Furthermore, there is strong evidence that NK cells are indispensably involved in controlling influenza infection and in inducing effective adaptive immune responses. Previous published research from our laboratory indicates that after influenza infection, NK cell cytotoxicity is increased in young mice but not in aged mice, while reduced NK cell cytotoxicity in aged mice is correlated with increased lung viral titers. In the following chapters we provide evidence for the age-related changes on NK cell responses to influenza infection as well as a detailed comparison of NK cell homeostasis, phenotype and function between young and aged mice and provide some insight into the specific defects.

This dissertation is composed of six chapters. Chapters 1 and 2 give the background information and review the current literature on the subject. Specifically, in chapter one the significance in maintaining high numbers of NK cells and high natural cytotoxicity in later ages is presented as well as the current understanding of NK cell, their mechanism of action and how they respond to influenza infection. Chapter 2 is an extensive literature review on the effects of aging on NK cells in both humans and mice. Aged NK cell numbers, phenotype and function in

resting conditions and upon activation with cytokines or influenza infection are evaluated, compared with that of younger subjects and summarized. A version of this review was written as a paper during a formal course offered by FSHN (HNF 890, Special Topics: Scientific *Writing*), under the direct supervision of Dr. Gardner and we plan to submit it for publication as a review on the effect of aging on NK cells.

The next two chapters provide the experimental data generated in order to address the main hypothesis that aged NK cell show defective activation and function. According to the specific aims, we characterized NK cell numbers, phenotype and function at resting conditions and after influenza infection. We compared the NK cells from aged mice (22 month) with that of adult (6 month) mice -referred also as young- after infections with the mouse adapted strain of H1N1 influenza A/Puerto Rico/8/34 (A/PR8). By comparing aged to adult mice we attend to reflect the observations made in human studies: people maintain their immune status from 20 years old until around 60-65 years old, but immune responses are dysregulated in older ages. We analyzed the percentages of NK cells as well as their maturation status in various tissues and compared their different functional properties to reveal specific defects on the circulation, development and function of NK cells.

Specifically, in chapter 3, the numbers, phenotype and function of NK cells have been compared in young and aged mice with a complete characterization of the response of pulmonary NK cells, the site of infection. These studies have given insight into some of the mechanism by which aged NK cells are impaired, and has already been published in *Mechanisms of Ageing and Development*, 132 (2011) 503-510. In chapter 4, we examine the homeostasis of NK cells in young and aged mice in resting conditions. We have provided data for the altered phenotype and maturation status of aged NK cells, and provide evidence for the stages where this

developmental defect exist and how it affects aged NK cell function. A manuscript summarizing these data is under preparation to be submitted for publication.

Finally, in chapter 5 the main conclusions and future directions are provided.

Overall, these studies are the first, to our knowledge to characterize in very details the phenotypic and functional differences among young and aged NK cells and to examine the development of NK cells in aged mice.

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LIST OF ABBREVIATIONS

ADCC	antibody dependent mediated cytotoxicity
AHCC	active hexose correlated compound
ANOVA	analysis of variance
APC	allophycocyanin
Bcl-2	B-cell lymphoma 2
Bim	Bcl-2 like protein 11
CCL	chemokine C-C motif
CCR	C-C chemokine receptor
CD	cluster of differentiation
CXCL	chemokine C-X-C motif
CXCR	C-X-C chemokine receptor
DC	dendritic cells
EOMES	eomesodermin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gr	gram
H2D	MHC haplotype in mice
HAU	hemagglutinin units
IFN	interferon
IL	interleukin
IU	international units

JAK	Janus kinase
KIR	killer cell immunoglobulin like receptors
KLRG1	lectin like receptor G1
LAK	lymphokine activated killer
LFA	lymphocyte function associated antigen
LIR	leukocyte inhibitory receptors
LTA	lymphotoxin A
LTB	lymphotoxin B
Ly49	killer cell lectin receptor subfamily A
Ly6C	lymphocyte antigen 6 complex
mg	milligram
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
ml	milliliter
mTOR	mammalian target of rapamycin
NCR	natural cytotoxicity receptors
NF- κ B	nuclear factor kappa B
ng	nanogram
NK	natural killer cells
NG2	killer cell lectin receptor subfamily C
NKP	natural killer progenitor cells
NKT	NK- T cells
NT	no target

p.i.	post infection
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
pg	picograms
PI3K	phosphoinositide 3-kinase
RPMI medium	Roswell Park Memorial Institute medium
S1P	sphingosine-1-phosphate
SEM	standard error of the mean
STAT	signal transducers and activators of transcription
T reg	regulatory T cells
TGF	transforming growth factor
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
β2M	beta-2 microglobulin
μl	microliter

CHAPTER 1

BACKGROUND

1.1 Influenza virus infection

Influenza virus is an enveloped RNA virus of the family of Orthomyxoviridae. The virus has two glycoproteins on its surface, hemagglutinin (H) and neuraminidase (N) that assist in viral attachment and release from infected cells and are used to subtype influenza viruses. Genetic changes through mutations, antigenic drift and genetic reassortment can alter influenza virulence, promote escape from the immune system and even alter tissue tropism and the profile of symptoms observed during an infection. Thus, circulating influenza viruses continue to be a constant threat for new pandemics. In the 20th century alone there were three influenza pandemics documented and studied in detail. The most deadly pandemic was the one in 1918 (H1N1) which is estimated to be responsible for 50 million deaths worldwide [1]. The next two occurred in 1957 (H2N2) and 1968 (H3N2) and claimed approximately 1 million lives each one. Last, in 2009 another influenza outbreak was characterized as a pandemic, but it did not cause a severe infection and high mortality rates as the previous pandemics; however it had a high incidence of disease, high rate of transmissibility similarly to previous pandemics [2] and high rates of hospitalization [3].

Apart for the threat of pandemics that affects the majority of the population, seasonal circulating strains of influenza cause a worldwide public health concern as well. The most recent statistics indicate that flu epidemics cause between 3 and 5 million cases of severe illness, and between 250,000 and 500,000 deaths worldwide (91). Although flu-related illness can seriously

affect all age groups, those with the highest risks for influenza-related complications are children under the age of 2, adults over 65, and individuals who are immunocompromised or have chronic medical conditions (62, 88). The only known preventative for influenza disease is yearly vaccination to protect against circulating strains of influenza virus. There is clear evidence, however, that vaccination is not always effective in protecting from influenza disease in vulnerable populations. For example, influenza vaccination is 70% to 90% effective in protecting healthy individuals from influenza specific disease. However, vaccination is much less efficacious in the elderly. Vaccination reduces influenza-induced illness by 60% and mortality by 80% in the elderly (62). Despite this, influenza and its secondary complications result in 200,000 hospitalizations and 36,000 deaths each year in the United States, and influenza is the fourth leading cause of death in individuals 65 years and older.

Humans typically contract influenza virus through inhaling aerosols containing the virus caused by coughs or sneezes and through direct contact by surfaces contaminated with nasal secretions or bird droppings. It creates an infection with symptoms commonly described as the flu syndrome; yet, other respiratory viruses can also cause flu-like diseases making diagnosis of influenza infections rather difficult. The most characteristic symptoms for influenza infection are fever, cough and myalgia [4]. Yet during influenza infection, one can also develop headache, sore throat, nasal congestion, weakness and loss of appetite [5]. Many times these symptoms are confused with the common cold thus influenza infection goes self-diagnosed and self-treated if mild. However, influenza infection is considered a greater threat than the common cold for its potential to cause life-threatening infections with the development of viral pneumonia. All the symptoms generated during influenza infection, are a result of the inflammatory or cellular immune response to influenza virus. Increased cough is a specific characteristic of the flu,

because influenza virus can cause substantial lung cellular damage, which causes edema and hemorrhage accompanied by capillary thrombosis and increased necrosis of the alveolar wall [6]. Hypercytokinemia [7] and infiltration of neutrophils and/or mononuclear cells [6] has also been observed in severe cases of influenza infection. Damage of the lung cellular structure causes severe hypoxia and capillary thrombosis and mechanical ventilation may be needed. Fever, headaches, myalgia, weakness and anorexia are associated with the release of cytokines IL-1, IL-6, TNF- α and interferons, while nasal congestion, and runny nose are associated with the effects prostaglandins, and bradykinin, a potent vasodilator peptide, upon encountering influenza virus as well as the infiltration of cellular debris and immune cells in the alveoli [5].

People with reduced immune status, chronic medical conditions, elderly and pregnant women are at higher risk in developing severe influenza infection and are recommended each year to receive vaccination [8]. However, when vaccination does not prevent influenza infection with common circulating strains, these populations become infected and are a higher risk for severe complications because of their disturbed immune status. When elderly contract influenza infection, they develop severe illness that can lead to secondary complications and death. This severity has been associated with a general compromise of the cytotoxic CD8 T cell responses [9] resulting to higher viral replication, delayed recovery and greater lung pathology. Although the age-related impairment of the adaptive immune responses to influenza infection has been studied in detail, studies on the contribution of innate immunity in controlling influenza virus infection are lacking. Importantly, when people get a viral infection, they have to rely on both the innate and adaptive cellular responses to be timely coordinated in order to clear the infection without major tissue damage. Yet, studies on the innate immune responses in the context of aging have received less attention.

Understanding the innate immune responses is not only relevant for the elderly but also for young people, as it was shown with the recent outbreak of the novel 2009-H1N1 influenza virus that vaccination was not even available. Information about the early innate immune responses to influenza infection could provide us with additional alternative therapeutic methods to increase resistance against infection and reduced their severity by limiting viral replication early on. In this dissertation we focus our studies in understanding the age-related defects of the natural killer (NK) cells, cells of the lymphocyte lineage that are categorized in the innate branch of the immunity.

1.2 Clinical significance of NK cells in elderly

Lymphocytes are considered major coordinators of the immune responses and exhibit humoral and cellular responses. Among the lymphocytes, B and T cells require several days to get activated and exhibit their responses while natural killer (NK) cells act very quickly during the early stages of an infection and exhibit cytotoxicity against viral infected cells. Thus, we have focused our studies in investigating the effect of aging on NK cell responses.

The increased incidence of infectious diseases and cancer in the elderly, in combination with the importance of NK cells in the elimination of tumors and virus-infected cells, suggests that NK cell function may be impaired in aged populations. A more direct correlation between NK cell function and survival from infection was supported by a study done in healthy elderly selected based on the SENIEUR protocol in Japan [10]. The authors showed that among parameters such as age, sex, performance status, different immune cell subsets, serum albumin concentration, and NK cell activity, it was only low NK cell activity that was strongly correlated with increased risk for development of an infection and short survival due to infection.

Furthermore, centenarians usually maintain an increased number of NK cells that exhibit increased cytolytic function. Thus, preserved NK cell activity is an important determinant of longevity in humans. Whether there is a causative effect among good health and preservation of NK cell activity is not known, however there is more evidence supporting that the preservation of good immunity gives an advantage to individuals to live a longer life.

Previous studies from our laboratory and others have shown that aged mice exhibit increased susceptibility to influenza infection, sharp weight loss [11], increased severity and mortality [11, 12] and delayed viral clearance [11, 13]. Interestingly, aged mice have detectable viral titers by the fourth day post infection, in contrast to young mice, at a time that the specific cytotoxic CD8 T cell responses are still absent [9]. Thus, the study of the effects of aging on NK cells is not just relevant but can also offer the basis for the development of alternative therapies. Nogusa et al. (2008) showed that aged mice had less NK cells infiltrating into their lungs and significantly reduced natural cytotoxicity after influenza infection [13], but a more detailed characterization of NK cell functional impairment in aged mice is needed.

1.3 NK cells and mechanism of activation

Natural Killer cells compose a subset of lymphocytes that can kill malignant or viral infected cells without the help of other immune cells or activation from cytokines and within minutes of recognition of their targets. For this independent and spontaneous killing function they were named natural killers and categorized in the innate branch of immunity. NK cells differ from other lymphocytes in their morphology, and the absence of classical antigen receptors. Although NK cells don't have unique antigen recognition receptors as a product of gene rearrangement, they can identify their targets and kill them through a complex system of

recognition by the coordinating binding or absence of binding of their cell surface receptors that are inherited in their germline. Binding of NK cell surface receptors with their ligands can activate or inhibit NK cell function and thus have been named activating or inhibitory receptors. Activating receptors detect the presence of ligands on cells in “stress” and transmit the signal for a *positive* function, such as cytotoxicity or cytokine production. Inhibitory receptors detect the presence of ligands on normal, healthy cells, and transmit a signal for *negative* function so that they will neither exhibit cytotoxicity nor cytokine production. Because the ligands for inhibitory receptors are mostly MHC-I molecules expressed by all the cells of the host, such negative regulation of NK cells provides tolerance to healthy self cells. On the other hand, many viral infected cells, or tumor cells have reduced MHC-I expression, resulting in diminished inhibitory signal on NK cells and allowing the signal from the activating receptors to become dominant. The integration of the intracellular pathways downstream the engagement of activating or inhibitory receptors is what dictates NK cells to be activated or not. During viral infections, additional mechanisms contribute in NK cell activation. A good example is the activation of NK cells during murine cytomegalovirus (MCMV) viral infection: MCMV virus encodes the m157 viral protein, which is an MHC-class I like protein in order to avoid T cell recognition; however, m157 is the ligand for Ly49H, an activating receptor on NK cells. Thus, during MCMV infection, Ly49H⁺ NK cells can recognize and kill MCMV infected cells. Similar examples of tumor or viral infected cell recognition are the expression of ligands for the activating receptor NKG2D, or the recognition of hemagglutinins in influenza infected cells by the NK cell activating receptor NKp46. Finally, during an infection there are many inflammatory signals that can activate NK cells independently from MHC-I ligands.

NK cells acquire their receptor repertoire during their development in the bone marrow. NK cell progenitors (NKPs) undergo different stages of development that have been characterized by the subsequential acquisition of various cell surface markers, including the inhibitory and activating Ly49 receptors and several integrins that are associated with the acquisition of functional capacity. Kim et al (2002) proposed that during development in the bone marrow, indicated cell surface markers characterize 5 distinct stages from the NKPs to mature NK cells [14]. Based on this classification, NKPs belong to “*Stage I*” of maturation and when they acquire the universal NK cell marker, NK1.1 and CD94/NKG2 belong to the “*Stage II*”. On “*Stage III*” they acquire the activating and inhibitory Ly49 receptors, but because they still cannot exhibit any function are characterized as immature NK cells. Next, at “*Stage IV*”, the expression of the integrin DX5 is increased and they start expanding in numbers and at “*Stage V*”, with the acquisition of additional integrins such as CD11b and CD43, are considered mature NK cells and exhibit fully functional capacities. Most of the NK cells found in the bone marrow are CD27⁺, but the majority of circulating NK cells in the periphery are CD11b⁺CD27⁻ and these NK cells are considered terminally mature.

While NK cells are still immature and during their development in the bone marrow, the interactions of the inhibitory Ly49 receptors with their host-MHC ligands are considered important in regulating the reactivity of NK cells, and the process has been named “licensing” [15]. NK cells that express at least one inhibitory receptor that recognizes self MHC is licensed to acquire potent functional activities e.g. cytokine production and cytolytic activity, in response to cross-linking of their activation receptors, while NK cells that do not express inhibitory receptors for self-MHC class I molecules are hyporesponsive in order to avoid self-auto-aggressiveness [15]. In C57BL/6 mice, Ly49C is the major inhibitory receptor known to interact

with the self-MHC class I ligand K^b and to provide licensing. Ly49I also has some reactivity to self-MHC class I ligand K^b and the CD94/NKG2A receptor interacts with D^b peptide presented by Qa-1 [16]. On the other hand, Ly49A has known strong reactivity with $H2D^d$ that is absent from C57BL/6 mice. Because of the stochastic way that these receptors are expressed on NK cells during development, it is possible to have a very heterogeneous NK cell population with different combination of Ly49 receptors that recognize different MHC-class I molecules independently of the host MHC haplotype. However, the licensing theory predicts that NK cells that do not express inhibitory receptors for MHC-I remain unresponsive [15].

After NK cells acquire their Ly49 receptors, immature NK cells undergo functional maturation during a developmental stage that corresponds with an increase of the cell surface density of maturation markers, and a significant expansion of their numbers in the bone marrow. NK cells acquire function after they express high levels of CD11b and CD43 [14]. During these late developmental stages, a reduction of the density of CD27 and an increase of KLRG1 on NK cell surface is observed making the $CD11b^+CD27^-$ NK cells the most differentiated NK cell subset. These cells have increased expression of S1P5 receptor and decreased expression of CXCR4 which facilitates their release to the periphery [17]. In addition, $CD11b^+CD27^-$ NK cells are considered the most differentiated NK cells, they compose the majority of NK cells circulating in peripheral blood and in non-lymphoid tissues, they have high potential for cytolytic functions and finally, they compose the majority of NK cells that produce $IFN-\gamma$ upon activation.

1.4 NK cells and influenza infection

Influenza virus infects epithelial cells, which in turn, produce cytokines and chemokines to alert immune cells of the ongoing infection and effectively initiate a systemic immune response. However, influenza virus uses its NS1 protein to inhibit an early alert of the immune system and replicates at high levels before the immune system starts its counterattack [18]. Even with a small delay, people don't usually die from an influenza infection although they exhibit more severe symptoms compared to other common respiratory infections. Part of the reason, is that influenza infection results in a strong inflammatory response with the production of several cytokines and chemokines from infected macrophages and endothelial cells. These cytokines can attract neutrophils, activate NK cells and macrophages, mature dendritic cells (DCs) and induce their circulation to lymph nodes where the T cells are getting activated. Among the cytokines that are produced, type I interferons (IFN) are significant inducers of NK cell response. Additionally, activated macrophages produce many chemokines and cytokines to propagate the immune responses including NK cell function. Dendritic cells process viral antigens to present to T-cells, and also produce cytokines that can activate and support proliferation of NK cells. Finally, NK cells can get activated directly from recognition of the infected cells through NK cell activating receptors or the absence of MHC-I molecules on infected cells. Activated NK cells kill virus infected cells and produce IFN- γ . Actually, IFN- γ production plays an important role in shaping the adaptive immune responses to clear the viral infection. Activated NK cells can also kill hyperactivated macrophages, or immature DCs thus playing an immunoregulatory role. While it is clear that many events are initiated simultaneously in the lungs during influenza infection we don't know how exactly NK cells are activated, neither which cytokines are more responsible nor their role in activating NK cells during influenza infection.

Recently our collaborators have demonstrated the importance of direct action of type I IFNs on NK cell activation during influenza virus infection. NK cells from influenza-infected mice that lacked the IFN α / β receptor had diminished function in comparison to NK cells from infected mice that lacked the IL-12 or IL-18 receptor (personal communication, Hwang I and Kim S). The absence of NK cell function was not related to the inability of DCs or other accessory cells to activate NK cells, but rather was related to direct effects of type I IFNs on NK cell activation, because adoptive transfers of NK cells that lacked IFN- α / β receptor into wild type mice did not result in activation of these NK cells upon influenza infection (personal communication, Hwang I and Kim S). Even though this study establishes a dominant role for type I IFN for NK cell function during influenza infection, it also revealed that type I IFNs are more important for cytolytic function rather than IFN- γ production. Thus, it is possible that other cytokines or recognition signals play some role in NK cell activation for cytokine production.

In agreement with a significant role for interferons on NK cell activation during influenza infection, Yu Jing (2007) showed that both IFN- α and IFN- γ are major inducers of activation of human NK cells during influenza infection [19]. Blocking antibodies against IFN- α , IFN- α R, or IFN- γ downregulated IFN- γ production, while induction of IFN- α production by plasmacytoid DCs with CpG, upregulated IFN- γ production from NK cells during *in vitro* infections of human PBMCs with influenza virus [19]. However, recombinant IFN- γ alone or in conjunction with influenza virus could not activate isolated NK cells; therefore, other accessory immune cells are implicated in NK cell activation. Indeed, NK cells were activated in the presence of IFN- γ when NK cells were stimulated with influenza virus in the presence of monocytes [19]. Thus, it is possible that IFN- γ presence during the early stages of influenza infection enhances NK cell activation. Several murine studies have also provided data to support an IFN- γ dependent early

resistance towards respiratory infections [20, 21], and more specifically, presence of high IFN- γ early during infection protects mice from death induced by influenza infection in an NK cell dependent manner [22]. Altogether, these data suggest that IFNs have a vital role in activation of NK cells early on during influenza infection.

1.5 NK cell recognition of influenza virus

Extensive evidence supports an important role for NK cells during influenza infections. Severe influenza infections have often been associated with depletion of NK cells specifically [23-25] and lymphopenia in general [23]. During the initial days of influenza infection, NK cells migrate in the lungs become activated and [12, 24] and demonstrate high cytotoxic activity [13, 26-28] followed by production of IFN- γ [29], an important anti-viral cytokine. NK cell depletion results in severe influenza infection [13, 30-32], higher viral replication [13, 30], reduced cytotoxic responses [33, 34], and reduced early IFN- γ production [30, 34]. The protective role of NK cells against influenza infection was more appreciated when influenza hemagglutinin was identified among the ligands of NKp46, an activating receptor found in all NK cells [35]. Further experiments showed that disruption of the gene encoding NKp46 resulted in lethal influenza infection in mice [36]. Thus, appropriate NK cells responses have an important impact for the outcome of influenza infection.

Studies done *in vitro* have provided us with more details on the interactions of NK cells with influenza infected cells and viral particles and the strategies that influenza virus has evolved to avoid NK cell responses. The most important finding was the identification of influenza hemagglutinin as a ligand for NKp46 and NKp44 [35]. NKp46 is expressed in the majority of NK cells but not in other immune cells, while NKp44 is not expressed in resting human NK cells

and is induced only upon activation [37]. Influenza hemagglutinin can interact with NKp46 directly without the requirement of other molecules, but whether recognition of influenza particles from NKp46 receptors on the NK cell surface involves other costimulatory molecules is not known. The extracellular region of NKp46 is composed of 2 Ig-like domains and the interaction with influenza hemagglutinin is located on the domain which is proximal to the membrane and in particular the sialic acid residues linked via $\alpha 2,6$ to the threonine 225 residue on NKp46 are essential for this interaction [38, 39]. Although this is true for most of influenza viruses (H1N1, H3N2), the H5N1 avian influenza virus requires interactions of H5 with NKp46 with threonine residues 216 and 225 [39]. Binding of the hemagglutinin expressed on transfected or infected cells with the NKp46 of NK cells can activate effector functions [35, 38], however H5 hemagglutinin binding only to NKp46 could not elicit NK cytotoxicity; it required the engagement of additional ligands with NKG2D, another activating receptor on NK cells, in order to lyse H5N1 infected cells [39].

Direct infection of isolated NK cells with influenza virus particles causes activation of NK cells with increased expression of CD69, CD107a and IFN- γ and a downregulation of NKp46 expression, possibly due to internalization of this receptor [40]. However, it was found that the influenza virion binds to sialic acids on NK cells and internalizes through clathrin and caveolin mediated endocytosis. Through this pathway, influenza virus could infect not only epithelial cells but also NK cells- although with reduced efficiency- and was able to replicate inside them [41]. Infection of NK cells by influenza viruses did not result in new infectious virus but rather it induced marked apoptosis of NK cells [41]. These data could be translated in clinical settings, where a marked decrease of NK cells is observed in severe cases of influenza infections [23-25]. Earlier it was mentioned that the help of additional activating receptors, such as

NKG2D, is important for NK cells to show cytotoxicity upon recognition of the influenza hemagglutinins. The costimulatory role of NKp46 and NKG2D was also supported in studies that showed that NK cell activation by influenza infected DCs was inhibited upon blocking antibodies for these two receptors [42]. Apart from NKG2D, additional activating receptors may be important. Indeed, while NKp46 is downregulated upon NK cell activation with live influenza virus, 2B4 expression is increased on NK cells in *in vitro* influenza virus infections [43]. Interestingly both the amount of 2B4 and the percentage of NK cells positive for this receptor were increased upon influenza infection. Induction of 2B4 was specifically increased on previously 2B4 negative NK cells, such as the CD56^{bright} and CD56^{neg} NK cells that in general have reduced cytotoxicity. NK cells that were 2B4^{bright} showed increased granule release after infection, indicating that influenza infection induced these subsets towards a phenotype that supports cytotoxicity [43]. *In vivo* influenza vaccination studies confirmed these results and indicated that although the reduction of NKp46 is transient and occurs within the first day post vaccination, the expansion of 2B4 NK cells was long lasting extending for two weeks post vaccination [43]. Increase in the expression of 2B4 could be related to effects on NK cells from cytokine activation, yet the role of IL-2 and IL-12 on 2B4 induction was excluded based on *in vitro* blocking experiments [43].

In general, although NK cells have the ability to recognize and kill influenza infected cells within minutes of target recognition, it is evident that influenza virus has evolved multiple ways to disrupt NK cell activation. First, the influenza encoded NS1 protein interferes with the initiation of the host's interferon response upon infection allowing the virus to multiply for almost two days without the activation of innate immune cells [18]. After that, robust host responses are initiated that can limit viral replication but can also lead to severe pathology.

Secondly, the virus impairs innate immune cell activation by infecting innate immune cells and causing their death [41]. Macrophages and DCs are a target for many viruses, however, the suggestion that influenza virus can non-productively infect NK cells and induce their apoptosis is of specific evidence that NK cells are important in fighting influenza infection, because influenza virus cannot infect other lymphocytes. Moreover, influenza virus results in functional impairment of NK cells as it was shown that NK cells from infected mice with influenza virus killed significantly less target cells and produced significantly less proinflammatory cytokines upon the engagement of their activating receptors [44]. The increased long lasting expression of 2B4 in influenza vaccinated individuals may have an inhibitory effect on NK cells, since it has been suggested that 2B4 can deliver inhibitory signals when the surface density of this receptor is high [45]. However, a more possible explanation was given by Mao et al (2010): binding of the viral hemagglutinin caused its internalization and downregulation of the ζ chain, which is associated with NKp46, thus reducing subsequent downstream signaling and cytotoxicity upon target cell stimulation through the NKp46 [46]. Finally, influenza virus augments NK cell inhibition by generating complexes of class I MHC complexes in infected cells that cause increased recognition of inhibitory NK cell receptors [47]. Overall, NK cells seem to have been an important barrier for influenza virus to overcome and influenza virus has developed several ways to impede their function.

1.6 Objective and hypothesis

Previous published research from our laboratory indicates that after influenza infection, fewer NK cells are recruited in the lungs of infected aged mice and while NK cell cytotoxicity is increased in young mice, it is significantly less in aged mice, and this defect correlates with

increased lung viral titers. However, there is a gap in our knowledge about the specific age-related defects on NK cell function, homeostasis and phenotype.

In this dissertation we test the following central hypothesis: **NK cells from aged mice show impaired function during an influenza virus infection or other stimulatory conditions compared to NK cells from young mice. The defect of aged NK cells is associated with an alteration of the distribution of NK cell subsets belonging to a different maturational stage, with aging to promote a reduction in the levels of the most functional mature NK cells. Therefore, it is possible that aged mice show defective generation of mature NK cells from the bone marrow, or reduced survival of mature NK cells in the periphery.**

Chapter 2 is an extensive literature review on what is known up to date, about the age-related changes on NK cell numbers phenotype and function from murine and human studies.

Chapter 3 describes the NK cell responses in young and aged mice during influenza infection and demonstrates that impaired NK cell function in aged mice is associated with an altered distribution of NK cells in different maturational stage.

Chapter 4 describes the homeostasis of NK cells in young and aged mice in resting conditions. In this chapter we demonstrate that aged mice exhibit a specific developmental defect in the generation of mature NK cells from the bone marrow, and that mature NK cells are more prone to apoptosis in aged peripheral tissues compared to young.

Chapter 5 summarizes all described data and outlines future studies.

The results of this work provide an important mechanistic understanding on the changes of NK cell subsets in aged mice and to our knowledge, this work is the first to characterize in detail the development of NK cells in aged mice.

CHAPTER 2

NATURAL KILLER CELLS IN ADVANCED AGE: A REVIEW OF MURINE AND HUMAN STUDIES

2.1 Abstract

In this review we summarize and compare findings by human and murine studies about the effect of aging on NK cell numbers, phenotype and function. In contrast to humans where NK cell numbers are preserved in the blood, aged mice have reduced NK cells in their peripheral tissues, but not in the bone marrow. However, aged mice have less mature NK cells in all peripheral tissues, including the bone marrow. The specific reduction of NK cells in aged mice could be associated with reduced survival in the periphery, or reduced maturation and exit of mature NK cells from the bone marrow. In contrast, elderly humans exhibit an accumulation of mature NK cells in their blood, but data concerning their distribution in other tissues are lacking. Further characterization of the phenotype of aged NK cells in humans and mice reveals certain similarities, such as altered inhibitory repertoire, accumulation of KIRs or Ly49s on immature NK cell subsets, and increased expression of phenotypic markers usually observed in immature or activated NK cells. Basal natural cytotoxicity is reduced or preserved in mice and humans, and it is strongly related with the effects of aging on the distribution of NK cell subsets. Stimulation of NK cells from young and elderly people with cytokines has generated conflicting data based on the various combinations of cytokines and the duration of stimulation. Short-term incubations reveal significant impairment of NK cell production of IFN- γ and chemokines, and - to a smaller extent - of natural cytotoxicity, while longer incubations can correct the impaired

natural cytotoxicity. Finally, aged NK cells after interferon stimulation show significant impairment, something that could have severe implications during viral infections.

2.2 Introduction

Natural Killer (NK) cells belong to the lineage of lymphocytes, but they function as cells of innate immunity. The other lymphocytes, B and T cells, are responsible for the adaptive immune responses that result in an effective clearance of an infection, by producing antibodies or killing of virus infected cells. However, they require several days to mount their responses, while the virus can replicate in high levels during the early stages, when innate immune cells are acting. On the other hand, NK cells can kill their target within minutes of activation and therefore, they could control viral replication at manageable levels before the adaptive immune responses are mounted. NK cells can be activated by cytokines that other innate immune cells release, or solely by the recognition of target cells through the integration of signals from a wide array of cell surface receptors that can be either activating or inhibitory. In contrast to B and T cells, NK cell receptors exhibit specificity, which is not a product of gene rearrangement but rather is inherited in their germline. Most of the studies on the effect of aging on the immune responses to viral infections or vaccination have focused extensively on the adaptive immune responses; yet, innate immunity has received less attention. Based on the fact that NK cells can kill cancer and virus infected cells and that the incidence of cancer and fatal infections increase with aging, it is of importance to consider how aging affects their function. In the following review we summarize the results of several studies, performed in humans or mice -including the data generated for the purpose of this dissertation- about the effect of aging on NK cell numbers, phenotype and function, and we will provide possible mechanisms.

2.3 The effect of aging on NK cell numbers

Murine studies on the effect of aging on NK cell numbers at resting conditions have been contradictory. Some reported that there is no difference on the numbers of NK cells between young and aged mice [13, 48-50], while others reported that aged mice have reduced NK cell numbers [51-53]. These differences may be explained by the use of non-physiological models of aging or inaccurate techniques to identify NK cells. Among those who reported that there was no change on basal NK cell numbers in aged mice, Dong et al. (2000) used the senescence prone SAM-P1 strain and performed comparisons between the SAM-P1 strain and its parental senescence regular SAM-R1 strain of the same age, but not across ages [48]; Koo et al. (1982) used early detection methods to identify NK cells that were not very specific for NK cells [49]; Plett et al. (2000a) reported that $NK1.1^{+}$ lymphocytes from spleens were not different among young and aged splenocytes but the $NK1.1^{+}$, $CD8^{+}$ cells in spleens were elevated in the aged mice [50]; yet, if $NK1.1^{+}$, $CD8^{+}$ cells are excluded from $NK1.1^{+}$ lymphocytes, NK cells are reduced in aged mice. Finally, having identified NK cells as $NK1.1^{+}CD8^{-}$, Nogusa et al. (2008) also reported no differences on basal NK cell frequencies [13]. On the other hand, recent studies show that aged mice have reduced NK cells in their periphery [[52] and data presented in this dissertation]. In our studies we used a very specific gating strategy for the identification of NK cells which contained lineage and NK cell markers [$CD3^{-}$, $CD19^{-}$, $NK1.1^{+}$, $NKp46^{+}$] to identify NK cells and we show that aged mice have reduced NK cells in most of their peripheral tissues with the exception of the lymph nodes, but they maintain similar numbers of NK cells in the bone marrow.

In contrast to murine studies, it is generally accepted that elderly maintain a high percentage of NK cells in circulation. Human peripheral blood from elderly had either similar [54] or higher NK cells [55-60] in comparison to samples from young people. There are certainly immunological differences between humans and mice, but one has also to consider that most of the results reported in humans are obtained from blood samples. Thus, limited information exists on the effect of aging on the distribution of NK cells in other tissues. Furthermore, comparisons among human and murine NK cells are impeded from the use of different markers to identify NK cells. Human NK cells are usually defined as $CD56^{+} CD3^{-}$, or $CD16^{+} CD3^{-}$ cells but murine NK cells do not express CD56, and usually they are defined mostly as $NK1.1^{+} CD3^{-}$ cells. Thus, it is difficult to make comparisons between human and mice concerning NK cell numbers.

As mentioned before, we observed a reduction of NK cells in aged mice in peripheral tissues but not in the bone marrow. This could be the result of decreased egress from the bone marrow, reduced generation of NK cells, decreased survival in the peripheral tissues or preferentially trafficking and accumulation to other tissues such as the lymph nodes. The possibility that aged NK cells are retained in the bone marrow is supported by the findings presented in the following studies: aged mice have higher percentage of NK cells in their bone marrow, most of which are immature NK cells. Since it is only when NK cells become mature in the bone marrow that they acquire the appropriate receptors to receive the egress signals and migrate to the periphery, the implications of reduced terminal maturation in the bone marrow may contribute to less NK cells in the periphery. We further show evidence that support the possibility that aged mice have reduced generation of mature NK cells. Less NK cells from the bone marrow of aged mice were identified as recently proliferating cells. Besides, previous evidence supports that aged NK cells are intrinsically impaired to proliferate in response to IL-2

[55] and *in vivo* labeling experiments in humans concluded that total NK cell production rates are impaired with aging [61] and immunoperoxidase labeling techniques in mice showed reduced production of new NK cells from the bone marrow of aged mice [51]. Although NK cell proliferation is reduced in the bone marrow of aged mice, we found that proliferation of NK cells in the spleens was similar independently of age. In accordance, when Ki67 staining was measured on NK cells from peripheral blood of elderly humans, it was also observed only a trend for reduced peripheral proliferation but not significance [62]. Thus, aging results in reduced bone marrow proliferation, while it may not affect the homeostatic peripheral proliferation.

Although reduced numbers of NK cells can be partly explained with the hypothesis of reduced generation of NK cells from the bone marrow, we provide evidence that aged mature NK cells have reduced survival rate in the periphery. Aged NK cells show reduced Bcl-2/Bim ratio, which was attributed to elevated Bim expression and not differences on the Bcl-2 expression. For NK cells, survival in the periphery depends, among other factors, on the presence of IL-15 and its trans-presentation through the IL-15R α receptor [63], which results in maintaining increased levels of the antiapoptotic molecule Bcl-2 [64]. Interestingly, there is evidence that circulating IL-15 is reduced in aged mice [65]. Finally, data supporting the hypothesis that aging alters trafficking of NK cells in the peripheral tissues are presented in the following studies and from Fang et al. (2010): aged NK cells do not migrate effectively to lungs or lymph nodes after viral infection [52]. Although, we show that uninfected aged mice have similar NK cell numbers in their lymph nodes in resting conditions in contrast to other peripheral tissues, an explanation for the reduced NK cells in the peripheral tissues could be that aged NK cells preferentially home to lymph nodes through the expression of specific chemokine receptors. In particular, human lymph nodes are considered home for a significant proportion of NK cells,

thus altered trafficking may affect their distribution and ultimately their function. In fact, in both humans and mice, aged NK cells had reduced expression of certain integrins, among which, CD62L, that could affect their migration to these tissues during inflammatory conditions. However, there are currently no studies addressing homeostatic distribution of NK cells in the peripheral tissues in aged humans, and we are the first to report these changes in aged mice.

2.4 The effect of aging on NK cell phenotype

NK cells are composed of several subsets identified based on a combination of functional assays and the expression of various surface markers. In mice, NK cells can be separated into four functional subsets based on the expression of CD11b and CD27 [66], or they can be identified as subsets in various maturational stages, as proposed by Kim et al.(2002) based on the subsequent acquisition of NK1.1, CD94/NKG2, Ly49s, CD49b, CD11b and CD43 and the downregulation of CD51 [14]. Furthermore, NK cells can be separated in subsets based on the expression of several activating and inhibitory receptors, their Ly49 receptor repertoire, or based on the expression of several markers uniquely identified on NK cells that reside in lymph nodes, thymus or liver. The most popular characterization scheme of NK cells uses the CD11b and CD27 classification. Based on this, four subpopulations of murine NK cells have been characterized: $CD11b^{-} CD27^{+}$, $CD11b^{+} CD27^{+}$, $CD11b^{+} CD27^{-}$ and $CD11b^{-} CD27^{-}$ NK cells. NK cells that are positive for CD11b are considered mature and are capable of natural cytotoxicity and cytokine production while NK cells negative for CD11b are immature and show reduced effector functions. NK cells that are $CD11b^{+} CD27^{+}$ have higher cytokine production capacity, and they can differentiate into $CD11b^{+} CD27^{-}$ that are considered terminally mature

NK cells, highly cytotoxic and compose the majority of NK cells found in circulation [67].

In the studies presented in this dissertation, we show that aged mice have reduced terminally mature, $CD11b^{+}CD27^{-}$ NK cells in all examined tissues. The reduction of $CD11b^{+}CD27^{-}$ NK cell percentage is not a result of an increase in the release of immature NK cells in the periphery but rather a specific reduction of the $CD11b^{+}CD27^{-}$ NK cell subset. Other recent studies have also confirmed that aged mice have less mature NK cells [52]. We further provide evidence for a maturational defect in aged NK cells based on the expression of additional maturational markers. Aged NK cells are composed of a high percentage of $CD11b^{-}$, $CD43^{low}$, $KLRG1^{-}$ and $CD51^{high}$ NK cells. These data support the hypothesis that in aged mice there is a defect in NK cell development that results in less circulating mature NK cells.

Besides altered maturational markers aged NK cells have a different phenotype in comparison to young NK cells. Among the markers we examined, aged mice had upregulated expression of NKG2A, an inhibitory receptor whose expression is inversely related with the expression of MHC-class I inhibitory receptors and is found predominantly in immature NK cells [68]. In addition, aged mice had significantly increased CXCR3 expression, a chemokine receptor for CXCL9-10 that is implicated in the recruitment of NK cells to sites of inflammation. CXCR3 expression is induced upon activation and is associated with increased expression of CD27, which was also observed in aged NK cells. Moreover, aged mice had increased expression of CD127, the IL-7 receptor, which characterizes NK cells that arise in the thymus [69]. Finally aged NK cells had increased expression of CD90.2 (Thy1) -a marker of liver NK cells- and recently suggested to characterize memory NK cells [70]. The increase of CD127 and CD90.2 on aged NK cells suggests that alternative pathways of NK cell development may exist

in aged mice. On the other hand, aged NK cells had reduced expression of CD62L, a cell adhesion molecule that functions as a homing receptor to secondary lymphoid tissues; its expression is increased with maturation and its reduced expression on aged NK cells may be implicated in the reduced migration to inflamed lymph nodes in aged mice [52]. At last, we show that aged mice have reduced expression of Ly6C; Ly6C is upregulated in activated T cells, and while resting NK cells have low levels of Ly6C, activated NK cells have increased Ly6C expression [71]. All the above-mentioned markers can change upon activation with various cytokines or changes on the other cell types thus, it is possible that these changes in aged mice contribute to the differential phenotype of NK cells. However, we observed that the markers upregulated in aged NK cells are mostly found on immature NK cells, while the markers downregulated with aging correspond to mature subsets, indicating that changes in the composition of the NK cell subsets may impact their overall expression by NK cells.

Other important cell surface receptors that regulate NK cell activation are the inhibitory and activating Ly49 receptors. To our knowledge, the data presented in the following chapters are the first to characterize the Ly49 receptor repertoire in aged mice. We observed that aged mice had not increased coexpression of the 2, 3, or 4 inhibitory receptors (Ly49C, Ly49I, Ly49A and Ly49G2) on the same NK cell, but the percentage of NK cells that do not express any of these receptors was higher, and the percentage of NK cells that expressed only one of them was reduced. The later, may simply reflect the significant reduction of Ly49I observed in the aged NK cells, because aged C57BL/6 mice have less NK cells expressing Ly49I, but more NK cells that express Ly49C and Ly49A. The differential expression of Ly49C and Ly49I may indicate altered expression of MHC-I molecules in the bone marrow, and therefore altered interactions of these inhibitory receptors with their ligands during development. The increased expression of

Ly49A may be explained by defects during NK cell development, because Ly49A is among the first to be expressed and before Ly49C/I, G2 and F [72], while its expression is under the control of a separate transcription factor that doesn't affect the expression of other Ly49s [73]. Moreover, we showed that less NK cells from aged mice express the activating receptors Ly49D and Ly49H. These receptors are acquired later during development in the bone marrow and after the acquisition of the inhibitory receptors [74]. Altogether, it is possible that the differences observed among young and aged NK cells reflect defective maturation of NK cells in aged mice. In support with this, we observed that in aged mice, immature NK cells have a higher expression of all Ly49s in comparison to young immature NK cells. Similar results have been obtained for NK cells lacking MHC-class I molecules such as the TAP^{-/-} or β 2M^{-/-} mice: those mice had higher percentages of immature CD11b⁻ CD27⁺ NK cells with increased expression of Ly49C/I [75]. Overall, age-related altered interactions of the inhibitory receptors with their MHC-class I ligands during development could affect the distribution of Ly49s in the CD11b/CD27 NK cell subsets.

Previously we discussed that aging affects NK cell phenotype of murine NK cells, but these data are difficult to translate in human NK cells because the classification of human NK cell subsets is distinct from that of murine NK cells. Human NK cells are defined based on the expression of CD56 and CD16 and are classified into two functional subsets, CD56^{bright} CD16⁻ and CD56^{dim} CD16⁺ NK cells. In humans, CD56^{bright} CD16⁻ NK cells are known to be potent cytokine producers and are found in higher percentages in secondary lymphoid tissues, while CD56^{dim} CD16⁺ NK cells exhibit potent cytotoxicity and compose the majority of peripheral blood NK cells. It is not clear whether there is a developmental connection between the two

subsets, however there is evidence suggesting that CD56^{bright} CD16⁻ NK cells can give rise to CD56^{dim} CD16⁺ both *in vitro* [76] and *in vivo* [77]. Additionally, it has been shown that CD56^{dim} have shorter telomeres compared to CD56^{bright} NK cells, supporting the notion that CD56^{bright} NK cells are less terminally differentiated cells [78]. Evidence for the existence of a developmental intermediate stage was recently published proposing that CD56^{bright} NK cells become first CD56^{bright} CD16⁺ cells and then CD56^{dim} CD16⁺ cells [79].

In humans, aging results in an overall reduction in the CD56^{bright} NK cells and an increase in CD56^{dim} NK cells in peripheral blood [57, 80-82]. These data are further supported by the finding that telomere length of NK cells is shortened with increasing age [83]. Overall, human studies suggest that aging brings a shift of NK cell diversity towards the most terminally differentiated stage of CD56^{dim}, at the expense of CD56^{bright} NK cells, in contrast to what is observed for murine aged NK cells. Additionally, among the CD56^{bright} subset, more CD56^{bright} NK cells have increased expression of CD16 in the elderly compared to young [79]. Although the CD56^{dim} subset increases with aging, it is suggested that CD56^{dim} NK cells are composed of several NK cells into various differentiation stages [68, 84, 85], while others showed that they are composed mostly from CD11b⁺CD27⁻ NK cells [86]. The specific reduction of CD56^{bright} NK cells in blood circulation of elderly can also be attributed to altered trafficking into the secondary lymphoid tissues, where they preferentially home, resulting in their

depletion from the blood. However, to our knowledge, no studies to date have addressed this possibility, as well as the CD11b/CD27 subset classification of NK cells in the elderly.

Recently a few studies characterized the phenotypic changes in aged NK cells in humans. In agreement with aged NK cells from mice, circulating NK cells from the blood of elderly were also found to have reduced KLRG1 expression [87]. However, in contrast to aged murine NK cells, but in accordance with the accumulation of CD56^{dim} NK cells with aging, human NK cells from elderly have reduced CD94 and NKG2A expression [56, 57]. Accumulation of CD56^{dim} NK cells that are CD62L^{low} has also been reported in elderly, and it was explained by the fact that CD62L^{low} NK cells showed characteristics of terminal differentiated subset of CD56^{dim} NK cells with reduced proliferative capacity; however, they showed increased IFN- γ production [88]. CD56^{dim} NK cells from elderly have also increased expression of CD57 [82], a marker of terminal mature NK cells that is also considered a senescence marker for T cells. However, increased expression of CD57 has also been associated with recently activated and proliferated NK cells after viral infection [89]. Finally, aged NK cells had higher expression of CD69 and slightly higher expression of CCR5, while their expression of CXCR1 was reduced [90].

Further studies on the effect of aging on the repertoire of inhibitory and activating receptors on human NK cells (killer-cell immunoglobulin-like receptors, KIRs, natural cytotoxicity receptors, NCRs) reveals that they are altered with aging, similar to what we have observed for aged mice and regardless the structural differences between human and murine NK cell receptors. Aged human NK cells have higher expression of LIR-1 and ILT-2 inhibitory receptors that recognize a broad range of classical MHC-I molecules [82]. Here, it is important to note that the specific binding of LIR-1 with its ligand is enhanced after influenza infection,

resulting in inhibition of NK cell activation [47] in relation to the fact that influenza virus induces specific clustering of MHC-I ligands of inhibitory receptors on the membranes of infected cells to inhibit their killing by NK cells [91]. Since aging results in increased expression of LIR-1 it is possible that individuals that have the MHC-I ligands for this receptor are more susceptible to influenza infection. Among other inhibitory NK cell receptors, the expression of KIR2DS3 and KIR2DL5 was associated with aging [57, 92]. Nevertheless, their role in inhibition of NK cell activity during influenza infection has not been examined. On the other hand, other KIR receptors, not characterized in the context of aging, are associated with influenza infection. Patients treated in the intensive care unit for the pandemic influenza A (H1N1/09) had a higher proportion of KIR3DL1/S1 receptors in the absence of their cognate ligands and a higher proportion of KIR2DL2/L3 ligand positive pairs. This suggests that in conjunction with its ligand, KIR3DL1 contributes to resistance to influenza infection, while, KIR2DL2/L3, contributes to susceptibility to influenza infection [93]. Increased expression of some of these markers on NK cells has also been observed after chronic viral infections, thus it is possible that human aging reflects a history of viral infections encountered in the past. In contrast, laboratory animals become older in a disease-free environment. Yet, there are some similarities on the phenotype of human and murine aged NK cells. Even though KIR expression is increased in human aged NK cells [56, 82], only the immature, CD56^{bright} NK cells have higher expression of KIRs [57, 92] while the most mature, CD56^{dim} NK cells have similar expression of KIR receptors.

Finally, among the examined natural cytotoxicity receptors (NCRs), aging of human NK cells has shown to reduce the expression of NKp30 and NKp46 [57]. The reduction of these receptors could have implications in NK cell function, since NKp30 takes part in the interactions

of NK cells with dendritic cells, and NKp46 is a major activating receptor that recognizes influenza hemagglutinins. Additional phenotypic differences among young and aged human NK cells include reduced expression of CD2 [87] and increased expression of CD16, while the expression of NKp44, NKp80, 2B4 or LAIR-1 remained unchanged with aging [82].

Overall, there are phenotypic differences among young and aged NK cells in both mice and humans. These differences could reflect a change in the distribution of NK subsets with aging or could reflect intrinsic changes within the subsets as a result of changes of the microenvironment and the history of viral infections. In the next chapters, we provide evidence supporting that aging affects terminal maturation of NK cells in the bone marrow of aged mice, while evidence about the effect of aging on human NK cell development is lacking. No age-related defect was detected during the early developmental stages when NK cells are still considered immature. Aged mice showed impairment only during the transition towards the latest stages of terminal maturation, where upregulation of CD11b, CD43 takes place. We propose that the observed reduced proliferation of NK cells in the aged bone marrow is a major block in the maturation of NK cells, resulting in the accumulation of immature NK cells that have increased Ly49 receptors but cannot become terminally mature. In humans it was proposed that NK cells remain in the bone marrow for a significant time - almost 10 days - after their expansion and before they go to the peripheral blood to mature [61]. Thus, it is possible that the microenvironment of the bone marrow in aged mice affects their terminal maturation of NK cells. Adoptive transfer experiments of bone marrow cells from young mice to irradiated young and aged mice would provide definite answers to this hypothesis.

To date, the regulation of terminal maturation of NK cells has been attributed to the functions of various transcription factors but they have not been investigated in the context of

aging. In the following paragraphs we will discuss which transcription factors these are and how they could affect terminal maturation of NK cells from aged mice. T-bet and EOMES are two major transcriptional factors associated with terminal maturation of NK cells. Deficiencies of either of these factors resulted in a block of *stage IV* of development with immature NK cells that have acquired CD49b but not CD11b [94]. In contrast, we observed that aged mice acquire CD49b normally, and similar percentages of NK cells in young and aged mice belong to the developmental *stage IV*; however, significantly less NK cells from aged mice could transit to the next developmental *stage V*, which is characterized by increased expression of CD11b and CD43. Nevertheless, others showed that the presence of both T-bet and EOMES as well as GATA-3, another transcription factor [95] is required for the later transition to CD11b⁺ KLRG1⁺ NK cells. Thus, it is possible that the dysfunction of more than one transcription factor is responsible for the altered maturation of aged NK cells. Furthermore, T-bet is essential for the upregulation of Blimp-1, another transcription factor involved in the maturation of NK cells towards the CD11b⁺CD27⁻ subset, and for driving NK cell proliferation in the bone marrow [96]. Finally, another, newly identified transcription factor, named Helios, is silenced upon maturation to CD11b⁺ NK cells, and this downregulation is depending on signals received earlier during development from the activation receptor NKp46 of NK cells [97]. Thus, it is possible that NK cell maturation in aged mice is regulated not only by transcription factors that are specifically implicated in NK cell terminal maturation but also by others acting at earlier stages of NK cell development or others with wider functions on immune cells. Indeed, hyperactivation of NF-κB, a master regulator of inflammation, has been implicated in blocking terminal maturation of NK cells and in reducing NK cell numbers and function in the periphery [98].

Because aging results in constitutive NF- κ B activation in various immune cells [99] and NF- κ B is a central player of inflammaging [100], it is very possible that hyperactivation of NF- κ B in aged NK cells to be responsible for the observed phenotype. Overall, studies on the effect of aging on the function of these transcription factors and their implications in maturation of NK cells need to be done.

In addition to transcription factors, certain extracellular signals, such as chemokines, cytokines, growth factors or interactions with bone marrow stromal cells may also be important in driving NK cell proliferation and terminal maturation in the bone marrow. Both CCR5 [101] and CXCR4 signaling [102] regulate NK cell proliferation in the bone marrow under physiological conditions and their deficiencies significantly reduce NK cell proliferation. Additionally, acquisition of Ly49s and terminal maturation of NK cells in the bone marrow require contact with stromal cells and the involvement of the Tyro3 family receptor signaling [103]. It has been shown that the downstream signaling of CXCR4 in T cells [104] and the Tyro3 family receptor signaling cascade [105] involves the phosphoinositol-3-kinase (PI3K) pathway. Absence of this signaling pathway can result in reduced NK cell numbers and terminal maturation. Indeed, mice lacking the PI3K catalytic subunits p110 γ and p110 δ [106] or p85 α [107] had reduced NK cells and altered terminal maturation. In the absence of P110 γ and P110 δ , mice had reduced early immature CD11b⁻ CD27⁺ and CD11b⁺ CD27⁺ NK cells but increased terminally mature CD11b⁺ CD27⁻ NK cells; however, absence of the p85 α subunit results in less NK cells at their final maturation stage, CD11b⁺ and CD43⁺, similar to what is observed in aged mice. Because aging has been shown to decrease the activity of PI3K upon activation [108], and the PI3K/Akt pathway is upstream of the inhibition of mTOR, a key player in regulating protein

synthesis, nutrient utilization and lifespan, future studies are needed to investigate the effect of these pathways in NK cell maturation and the implications of aging.

2.5 The effect of aging on NK cell function

Since NK cells are involved in tumor surveillance, and cancer incidence is increased with aging, many studies examining NK cell cytotoxicity in the elderly have been published. In the following paragraphs we review how aging affects NK cell function, NK cell activation, and cytokine production upon stimulation with tumor cells, and cytokines. We will also refer to some studies and findings from our work on the effects of aging on NK cell responses to influenza infections.

Natural cytotoxicity

Resting NK cells can exhibit natural cytotoxicity and kill tumor cells without previous activation with cytokines or helper cells. Upon recognition of target cells, NK cells get activated to release granules and to produce cytokines. Not all tumor cells are susceptible to recognition and killing by resting NK cells, therefore, only susceptible tumor cells lines, such as YAC-1 cells for murine studies and K562 cells for human studies, are used to examine natural cytotoxicity. In mice, many studies report that spleen cell suspensions from aged mice have reduced natural cytotoxicity in the absence of stimulatory conditions [49, 53, 109-119]. However, studies from our laboratory have shown that aging did not result in significant impairment of NK cell degranulation in unstimulated co-cultures with YAC-1 cells [11, 13, 50, 120]. Indeed, the reported impairment of NK cell killing in aged mice was shown in some cases as just as a slight reduction, while the reported not chance was the result of a failure to detect statistical significant

differences, although aged mice still show slightly lower natural cytotoxicity. It is possible that these differences are derived by the age of young animals that aged mice are compared with; aged mice are usually 22-24 months old, while young mice are 2 to 6 months old. It was shown that natural cytotoxicity of 6 months old is lower than that of 2 months old [50], making it harder to find significant differences when 6 months old mice are compared to 22 months old. Although there are contradicting reports for the killing function of NK cells from spleens, NK cells from bone marrow [121], blood [122] and lungs [13] of aged mice did not show differences in the natural cytotoxicity. Thus, in general, natural cytotoxicity of NK cells may only slightly be lower in aged mice and it is not known whether this results in significant impairment in immunosurveillance. Studies on the content of cytotoxic molecules in NK cells of young and aged mice showed that in the absence of any stimulus NK cells from the lymph nodes and spleens of young and aged mice had similar staining of granzyme B [52, 123] while splenocytes from aged mice had increased perforin staining [123]. On the other hand, the ability of resting aged NK cells to produce cytokines upon target cell recognition was significantly impaired. In the following chapters we show that splenic NK cells produced significant less IFN- γ upon stimulation with YAC-1 target cells but the ability of resting NK cells to produce cytokines upon target recognition has received little attention.

Human studies on natural cytotoxicity in the elderly have also reached diverse conclusions: NK cells from elderly have similar [54, 57, 59, 124, 125], increased [126] or decreased [82, 108, 127, 128] natural cytotoxicity against K562 target cells. In several reviews [129-132] it is suggested that these differences are related to the criteria used to select the studies participants. When general good health is used as an inclusion criteria regardless the age, it is more likely to observe that NK cell cytotoxicity is reduced, while when optimal health criteria

are used, as the ones established by the SENIEUR protocol, it is more likely to observe no differences [54, 59, 125, 133] or even higher NK cell cytotoxicity [126]. Concerning the cytolytic machinery of aged human NK cells, there is a report that NK cells from elderly have reduced perforin expression [134]. Although this comes in contrast to the data presented by Nogusa et al. (2012) [123] it is important to consider that different tissues have been examined and these results refer to different species. In all of the human studies, the usual source of samples is peripheral blood samples and it is possible that in humans, similarly to mice, NK cells from other tissues are affected differently by aging.

Basal NK activity reflects the ability of NK cells to recognize susceptible target cells. This process is mainly affected by the interactions of inhibitory and activating receptors with their ligands. The binding and the establishing of a cytolytic synapse through integrin and adhesion molecules allows for the transmission and integration of the signaling initiated by the binding of NK cell activation receptors leading to the release of cytolytic enzymes that are already preformed and stored in granules in the cytoplasm of NK cells. The observed defective natural cytotoxicity with aging is correlated with reduced numbers of NK cells-at least in mice-, reduced ability of NK cells to bind to their targets [113], reduced ability to generate total inositol monophosphates and in particular inositol triphosphates [108] and finally, reduced expression of perforin [134]. However, the ability of NK cells to integrate activation signals and initiate cytotoxicity or transcription of genes, can only be assessed by evaluating the function of specific activating receptors or by activation of NK cells in an inflammatory environment such as with stimulation of cytokines.

Activation of NK cells through ligation of their receptors

Natural killer cells can induce lysis of cells that are opsonized with antibodies through the engagement of CD16, a receptor on NK cells recognizing the Fc portion of antibodies. This function is named antibody dependent cell mediated cytotoxicity (ADCC) and is a major pathway by which NK cells exert their cytotoxic functions. Many studies have assessed ADCC in aged NK cells and have shown that aging does not affect the ability of NK cells to kill cells through this pathway [82, 108, 135, 136].

In contrast to ADCC, a few studies have assessed the activation of NK cells through the engagement of other activating receptors. In the studies presented in this dissertation we show that NK cells from spleens of aged mice produced less IFN- γ upon ligation of NKp46 or NK1.1 with their plate bound antibodies. In relation to influenza infection, reduced activation through NKp46 could have significant implications in the ability of NK cell to get activated by the influenza hemagglutinins. We further show that in uninfected mice, splenic NK cells are more responsive than lung NK cells, and that only after influenza infection, IFN- γ production is increased in lung NK cells; yet, a significant impairment on the ability of lung NK cells to produce IFN- γ after influenza infection is observed in aged mice. Because we only assessed the ability of NK cells to produce IFN- γ through NKp46 receptor activation but not degranulation, it is important that future studies address all aspects of NK cell activation and there is a great need to evaluate the intracellular signaling events that result in reduced cytokine production with the engagement of different activating receptors in aged mice.

In the following studies, we further undertook the task to investigate whether this reduced NK cell function is related to age-related phenotypic differences on NK cells. As discussed earlier, splenic NK cells from aged mice have altered Ly49 receptor repertoire. To examine what

the contribution of such an altered phenotype on NK cell function is, we activated NK cells with plate-bound NK1.1 antibody, a standard assay to examine licensing of NK cells. We suggest that the impaired NK cell function observed in aged mice is not so much related to licensing, i.e the expression of inhibitory receptors for self MHC, but rather is related to their defective terminal maturation: even licensed NK cells in aged mice exhibit reduced IFN- γ production during stimulation with anti-NK1.1 antibody, and licensed NK cells in aged mice consisted of more immature NK cells.

Activation of NK cells with cytokines

In contrast with the study of specific receptor-mediated responses, the effect of cytokines on activation, proliferation and function of aged NK cells has been extensively examined. The protocols vary significantly with the use of different cytokines to stimulate NK cell function and with different lengths of time for the *in vitro* incubations. Short term *in vitro* incubations maintain the initial cell phenotype isolated from the freshly collected samples, do not result in extensive death or proliferation and examine the ability of NK cells to carryout transcription and translation of functions initiated by the cytokines. In contrast, long term *in vitro* incubations result in expansion and change of the phenotype of the freshly isolated single cell suspensions and examine the differentiation potential, survival and death rates of the incubated cells as well as function in the long presence and abundance of cytokines.

The effect of aging on IL-2 stimulation of NK cell function has been characterized in the context of lymphokine activated killer cell (LAK) activity and in short term incubations as activation of natural cytotoxicity and production of IFN- γ . LAK activity is described when freshly isolated splenocytes in mice and PBMCs in humans are incubated with IL-2 for several

days, and acquire the ability to kill a wider variety of tumor cells compared to freshly isolated NK cells. The majority of the cells that exhibit LAK activity are similar to NK cells and it is thought that are derived from NK cell precursors in the initial single cell suspensions [137]. LAK cells attract significant clinical attention for immunotherapies against cancers. Thus, there are very early reports on the effect of aging in inducing LAK activity against resistant tumor cell lines. Incubation of murine aged splenocytes with IL-2 for 5 days resulted in LAK cells that exhibit similar cytotoxicity against fresh tumor cells with that of young cells, yet LAK activity peaked earlier in young splenocytes than in old [115]. However, studies on aged associated decline of LAK activity of murine splenocytes against a variety of other tumor cell lines has also been reported [138]. These differences probably reflect different periods of incubation with IL-2. Even for natural cytotoxicity, short term incubations with IL-2 resulted in reduced natural cytotoxicity against YAC-1 cells. However, longer incubation periods were able to correct this defect [53, 113, 139]. The similar but delayed LAK activity in splenocytes isolated from aged mice was attributed to a phenotypic difference between LAK precursors in aged mice from that of young mice. The majority of LAK precursors in young mice are considered to be mature NK cells and aged mice have reduced frequency of these precursors [53]. However, Kawakami et al. (1987) demonstrated that in aged mice, immature $CD90.2^{+}$ ($Thy1^{+}$) cells showed LAK activity while in young mice LAK activity was mostly restricted to mature NK cells [115]. The authors further elaborated to suggest that freshly isolated splenocytes from aged mice contained significant numbers of inactive pre-NK cells, which could differentiate into LAK cells so that it compensated for the lack of LAK activity from mature NK cells in the spleens of aged mice. In addition, they showed that LAK activity from bone marrow, which is comprised mostly from immature NK cells in both young and aged mice, was independent of age [121]. Indeed, LAK

cells primarily consisted of CD90.2⁺ NK cells in both young and aged mice in contrast to spleens, where there is a preferential accumulation of CD90.2⁺ NK cells in the aged mice [121]. Altogether these data suggested that splenic NK cells in aged mice consist of more immature, pre- NK cells that do not show natural cytotoxicity but upon long term incubation with IL-2 can differentiate into cytotoxic cells and exhibit LAK activity. Data from our laboratory presented in the following studies confirm that aged mice have reduced mature NK cells, and they further suggest that the immature aged NK cells can compensate for the cytotoxic function induced by influenza infection, but do not compensate for the reduced IFN- γ production, which is mostly restricted to mature NK cells.

In humans, it was shown that LAK activity of PBMCs was reduced with aging [54, 140] and similarly to mice, short term incubation of aged PBMCs with IL-2 resulted in less natural cytotoxicity against sensitive tumor cell lines [54, 82, 134]. NK cells from peripheral blood of elderly people have reduced CD56^{bright} NK cells, and increased most differentiated CD56^{dim} NK cells. However, under long-term incubation with IL-2, the CD56^{bright} subset mediates significantly greater levels of LAK activity in comparison to the CD56^{dim} subset [137, 141]. Furthermore CD56^{bright} NK cells express the high affinity IL-2 receptor, CD25, and respond vigorously to IL-2, while the CD56^{dim} subset does not express CD25 and responds less vigorously to IL-2. Thus the observed reduction of CD56^{bright} NK cells in the elderly may have implications for their LAK activity. Finally, IL-2 stimulation resulted in significantly reduced IFN- γ production by NK cells isolated from elderly humans [142]. These data indicate that the effects of aging on NK cytotoxicity induced by IL-2 are reflective of the altered subset

distribution; yet, IFN- γ production from aged NK cells is significantly impaired reflecting possible intrinsic intracellular defects.

After IL-2 binding with its receptor, several intracellular signaling pathways are activated. On receptor ligation, the JAK1 and JAK3 kinases are recruited to the cytoplasmic portion of the IL-2 receptor and phosphorylate the IL-2R β and the common cytokine receptor γ chain respectively. Once JAKs are activated they phosphorylate and activate STAT5 or STAT3 that translocate to the nucleus and perform essential functions for lymphocyte development and differentiation. Albright et al. (2004) illustrated that stimulation of LAK cells from aged mice with IL-2 or IL-15 showed reduced STAT5A and STAT5B translocation into the nucleus compared to young mice [118]. These data indicate that there are some intrinsic defects on the intracellular signaling pathways in aged NK cells and future studies should focus in identifying them.

When NK cells are activated they do not only produce IFN- γ or release granules, but they also produce chemokines. Two studies have investigated the effect of aging on NK cell chemokine production [90, 118]. Purified, aged NK cells stimulated with IL-2 and IL-12 released significantly less CCL-3, CCL-5 and CXCL8 in their supernatants [90]. Similarly, LAK cells from aged mice generated in long-term cultures with high concentrations of IL-2 had reduced mRNA of XCL1, CCL-4 and CCL3, CCL5 and no detectible mRNA of CCL-3, CXCL-2 or CXCL-10 while LAK cells from young mice produced significantly more transcripts of all of these chemokines [118]. Further activation of these LAK cells with IL-2 for 3 or 7 hours after a resting period of 12 hours maintained the same levels of transcription in young but not in aged cells [118]. More interesting was the effect of additional stimulation of LAK cells with IL-15 for 3 hours after a resting: all of the above mentioned chemokines were substantially elevated in

young cells, but only XCL1 and CCL4 somewhat increased in the aged LAK cells [118]. Thus, aging results in altered chemokine transcription from NK cells in stimulated cultures. These findings may be of particular importance during an *in vivo* infection, when a timely and rapid migration of immune cells is required to initiate effective immune responses. Moreover, these studies indicate that aging affects more than just natural cytotoxicity and IFN- γ production, and future studies should undertake a holistic approach in studying NK cell function in the context of aging.

With the exception of IL-2, the effect of other cytokines on the activation of NK cells in the context of aging has received little attention. Interleukin-12 has been used along with IL-2 to enhance LAK activity and to evaluate NK cell function. Aged murine splenocytes incubated with various doses of IL-12 for 20 hours resulted in enhancement of their natural cytotoxicity, however in reduced levels compared to young mice [139]. Similarly, in human studies, an 18-hour incubation of PBMCs with varying doses of IL-12 resulted in slightly reduced natural cytotoxicity against K562 cells, and in significantly less LAK activity against resistant Daubi cells [54]. Moreover, incubation of murine splenocytes with IL-12 alone for 4 or 24 hours resulted in reduced IFN- γ production from NK cells [143]. In the later study, IL-12 incubation for 24 hours resulted in only slightly reduced NK cell cytotoxicity, and aged mice had significantly higher perforin but similar granzyme B content in comparison to young mice. Whether this indicates that young mice had already degranulated and aged mice did not, needs further examination.

Nogusa et al.(2012) provided additional insight into the effect of cytokines in NK cell function [123]. Incubation with IL-12 alone was more efficient in inducing IFN- γ production from murine splenocytes than IL-18 or IL-2 and after 24 hours incubation, IL-2 induced IFN- γ

was comparable to that of IL-15. These studies further showed that there was a synergistic effect on IFN- γ production when IL-12 was added along with IL-18, which was even higher when IL-12 was added along with IL-18 and IL-2. In all of these combinations, aged NK cells showed enhanced but still less IFN- γ production in comparison to young NK cells. However, in human studies, a stimulatory combination of IL-12 and IL-18 resulted in only a modest impairment of NK cell ability to produce IFN- γ [82]. In contrast to the effects on IFN- γ production, incubation with IL-12 was not as efficient in enhancing natural cytotoxicity as incubation with IL-2 or IL-15. Aged NK cells incubated with either IL-2 or IL-15 demonstrated reduced natural cytotoxicity compared their young counterparts, which was associated with reduced granzyme B content.

In the following studies we provide data on the effect of the stimulatory combination of IL-18 with IL-2 on the function of aged NK cells. We show that uninfected NK cells from the spleens of aged mice produce less IFN- γ , while this combination did not stimulate resting NK cells from lungs of either young and aged mice. Similarly to NKp46 activation discussed above, only after infection, lung NK cells were responsive to IL-18 and IL-2 stimulation, and a significant age-related impairment in IFN- γ production was observed. Similarly to [143] we show that after stimulation of resting NK cells with this combination, aging resulted in significantly less NK cells that were positive for granzyme B. We observed that the reduced function was not related to the ability of each subset to function, but rather to the reduction of CD11b⁺ CD27⁻ NK cells as an absolute numbers in the peripheral tissues. Thus, the impairment of aged NK cells to respond to short-term stimulation is related to their altered distribution, while longer times of incubation may promote differentiation and result to increased responsiveness of NK cells.

In contrast to previously mentioned cytokines, the effect of type I interferons and PolyI:C has received quite a lot of attention because they are potent inducers of NK cell cytotoxicity and are induced during viral infections. Studies in mice have shown that IFN α/β or PolyI:C induced natural cytotoxicity in both young and aged NK cells, but NK cells from aged mice did not reach the same high level as NK cells from young mice [112, 113, 116, 120, 144]. This reduced induction of NK cell activity after interferon treatment was related to higher suppressor macrophage activity and high prostaglandin E-2 (PGE₂) production in aged mice, because when macrophages were depleted from aged splenocytes, natural cytotoxicity of aged NK cells reached similar levels as that of young [114, 144]. Plett et al. (2000b) showed that although aged mice had increased expression of IFN α/β receptor and increased binding of IFN α/β to its receptor, these findings were not reflected as increased natural cytotoxicity when aged splenocytes were activated with IFN α/β , but rather were associated with increased apoptosis [120]. Activated aged NK cells had higher expression of Annexin V and CD95, a Fas ligand associated with apoptotic death. Thus, fewer NK cells in aged mice exhibit natural cytotoxicity. In elderly humans, NK cells also showed reduced natural cytotoxicity when incubated for short periods with IFN α [125] and for 18 hours with low doses of IFN α [145] but they showed similar cytotoxicity to that of younger people when incubated for long periods with higher doses of interferons [145]. These data suggest that the kinetics of NK cell activation through interferons is altered with aging, something that could result in complications during the immune responses to a viral infection.

The effect of aging on NK cell responses to viral infections in general and in particular to influenza infection is not well established. Low NK cell activity is significantly correlated with increased risk for development of an infection and short survival due to infection in healthy

elderly [10]. Influenza vaccination studies in humans, have shown that NK cells are the major source of IFN- γ post vaccination [146], while aged individuals exhibit reduced NK cell cytotoxicity after vaccination [147]. Studies by Yu Jing (2007) went further to investigate how aging influences human NK cell activation with respect to IFN- γ production in response to *in vitro* influenza infection. These studies showed that aged NK cells produced significantly less IFN- γ upon influenza infection and that both soluble factors and cell-contact activation of NK cells were defective with aging [19]. In particular, IFN- α production by plasmacytoid DCs and IFN- γ production by T-cells were identified as crucial factors in activating NK cells and their production was dramatically impaired in aged individuals contributing to downregulation of activation and IFN- γ production by NK cells [19].

Existing murine studies are less detailed on the effect of aging on NK cell responses to influenza infection. Our laboratory has previously shown that aged mice lose significant weight during influenza infection [11, 13], have less NK cells infiltrating in their lungs, exhibit reduced natural cytotoxicity after influenza infection [13], show delayed clearance of the virus [11, 13] and reduced survival [11, 148]. Additionally, other independent studies showed that aged mice have delayed infiltration of NK cells in their lungs, and reduced activation [12, 148]. In the following studies we show that *ex vivo* co-culture with YAC-1 cells of cells isolated from the lungs of infected aged mice, resulted in almost half the magnitude of degranulation and IFN- γ production of what is observed for young mice. We also show that influenza infection resulted in exaggerating the basal defect of aged NK cells concerning IFN- γ production after stimulation with abundant cytokines and after receptor mediated activation, revealing that aged NK cells are intrinsically defective. We further suggest that the reported differences in NK cell function are reflective of the subset composition of NK cells with aging. Lung NK cells from aged mice were

composed of less mature NK cells, and during the next couple of days of the infection their percentages were even further reduced. Thus, aged mice had impaired ability to supply mature NK cells at the site of infection when they were needed the most.

2.6 Conclusions

Although the effect of aging on the adaptive immune responses has received extensive attention, in part because of the reduced responsiveness of elderly people to vaccination, there is no doubt that aging affects all types of cells, including cells of innate immunity. In this review we have discussed current findings of the effects of aging on NK cells. The increased incidence of cancers and infectious diseases in the elderly, in combination with the importance of NK cells in the elimination of tumors and virus-infected cells, suggests that preservation of good NK cell function during late ages is vital. Indeed, low natural cytotoxicity is strongly correlated with increased risk for development of an infection and short survival, while centenarians usually maintain increased NK cell numbers with increased cytolytic activity.

In this review we have summarized and compared findings provided by several human and murine studies, including those generated for the purpose of this dissertation, and discussed how aging affects the numbers, phenotype and function of NK cells providing possible mechanisms. Briefly, in contrast to humans where NK cell numbers are preserved in the blood, aged mice have reduced NK cells in their peripheral tissues, but not in the bone marrow. Moreover, aged mice have less mature NK cells in all peripheral tissues, including the bone marrow. The specific reduction of NK cells in aged mice could be associated with reduced survival in the periphery, or reduced maturation and exit of mature NK cells from the bone marrow. Aged mice show in fact defective terminal maturation which results in the accumulation

of immature NK cells in the bone marrow. In contrast, elderly humans exhibit an accumulation of mature NK cells in their blood, but data concerning their distribution in other tissues are lacking. Further characterization of the phenotype of aged NK cells in humans and mice reveals certain similarities, such as accumulation of KIRs or Ly49s on immature NK cell subsets, and increased expression of phenotypic markers usually observed in immature NK cells. Based on the differences of NK cell identification and subset classification in humans and mice, it is possible that similar effects occur in both species, and only with harmonization of the phenotypic analysis of NK cells for both species could we investigate this possibility. Finally, related to the effects of aging on NK cell cytotoxicity, basal natural cytotoxicity is reduced or preserved in mice and humans, and it is strongly related with the effects of aging on the distribution of NK cell subsets. Stimulation of NK cells from young and elderly people with cytokines has generated conflicting data based on the various combinations of cytokines and the duration of stimulation. It is accepted that short-term incubations reveal significant impairment of NK cell production of IFN- γ and chemokines, and - to a smaller extent - of natural cytotoxicity, while longer incubations can correct the impaired natural cytotoxicity. In particular, based on the effect of interferon stimulation on NK cells it is inferred that during viral infections aged NK cells cannot perform well.

Overall, the recent advances in our knowledge about NK cells, their development and mechanism of function, in addition to the availability of multicolor flow cytometric analysis, microarrays and advances in our understanding of the intracellular pathways, there is an excellent opportunity to assess NK cell diversity and various aspects of their function or intracellular pathways in small number of samples. Furthermore, future studies need to use several markers commonly identified in mice and humans to allow for cross species

comparisons. Finally, little overlap exists among current research on the biochemistry of cellular senescence and metabolism with the study of immune cells. There is a great need for the field of immunesenescence to incorporate current theories of cellular senescence and altered metabolism with aging in the function of aged immune cells alone, or, in the presence of surrounding immune and non-immune cells.

CHAPTER 3

NATURAL KILLER CELL FUNCTION IS ALTERED DURING THE PRIMARY RESPONSE OF AGED MICE TO INFLUENZA INFECTION

Beli et al. (2011) Ref: [149]

3.1 Abstract

Influenza is a public health concern, especially for the elderly. While influenza vaccination is efficacious in the young, it offers only limited protection in the elderly. Thus, it becomes imperative to understand age-related changes in the primary response to influenza infection. This study identified potential age-related defects in natural killer (NK) cell function during influenza infection. We showed that NK cells from aged mice were reduced and had impaired function and altered phenotype in lungs during influenza infection. Aged NK cells demonstrated decreased IFN- γ production, but not degranulation, after influenza infection. However, after *ex vivo* activation with YAC-1 cells, aged NK cells demonstrated both reduced IFN- γ production and degranulation. IFN- γ was also reduced in aged NK cells after activation with anti-NKp46 and soluble cytokines. IFN- β , and IL-12p40 mRNA expression was not significantly different from that observed in adult mice. Analysis of NK cell subsets indicated that aged mice had more immature and less terminally mature NK cells. These data suggest that aging affects the numbers, function and phenotype of NK cells. Thus, these defects in NK cell function could impair the ability of aged mice to induce a strong antiviral immune response during the early stages of the infection.

3.2 Introduction

Influenza is a public health concern worldwide as it affects young children, the elderly and immunocompromised people. In humans, seasonal influenza infection usually results in a mild upper respiratory infection, but in immunocompromised it can result in critical illness and fatal outcomes. Elderly are particularly susceptible to current seasonal influenza strains, and almost 90% of the fatal cases with underlying pneumonia and influenza deaths occurred among persons over 65 years old [150]. Fatal cases have also been reported for infections with virulent influenza strains, resulting in a lower respiratory tract infection and aberrant immune responses leading to severe lung damage [151]. Similarly, in elderly humans pulmonary complication of viral pneumonia with seasonal influenza infections have also been reported [152, 153]. Vaccination is the primary strategy to prevent seasonal influenza infections, yet the elderly exhibit impaired antibody responses and remain vulnerable to flu infections [154]. In the absence of efficient protection, influenza infection proceeds like an acute infection, and the status of the host's immune system is a key factor for controlling viral replication and preventing infection associated tissue damage. In contrast to healthy humans, in mice, influenza infection shows symptoms of lower respiratory infection. In mice, the majority of influenza virus replication, occurs in the lungs during the early days of the infection [155] and if controlled in a timely manner, severe lung damage can be avoided.

Previous studies from our laboratory, showed that aged mice exhibit increased susceptibility to influenza infection, demonstrated by sharp weight loss [11] and increased viral titers in their lungs four days post infection, when young mice have decreased titers [11, 13]. Most importantly, aged mice show increased susceptibility during the first four days of infection, and before the specific cytotoxic CD8 T cell responses are mounted [9]. Based on this evidence,

we hypothesized that impaired innate immunity in aged mice may contribute to increased susceptibility.

Natural killer (NK) cells, a subset of lymphocytes of the innate immune system that can kill infected cells within minutes after they get activated and produce a wide array of cytokines and chemokines, are known for their antiviral functions. They further comprise a larger percentage of the lymphocyte population in the lungs than in other tissues, indicating an important role for them as a first line of defense. Extensive evidence further supports an important role for NK cells during influenza infections. Severe influenza infections have often been associated with depletion of NK cells specifically, and lymphopenia in general [23]. During the initial days of influenza infection, NK cells in the lungs become activated and demonstrate high cytotoxic activity, [13, 28] followed by production of interferon (IFN)- γ [29], an important anti-viral cytokine. NK cell depletion results in severe influenza infection [13, 30], higher viral replication [13, 30], reduced cytotoxic responses [33, 34], and reduced early IFN- γ production [30, 34]. Furthermore, NKp46, an activating receptor on NK cells that recognizes influenza hemagglutinin [35] was shown to play a critical role for survival during influenza infection [36]. Thus, appropriate NK cells responses possibly have an important impact for limiting virus replication and regulating the transition from innate to adaptive immunity at that time point.

The effect of aging on NK cell responses to influenza infection is not well established. Low NK cell activity is significantly correlated with increased risk for development of an infection and short survival due to infection in healthy elderly [10]. Human studies have shown that healthy elderly have either preserved [54, 59, 124, 147], or reduced basal natural cytotoxicity [58, 108, 132, 156] compared to healthy young adults. Moreover, after NK cells are stimulated with influenza vaccine [147], aged individuals exhibit reduced NK cell cytotoxicity.

Similarly, in murine studies, basal NK cell responses do not change significantly with age; however after influenza infection, aged mice have less NK cells infiltrating in their lungs, and exhibit reduced cytotoxicity [13]. Despite these observations, little is known regarding age-related defects in NK cells as they are related to increased susceptibility to influenza infection.

The scope of this study is to investigate whether aging affects NK cell numbers, function and phenotype, during influenza virus infection. Our results show that aged mice have reduced NK cells in lungs and spleens and demonstrate a particular defect in IFN- γ production. Under different stimulation signals, degranulation is also impaired in aged mice. This reduced NK cell ability is related to changes in NK cell subsets. Overall, our data suggest that after influenza infection, aged mice have reduced mature NK cells in their periphery during the early stages of the infection.

3.3 Materials and Methods

Mice, Virus and Infection

Male C56BL/6J young (6 month) and aged (22 month) mice were purchased from National Institute on Aging colony (Charles River Laboratories, Wilmington, MA, USA). Mice were acclimated for at least one week before infected and housed in micro-isolator cages in a Biosafety level -2 room at the Michigan State Research Containment Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International certified facility. All animal procedures were approved by the Michigan State Institutional Animal Care and Use Committee and were in accordance with National Research Council guidelines. The mouse adapted strain of H1N1 influenza A/Puerto Rico/8/34 (A/PR8) was used for all of our infections. Mice were anesthetized with an intraperitoneal injection of avertin (2,2,2-tribromoethanol,

Sigma-Aldrich, St. Louis, MO, USA) and infected intranasally with 0.025ml of 75 Hemagglutination Units (HAU) of influenza virus diluted in sterile PBS (Sigma-Aldrich). Viral titer was determined by hemagglutination assay. Mice were weighed and observed daily for sickness.

Cell preparation

Lymphocytes were isolated according previously described protocols [9, 13] with minor modifications. Preparation of single cell suspensions from lungs involved incubation of minced lung tissue at 37 °C for 2 h in digestion buffer [1 mg/ml Collagenase A, (Sigma-Aldrich), and 80 Kuntz units /ml of DNase I, (Roche, Indianapolis, IN, USA) in RPMI-1640 media, (Sigma-Aldrich), with 5% FBS (Atlanta Biologicals, Lawrenceville, GA, USA)], grinding through a 40µm cell strainer, separation with density gradient using Histopaque-1083 (Sigma-Aldrich) and ACK lysis to remove remaining red blood cells. Single cell suspensions from Dounce homogenized spleens were directly lysed with ACK lysis buffer to remove red blood cells.

Flow cytometry and FACS analysis

For cell surface, one million, and for intracellular staining, two million cells were stained with the appropriate antibodies on ice for 30 min according previously published methods [157]. All fluorochrome-conjugated monoclonal antibodies were purchased either from Pharmingen (La Jolla, CA, USA) or ebiosciences (San Diego, CA, USA). Combinations of the following antibodies were used for staining: APC- or PE-Cy7- NK1.1 (PK136); PerCPCy5.5- CD3e (145-2C11); PerCPCy5.5- CD19 (1D3); FITC-, eFluor647-, or AlexaFluor700- NKp46 (29A1.4), APC-Cy7-, eFluor450-, Alexa Fluor700- CD11b (M1/70), PE- CD27 (LG.3A10), FITC-

CD107a (1D4B), PE-Cy7- IFN- γ and CD16/32 (2.4G2). Samples were acquired on a LSR II flow cytometer (BD biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The lymphocyte population was gated based on forward and side scatter and NK cells were identified as CD3/CD19 negative and NK1.1/NKp46 positive cells within the lymphocyte gate. Numbers of NK cells were calculated by extrapolating the percentage of NK cells from the live gate to the total number of cells isolated from each tissue, determined by trypan blue staining.

In vitro stimulation

Activations with YAC-1 cells, cytokines and plate bound antibodies were performed in RPMI 1640 medium containing 10% heat inactivated FBS. For YAC-1 stimulation, 2×10^6 isolated cells were incubated with 2×10^5 YAC-1 cells in the presence of brefeldin A, (1 μ l/ml) and monensin (0.66 μ l/ml) as well as 8 μ g/ml FITC CD107a antibody (Pharmingen) for 4 hours in a 37°C, 5% CO₂, incubator. For NKp46 activation, we coated ultra-high binding polystyrene microtiter strips (Thermo-Fisher, Pittsburgh, PA, USA) with 1 μ g/ml FITC-NKp46 (ebiosciences) for 80 min at 37°C. Unbound antibody was washed two times with PBS and 2×10^6 isolated cells incubated in duplicates with brefeldin A for 7 hours. For cytokine stimulation, we incubated 2×10^6 isolated cells with a combination of IL-2, 1000 IU/ml, (provided by Dr Kim's laboratory) and IL-18, 20 ng/mL (MBL, Woburn, MA, USA) for 7 hours.

RNA isolation, Real Time PCR

Excised tissues for PCR analyses were stored immediately after harvesting in Tri Reagent

(Sigma-Aldrich) snap-frozen in liquid nitrogen until later processing. RNA was isolated using the manufacturer's protocol. Real-time PCR for IFN- β , IL-12 p40 and IFN- γ performed on an ABI PRISM 7900HT Sequence Detection System, using Taqman One-Step Real-time PCR Master Mix and Assays-on-DemandTM primer/probe gene expression products (Applied Biosystems, Carlsbad, CA, USA). Relative quantification of pro-inflammatory cytokine gene expression was carried out using GAPDH endogenous control and the $\Delta\Delta\text{Ct}$ method. All the results were expressed relative to young uninfected mice.

Statistical Analysis

All statistics were generated using Sigma Stat 3.11 (Systat Software Inc, Chicago, IL, USA). Student's t-test was used to compare young and aged NK cell responses. Two-way ANOVA was used to compare the effect of age and time post infection. Significance level for the two tests was $p < 0.05$. The Holm-Sidak method was used for pair wise multiple comparisons adjusting the overall significance level to $p < 0.01$.

3.4 Results

Aged mice have reduced lung and spleen NK cells

We examined the kinetics of NK cells in the lungs and spleens of infected young and aged mice before and after infection (p.i.). At baseline, aged mice had reduced percentage of NK cells in their lungs in comparison to young mice (Figure 1A), that is further demonstrated as reduced absolute numbers (Figure 1B), even though aged mice have more total cells in their lungs at that time (data not shown). Similarly, in spleen before infection, aged mice had reduced percentages and absolute numbers of NK cells compared to young mice (Figure 1C, 1D), but had

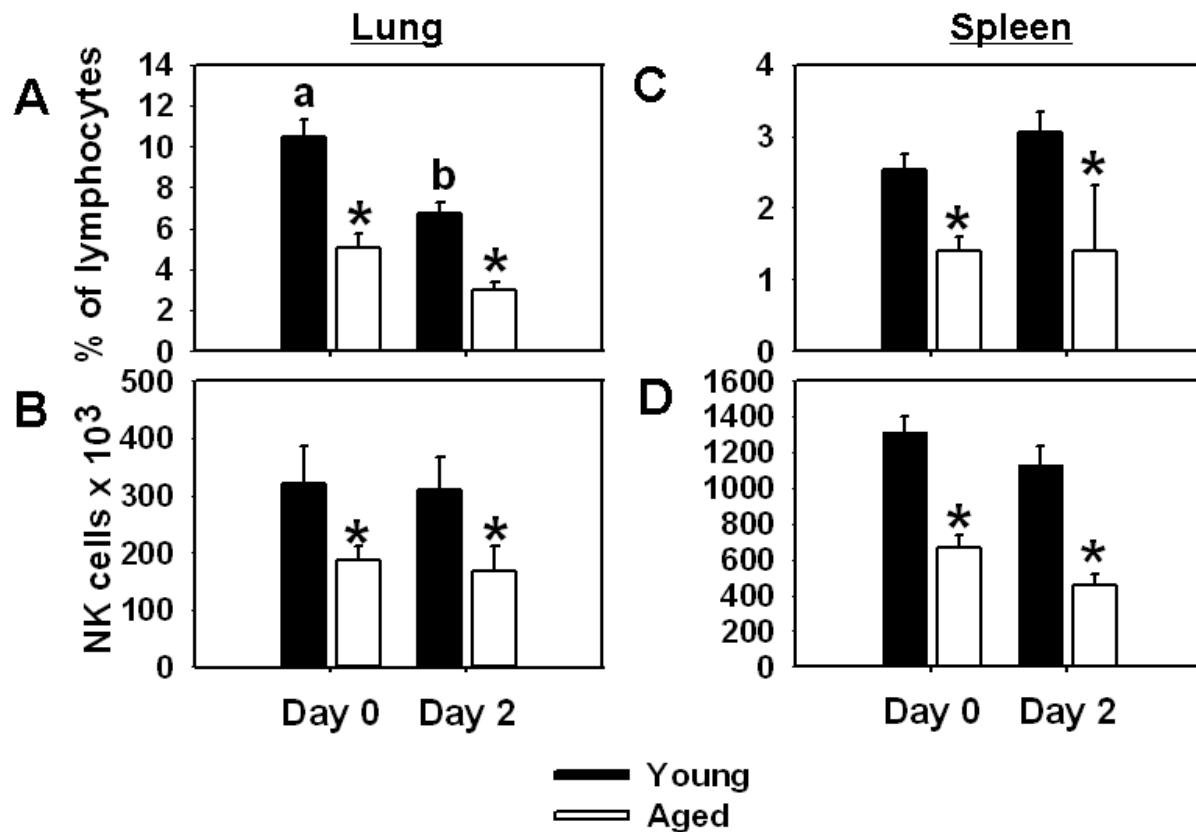


Figure 1. Aged mice have reduced NK cells in lungs during influenza infection. Single cell suspensions from lungs and spleens of uninfected and infected young and aged mice were stained and NK cell percentages (A, C) and NK cell numbers (B, D) were determined according the methods. Bars represent means \pm Standard Error of Mean (SEM); data shown are combined from three performed experiments, n=5 per group per experiment. Asterisks, (*), indicate statistical differences between young and aged mice at that time point (t-test, $p < 0.05$). Different letters indicate changes during infection within the same group (Two way ANOVA).

similar total splenic cells (data not shown). Two days after influenza infection, the percentage of NK cells in the lungs of both aged and young mice was lower than in baseline, and started to increase by day 4 but aged mice had consistently lower NK cells than young mice (Figure 1A). Infiltration of other cells may explain the reduction of the relative percentages of NK cells because the total cell numbers were higher in the lungs of infected mice independently of the age (data not shown). Yet, the absolute numbers of NK cells in the lungs of aged mice remained at lower levels than in young mice after infection (Figure 1B). In contrast with lungs, in the spleens of infected mice, the number of total cells was reduced compared to baseline (data not shown), the NK cells percentages remained unchanged (Figure 1C), and the absolute NK cell numbers reflected the reduction of total cellularity after infection (Figure 1D). At all times aged mice had reduced NK cells in both their lungs and spleens compared to young mice.

NK cell function during influenza infection is impaired in aged mice

Early in the course of infection, NK cells exhibit peak natural cytotoxicity in the lungs of infected mice. We have reported previously that aged mice exhibit dramatically reduced killing of target cells after influenza infection [13]. To further assess age-related changes in NK cell function, lung and splenic cell suspensions from uninfected and infected young and aged mice were incubated with YAC-1 cells. Degranulation was measured with the CD107a surface mobilization assay and cytokine production with intracellular staining for IFN- γ . Before infection, both aged and young NK cells from the lungs showed little degranulation and minimal IFN- γ production after activation with YAC-1 cells, with marginally less ($p = 0.057$) aged NK cells to degranulate (Figure 2A) and no age-related statistical differences on basal IFN- γ production. These findings were consistent with the absolute NK cell numbers that underwent

degranulation or produced IFN- γ at baseline (data not shown). In spleens of uninfected mice, no relative age-related differences ($p = 0.057$) were observed for degranulation (Figure 2C); however, IFN- γ was statistically reduced for aged splenic NK cells (Figure 2D). The absolute numbers of NK cells positive for CD107a or IFN- γ were significantly less in aged compared to young mice in baseline (data not shown), possibly reflecting the combination of reduced NK cell percentages and numbers present in the spleens of aged mice. After influenza infection, the portion of effector NK cells (Figure 2A, 2B) was increased significantly in the lungs of young mice but not significantly in the aged lungs. The percentage (Figure 2A, 2B) and number (data not shown) of infected lung NK cells degranulated and produced IFN- γ upon stimulation with YAC-1 cells, were significantly reduced in aged compared to young mice. Influenza infection did not significantly change the responsiveness of splenic young NK cells to YAC-1 stimulation; however, a significant impairment of aged NK cells to degranulate and produce IFN- γ was observed after influenza infection (Figure 2C, 2D) and at all times, significantly less absolute numbers of aged NK cells degranulated or produced IFN- γ in the spleens of infected mice (data not shown). Overall, basal relative activation of NK cells by YAC-1 cells was only slightly reduced in the spleens of aged mice, however, a significant age-dependent impairment of NK activation through contact dependent mechanisms with target cells was revealed after influenza infection.

We further assessed spontaneous NK cell function during influenza infection. Cell suspensions incubated without additional stimuli for 4 hours to allow time for CD107a staining. At baseline, very little background degranulation and IFN- γ production was observed in both lungs and spleens of young and aged mice (Figure 3). After influenza infection, NK cells from both young and aged mice showed increased effector functions only in the lungs (Figure 3A,

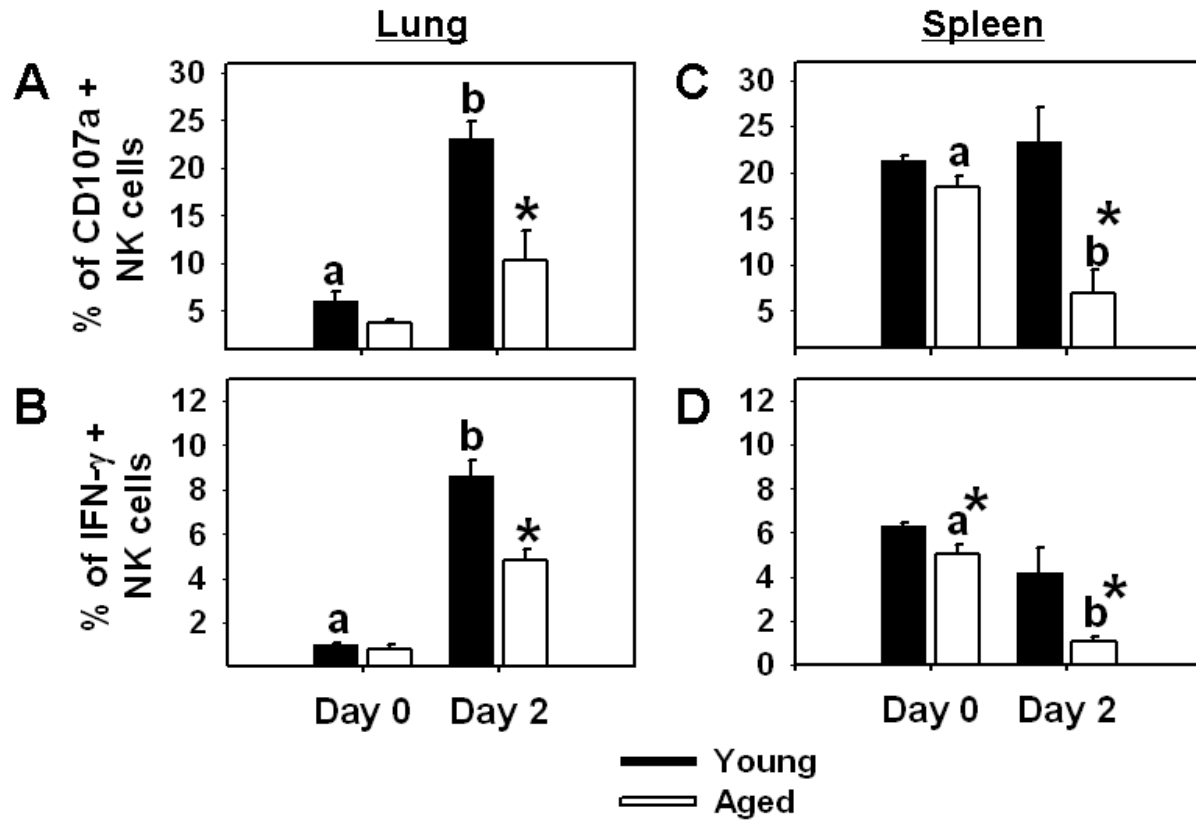


Figure 2. Aged NK cells have impaired function against YAC-1 cells after influenza infection. Single cell suspensions from lungs and spleens of uninfected and infected young and aged mice were incubated with YAC-1 cells at an effector to target ratio of 10:1 in the presence of brefeldin, monensin and FITC-CD107a antibody for 4 hours. Bars represent means \pm SEM of CD107a+ and IFN- γ + NK cells; data shown are from one representative of two performed experiments, $n=5$ per group per experiment. Asterisks, (*), indicate statistical differences between young and aged mice at that time point (t-test, $p < 0.05$). Different letters indicate changes during infection within the same group (Two way ANOVA).

3B), and not in the spleens (Figure 3C, 3D). Influenza infection resulted in an increase of the proportion of NK cells that degranulated with no observed age-related differences ($p = 0.193$) (Figure 3A), even though, aged mice had significantly reduced absolute numbers of CD107a positive NK cells in their lungs (data not shown). However, both the percentages (Figure 3B) and numbers of aged NK cells that produced IFN- γ after influenza infection (data not shown) was significantly reduced in aged mice compared to young. No differences observed in spleens, apart from the fact that after infection the absolute numbers of NK cells that degranulated and produced IFN- γ was significantly reduced in aged mice (data not shown), reflecting their reduced total NK cell numbers (Figure 1D). Overall, after influenza infection, an age-specific defect for IFN- γ production was detected, while the absolute magnitude of both functions was also reduced in aged mice reflecting their reduced NK cell numbers.

Because IFN α/β is an important activator of NK cell cytotoxicity and IL-12 is shown to regulate IFN- γ production from NK cells [158], we analyzed the expression of these two cytokines in the lungs of infected mice. As it is shown in table 1, no statistical differences were found among young and aged lung expression of these two cytokines at day 2 p.i.. IFN β mRNA expression was significantly up regulated in both aged and young mice, while IL-12p40 expression was minimally increased in young and not at all in aged. Even not statistically different, aged lungs had slightly less mRNA expression of these two important activating factors, which could have significant biological consequences. A more detailed assessment of the lung microenvironment is supplied in the tables presented in the appendix A.

To examine the specific defect of aged NK cells to produce IFN- γ upon infection, we stimulated NK cells with certain stimuli: (a) a ligand for an activating receptor, and (b), with cytokine stimulation. We used plate-bound anti-NKp46 antibody and a combination of cytokines

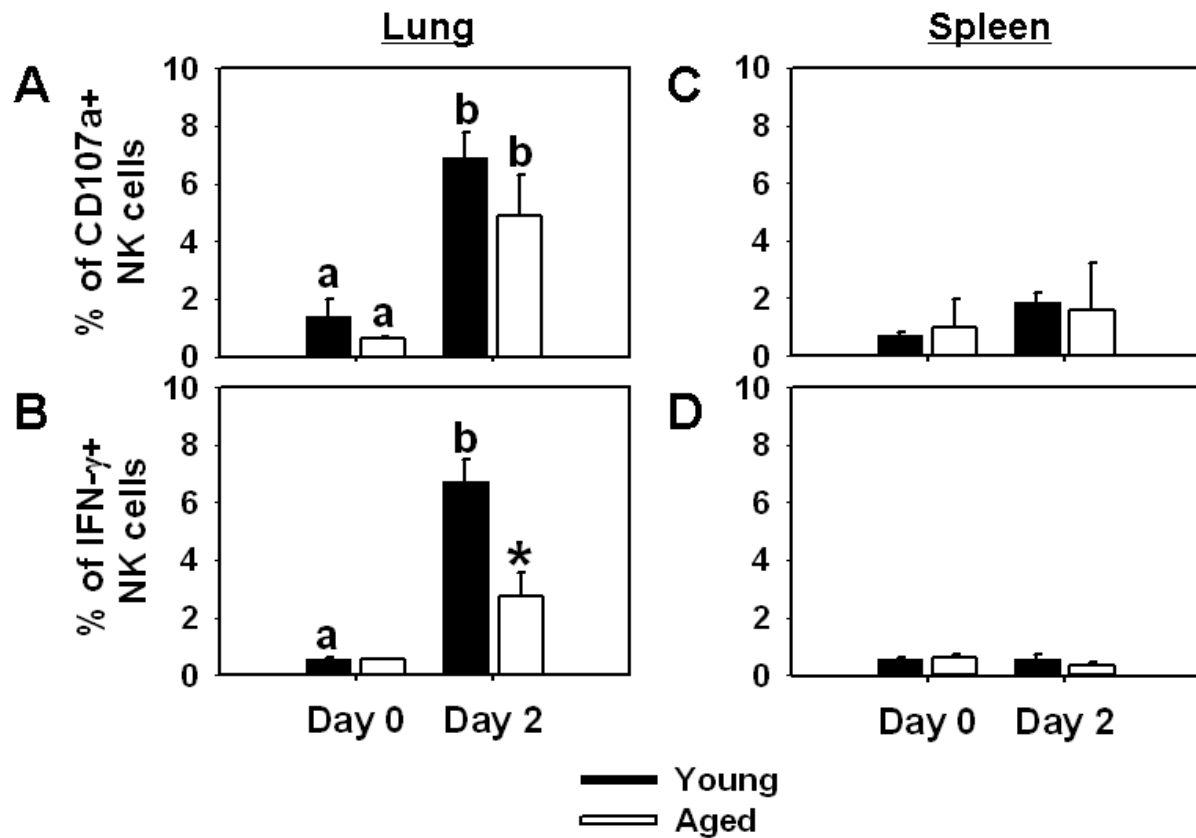


Figure 3. Aged NK cells have reduced IFN- γ production during influenza infection. Single cell suspensions from lungs and spleens of uninfected and infected young and aged mice were incubated in the presence of brefeldin, monensin and FITC-CD107a antibody for 4 hours. Bars represent means \pm SEM of CD107a+ and IFN- γ + NK cells; data shown are from one representative of two performed experiments, n=5 per group per experiment. Asterisks, (*), indicate statistical differences between young and aged mice at that time point (t-test, $p < 0.05$). Different letters indicate changes during infection within the same group (Two way ANOVA).

Table 1. Relative expression of mRNA in the lungs of day 2 infected mice, normalized to uninfected young lungs¹

	Young	Aged	<i>t</i> -test
IFN- β	221.83 \pm 64	169.02 \pm 47	$p = 0.518$
IL-12 p40	1.37 \pm 0.4	0.81 \pm 0.2	$p = 0.563$
IFN- γ	2.73 \pm 0.8	2.42 \pm 0.9	$p = 0.802$

¹ data are means \pm SEM, n=5 per group

(IL-2 and IL-18). At baseline, age-related defects were only observed in the spleens (Figure 4C, 4D) and not in the lungs (Figure 4A, 4B). Both the relative proportions and absolute numbers (data not shown) of splenic NK cells that produced IFN- γ after activation with either anti-NKp46 or IL-2 and IL-18 were significantly reduced in aged mice compared to young. After influenza infection, NK cells from the lungs of young mice produced increased IFN- γ upon engagement with the anti-NKp46 antibody (Figure 4A) and in the presence of IL-2 and IL-18, (Figure 4B), but in aged mice influenza infection did not result in a similar increase of neither percentages, (Figure 4A, 4B) nor numbers of IFN- γ producing NK cells (data not shown). In the spleens, the age-related defect observed at baseline was also retained after infection, with aged mice having significantly reduced NK cells that produced IFN- γ upon stimulation with NKp46 (Figure 4C) and with cytokines (Figure 4D). Overall, even in an equivalent stimulatory environment (specific cytokine, or ligation of a specific activating receptor) aged NK cells produce less IFN- γ , indicating that there is an intrinsic functional defect among aged NK cells.

Aged mice have reduced terminal mature NK cells in their periphery

NK cells can be subdivided into subsets using CD11b and CD27 staining (Figure 5a). These subsets represent subpopulations of murine NK cells circulating in the periphery that exhibit distinct functions and differential maturation status [66]. NK cells that are positive for CD11b (R2, R3) are considered mature and are capable of natural cytotoxicity and cytokine production while NK cells negative for CD11b (R1) are immature and show reduced effector functions [14]. NK cells that are CD11b⁺ CD27⁺ (R2) can differentiate into CD11b⁺ CD27⁻ (R3), which are terminally mature NK cells, and consist the majority of NK cells found in circulation [67].

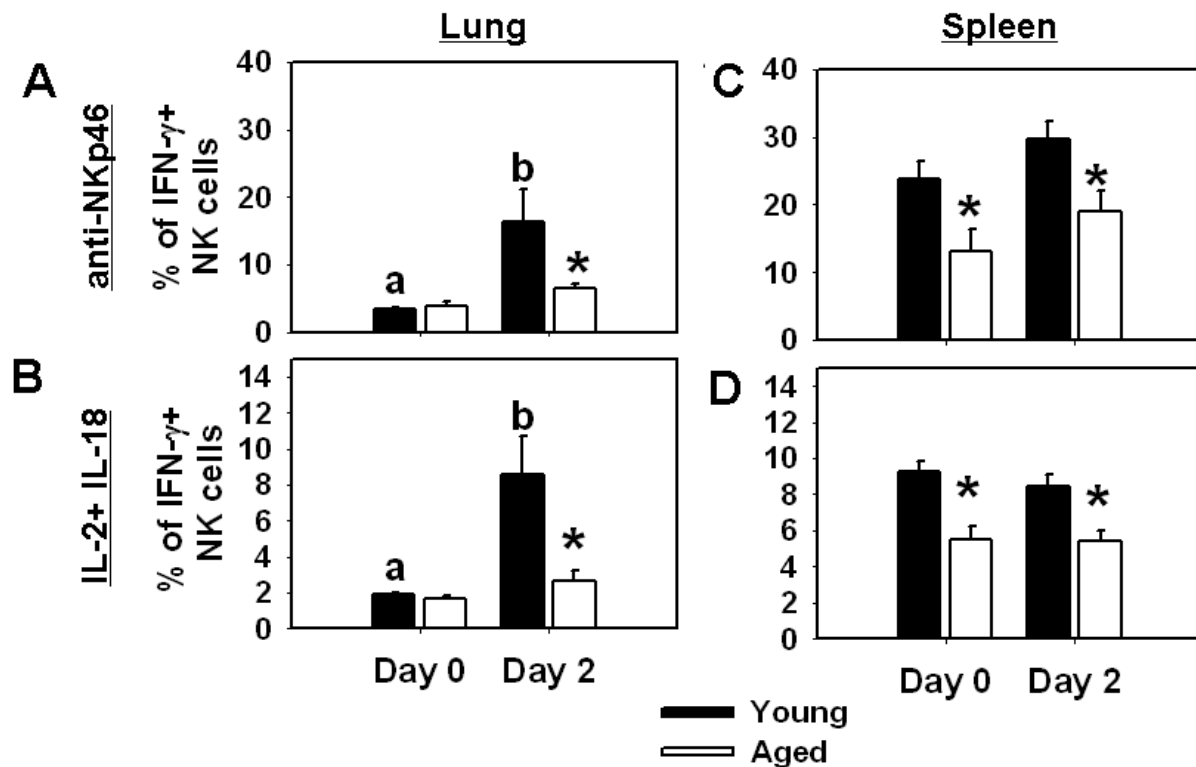


Figure 4. Aged NK cells produce reduced IFN- γ upon stimulation with anti-NKp46 and cytokines after influenza infection. Single cell suspensions from lungs and spleens of uninfected and infected young and aged mice were incubated with plate bound anti-NKp46 antibody (1 μ g/ml), and a combination of IL-2 (1000IU/ml) and IL-18 (20ng/ml). Bars represent means \pm SEM of IFN- γ + NK cells; data shown are from one representative of two performed experiments, n=5 per group per experiment. Asterisks, (*), indicate statistical differences between young and aged mice at that time point (t-test, p < 0.05). Different letters indicate changes during infection within the same group (Two way ANOVA).

We compared the distribution of NK cell subsets in the lungs and spleens of uninfected and infected, aged and young mice to examine whether the observed defect of aged NK cell responses to influenza mirrors a reduced portion of the mature (R3) NK cell subset. At baseline, NK cells in the lungs of aged mice had more immature (R1) (Figure 5B.i) and intermediate (R2) NK cells (Figure 5B.ii), and significantly less terminally mature (R3) NK cells (Figure 5B.iii) compared to young mice. Because of the increased cellularity of uninfected aged lungs (data not shown), the distribution of NK cells in the spleens of young mice, in the spleens of aged mice the percent of most-mature (R3) NK cells was reduced significantly (Figure 5B.vi). Similarly to the lungs of infected aged mice, in the spleens of infected aged mice significantly less NK cells belonged to each subset, with the most severe deficit to be shown for the (R3) NK cells (data not shown). Overall, after influenza infection, aged NK cells were comprised of increased proportion of immature NK cells (R1), and reduced proportion of mature NK cells (R3) suggesting that reduced effectors' functions observed earlier could reflect a change on the population of NK cells in the aged mice.

3.5 Discussion

We previously showed that influenza infection is more severe in the absence of NK cells [13]. Thus, the presence of more NK cells the first days of infection may be essential to recover from infection. In this study we showed that aged mice have less NK cells compared to young before and two days post infection. Decreased NK cells in lungs and spleens at baseline could be a result of reduced production of NK cells from the bone marrow, altered migration or reduced survival of NK cells in the aged microenvironment. It has been reported that healthy elderly have lower proliferation and production rates of NK cells under basal conditions [61]. In addition

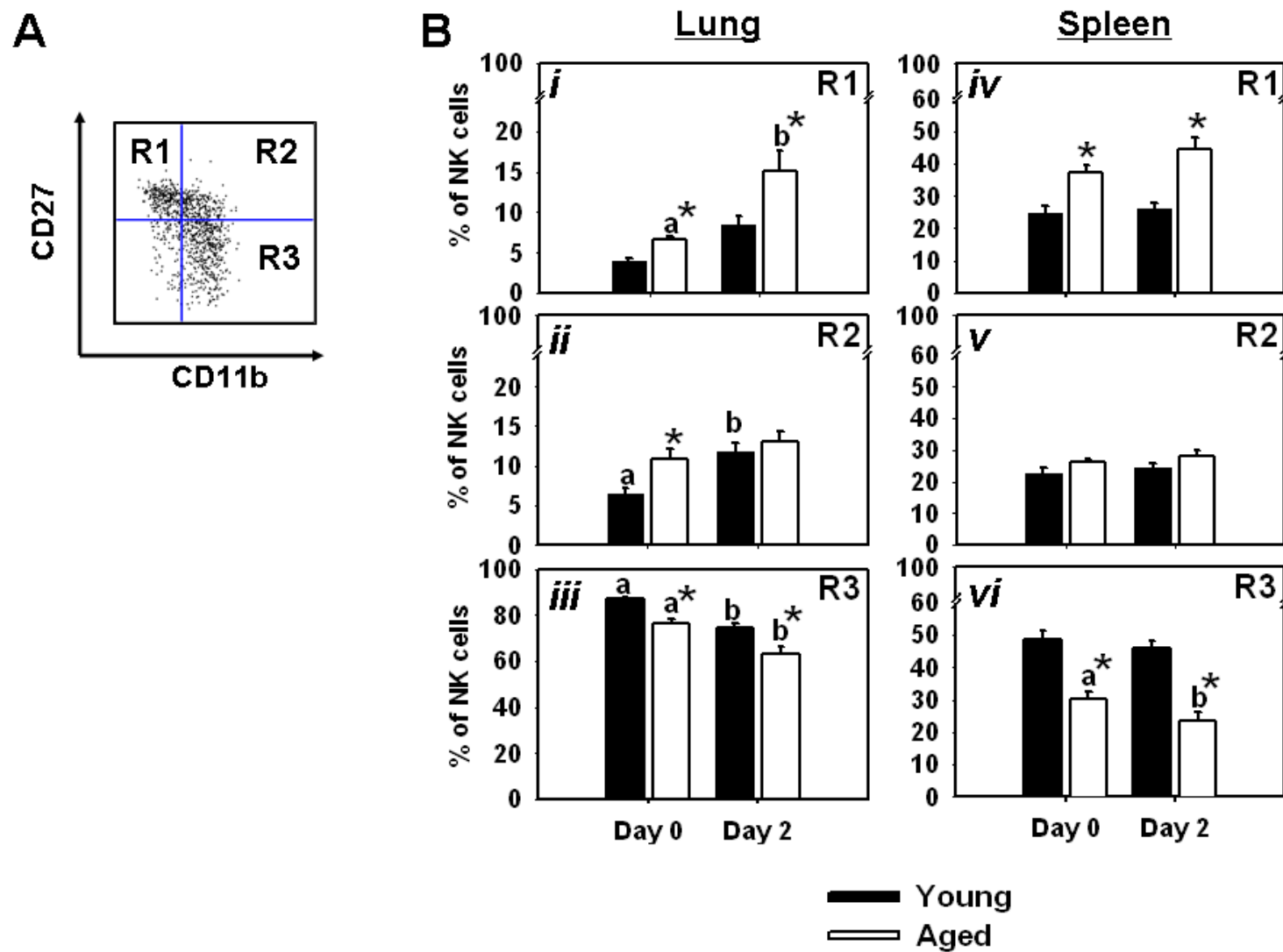


Figure 5. Aged mice have more immature and less functionally mature NK cells.

Figure 5 (cont'd). Single cell suspensions from lungs and spleens of uninfected and infected young and aged mice were stained for NK cell subsets based on the CD11b and CD27 expression. (A) Representative flow chart of NK cell subset distribution in the spleen of one young mouse. (B) NK cell subsets distribution in lungs (*i-iii*) and spleens (*iv-vi*). Bars represent means \pm SEM; data shown are combined from three performed experiments, n=5 per group per experiment. Asterisks, (*), indicate statistical differences between young and aged mice at that time point (t-test, $p < 0.05$). Different letters indicate changes during infection within the same group (Two way ANOVA).

aging affects migration of mature NK cells in the inflamed tissues during viral infection [52]. Finally, aging results in a reduction in circulating IL-15 in mice [65], and IL-15 strongly regulates homeostasis of NK cells in the periphery [64]. Moreover, aged NK/LAK cells have intrinsic defects in their activation after incubation with IL-15 [118]. Decreased NK cells in lungs and spleens after influenza infection could be a result of the previous mentioned factors in addition to increased apoptotic death and defects in homing to the lungs. In fact, influenza virus can infect and induce apoptosis of NK cells [41, 44], but it is not known whether apoptosis during influenza takes place more in the aged than in the young mice. Moreover, we (unpublished data) and others [22] have observed very low rates of proliferation in the lungs of influenza infected mice the first two to three days p.i. Finally, homing of aged NK cells to lymph nodes after infection with mouse pox virus was shown to be defective in recent studies [52]. Taken together, the observed reduced numbers of NK cells in the aged lungs, may result in an environment less equipped to control a respiratory infection, with potential detrimental outcomes for the host. More studies to understand the factors affecting homeostasis of NK cells in the aged environment are necessary.

NK cells regulate host immune responses to viral infections through two major effector functions, natural cytotoxicity and cytokine production [158]. During influenza infection, NK cells get activated to kill [13, 29] and produce IFN- γ between 2-3 days p.i. [29], before specific T cell responses are developed. The importance of enhanced natural cytotoxicity is supported by studies showing that NK cells can kill influenza (A/PR8) infected cells *in vitro*, a process mediated by the engagement of viral hemagglutinin with the NKp46 receptor on NK cells [39]. However, the *in vivo* contribution of natural cytotoxicity in controlling influenza infection is not well defined, although NKp46 has been shown to be essential for survival of mice from influenza infection [36]. Regardless, NKp46 engagement results not only in increased NK cell killing but also increased production of cytokines [35]. The importance of a strong IFN- γ production early during influenza infection is evidenced by increased survival in mice injected with IFN- γ early during influenza infection resulting in higher activation and proliferation of NK cells [22]. However, consistent with others [22], influenza infection does not result in high levels of IFN- γ production by lung NK cells in the early days of acutely infected mice. Nevertheless, influenza virus is one of the few known viruses to promote IFN- γ production by NK cells [158], and this response is abrogated when NK cells are absent [34]. Our present study indicates that during influenza infection NK cells from the lungs of infected young mice are activated to degranulate and to produce IFN- γ upon influenza infection and aging interfered specifically with only IFN- γ production.

During influenza infection, activation of NK cells reflects the integration of multiple signals from the microenvironment through NK cell receptors. NK cells bear cell surface receptors that can be activating or inhibitory [159] and their engagement dictates whether NK cells will be activated or not. Additional activation signals from produced cytokines during

influenza infection can override the effects the engagement of inhibitory receptors on NK cells and significantly enhance their activation and IFN- γ production [160]. Early during influenza infection, stimulatory cytokines for NK cells are produced, such as, type I interferons (IFN α/β), IFN- γ [22], IL-18 [161], IL-12 [34], and IL-15 [162]. Aging results in altered cytokine milieu and in various models, aged mice had reduced levels of type I IFNs [163], IL-12 [12], IFN- γ [12], and IL-15 [164] in their serum or lungs after influenza infection. Although we did not observe statistical significant differences on IL-12p40 and IFN- β mRNA expression in the lungs of infected mice, both were slightly reduced in aged lungs compared to young. Endogenous IL-12 has been related to IFN- γ production by NK cells [34] but influenza infection induced only 1.4 times more IL-12p40 mRNA in the lungs of infected young mice yet not increased at all in the lungs of aged mice. Because IL-12 is highly efficient in low concentrations, this small difference may have important biological consequences *in vivo*. On the other hand, IFN- β has been linked to endogenous NK cell cytotoxicity and was rapidly up regulated to high levels in both mice, with slightly higher levels in young mice. We did not observe a statistical significant impairment of NK cell degranulation during influenza infection *in vivo*, yet aged NK cells showed slightly reduced degranulation. We acknowledge that further studies need to be done to establish the differences in the lung microenvironment between young and aged mice. Preliminary studies in our laboratory show that aged infected mice have increased expression of proinflammatory cytokines such as IL-1 β , and TNF α as well as chemokines of the Macrophage Inflammatory Protein family (Appendix A) but the role of these cytokines in NK cell activation *in vivo* is not well established. We have attempted to examine the differences of the inflammatory cytokine environment in the lungs of young and aged mice with PCR arrays but these were preliminary experiments and are shown and discussed in the Appendix A.

Previously we have shown that after influenza infection aged NK cells exhibited reduced natural cytotoxicity, measured by the chromium release assay with YAC-1 cells target cells [13]. In the present study, YAC-1 targets were used to stimulate NK cells to measure NK cell activation directly. The killing of YAC-1 cells is mediated through NK cell activating receptor (NKG2D) engagement and missing self-recognition on the surface of YAC-1 cells resulting in activation of NK cells, degranulation and IFN- γ production. We observed that after influenza infection, aged mice had almost half the percentage of degranulating and IFN- γ producing NK cells in their lungs compared to young mice indicating that aged NK cells are not equally stimulated by target cells. This reduced response of aged NK cells to YAC-1 stimulation may simply reflect that aged mice have relative less NK cells in their lungs after influenza infection, which affects the effector to target ratio in the mixed co-culture. However, there may also be an age-related defect in NKG2D mediated activation of NK cells [NKG2D signals through intracellular pathways shared by T cells [165] that are shown to be defective in aged T cells [166]].

To further test the defects of aged NK cells in IFN- γ production we incubated NK cells with abundant activating signals such as plate-bound anti-NKp46 antibody and cytokines. We observed that after influenza infection, less aged NK cells produced IFN- γ with both anti-NKp46 and cytokine stimulation. Specifically for influenza infection, reduced activation upon NKp46 ligation could result in detrimental outcome because of the important role NKp46 has on protection from influenza infection. While there was no difference in the mean fluorescence intensity (MFI) of NKp46 on NK cells from young and aged mice (data not shown), the data in this paper suggest impaired ability of this receptor to induce functional responses upon ligation in aged mice. The intracellular signals triggered by NKp46 binding include CD3 ζ chain, Fc ϵ RI γ

receptor and ITAM phosphorylation [159]. It has been shown that CD3 ζ chain is down regulated upon high levels of hemagglutinin present [46], and thus, it is very interesting to correlate the presence of hemagglutinin antigens with NK cell activity in aged mice. While not well characterized in aged NK cells, reduced phosphorylation and activity of key kinases, common for both NK and T cells, were reduced in aged T cells after activation [166].

Because NK cell is not a homogeneous population but is comprised of subsets with variant functional abilities, another explanation for the reduced activation observed in aged mice, may be the presence of less functional NK cell subsets in aged mice. Mature NK cells are considered those who acquire function, which means they can degranulate and produce cytokines. Kim et al. (2002) proposed that NK cells acquire function at a maturational stage where they express high levels of CD11b and CD43 [14] and Hayakawa et al. (2006) proposed the CD11b/CD27 murine subset classification [66]. Additionally, it was shown that upon *in vivo* activation with type I interferon, IFN- γ production was mostly restricted to the mature R3 subset, while NK cells that are R2 were able to exert both functions (IFN- γ production and degranulation) and finally the immature R1 NK cells could only degranulate but not produce any IFN- γ [167]. Thus, the specific defect that we observed for NK cell IFN- γ production but not for degranulation upon influenza infection in aged mice could be partly explained by the reduced proportion of R3 and R2 but increased R1 NK cells in the aged lungs. However, when NK cells were stimulated with target cells, aged NK cells showed impairment in both functions indicating that intrinsic defects may also affect the proper function of aged NK cells.

Although human studies report that aging results in the accumulation of terminally differentiated lymphocytes in elderly people [81], our data indicate that the most mature NK cells are reduced in aged mice, in confirmation with previous reports in aged mice [52]. In

agreement with our results, Fang et al. (2010) showed that aged mice have reduced numbers of NK cells in circulation, specifically mature (R3) NK cells, unable to migrate to the lymph nodes after infection with mouse pox virus [52]. These aged NK cells did not have any changes in traditional NK cell surface markers, but only reduced CD11b and CD62L expression. Preliminary studies by our laboratory confirm these results and suggest that aged NK cells have a late maturational defect on the acquisition of integrins, a step that is associated with acquisition of functional abilities [14] and possibly with homeostasis of NK cells in the various tissues. Further studies are currently underway to elucidate the phenotypic differences among young and aged mice and to compare their responses during influenza infection.

3.6 Conclusions

At the early stages of a pulmonary infection, lung resident immune cells are crucial for the development of a strong antiviral response; activation and trafficking of peripheral immune cells into the lung requires some time. In this study, we showed that aged mice have less NK cells in their lungs (and spleens) before and early after influenza infection, with impaired function and altered phenotype, something that could explain their overall susceptibility to influenza infection. Depending on stimulation, we observed age related defects for NK cell functions: when NK cells stimulated by cell-contact dependent mechanisms with target cells, aged NK cells from both lungs and spleens showed reduced degranulation and IFN- γ production; however, during influenza infection, spontaneous degranulation was preserved in the aged mice, and only IFN- γ production was severely impaired; when the same amount of cytokines or anti-NKp46 antibody was used to stimulate NK cells, aged mice showed reduced IFN- γ production, indicating intrinsic to NK cell defects. These defects could be partly explained by an altered NK

cell phenotype observed in aged mice, with more immature and less terminally mature NK cells to respond to influenza virus. Overall we showed that aging affects the numbers, the function and phenotype of NK cells. All these together could complicate early viral replication and the induction of a strong antiviral immune response to resolve the infection quickly, resulting in prolonged immune responses and detrimental pathology for the aged mice.

CHAPTER 4

THE EFFECTS OF AGING ON MURINE NATURAL KILLER CELL SUBSET HOMEOSTASIS AT BASAL LEVELS

4.1 Abstract

It is well accepted that adaptive immunity is compromised with aging, but less is known about innate immunity. We compared the natural killer (NK) cells from young and aged mice to reveal age-related defects in their tissue and subset distribution, development and function. Our findings indicate that aged mice have reduced NK cells in most peripheral tissues but have increased NK cells in the bone marrow. Analysis of NK cell subsets based on CD11b and CD27 expression revealed that the reduction of NK cells in the periphery was attributed to a specific reduction of the most mature CD11b⁺ CD27⁻ NK cells and the accumulation of NK cells in the bone marrow was attributed to a specific increase of the immature, CD11b⁻ CD27⁺ NK cells. A detailed analysis of the stages of NK cell development in the bone marrow revealed that NK cells normally go through the *stages I-IV* of development, but they accumulated in *stage IV* and could not mature further. We related this defect with the reduced levels of proliferating NK cells in the bone marrow of aged mice. We further showed that CD11b⁺ CD27⁻ NK cells had reduced survival in the aged peripheral tissues. Finally, with an extensive phenotypic characterization of young and aged NK cells we have shown that impaired NK cell function is not related with their altered Ly49 receptor repertoire, but rather with their terminal maturation status. Indeed, we show that in aged mice, NK cells that had acquired the appropriate Ly49 receptors were less

responsive. Licensed NK cells composed of more immature NK cells with increased expression of all the Ly49 receptors examined. Overall, we provide data that support an age-related defective development of NK cells.

4.2 Introduction

Studies on immunosenescence have primarily focused on the impairment of the adaptive immunity, in part because of the reduced responsiveness of elderly people to vaccination [168]. It is well accepted that adaptive immunity is compromised with aging, but less is known about innate immunity. Natural killer cells (NK) belong to the lymphocyte lineage and are known for their spontaneous killing of tumor cells and their antiviral properties. In contrast to the rest lymphocytes, NK cells can kill target cells upon first recognition within minutes and they do not meet several hallmarks of adaptive immunity, factors that contributed to their characterization as components of the innate immunity. Yet, current observations suggest that similar properties are shared between NK cells and the rest lymphocytes [71, 169, 170], and thus it is possible aging may affect all lymphocytes similarly. The increased incidence of infectious diseases and cancer among the elderly, suggests that NK cell function is impaired in advanced ages. Indeed, low NK cell activity is strongly correlated with increased risk for development an infection and short survival, while preserved NK cell activity is an important determinant of longevity [10].

Despite the phenotypic differences of NK cells from humans and mice, murine models have been largely determining our knowledge about immunosenescence. It is generally accepted that in mice peripheral NK cell numbers are reducing with advanced age [51-53]. Furthermore, recently it was shown that aged mice have reduced mature NK cells [52, 149]. Based on the above, we hypothesized that aging impacts NK cell development and their distribution in the

peripheral tissues. Thus, we focused our studies to investigate in detail the effect of aging on the development of NK cells in the bone marrow, maturation status, subset distribution and NK cell function.

The current understanding of murine NK cell development is that NK cells are produced in the bone marrow and seed the peripheral tissues with mature NK cells. Although immature NK cells can be found in liver, thymus, spleen and lymph nodes, an indication that additional developmental pathways exist, the bone marrow is the primary site for NK cell generation. NK cell progenitors (NKPs) undergo several stages of development in which they acquire different cell surface markers, the inhibitory and activating Ly49 receptors and integrins [14]. When NK cells are still immature, they acquire inhibitory and activating receptors of the Ly49 family that recognize MHC class I molecules. The interactions of Ly49 inhibitory NK cell receptors with their ligands both during development as well as in the periphery play an important role in tuning and maintaining the reactivity of NK cells [171] and this process has been named “licensing” [15]. Thus, NK cells that are licensed, express at least one inhibitory receptor that recognizes self MHC and display potent functional activities, while NK cells that are unlicensed, do not express inhibitory receptors for self MHC molecules and are hyporesponsive in order to avoid self auto-aggressiveness [15]. In C57BL/6 mice, Ly49C is the major inhibitory receptor known to interact with the self MHC-I ligand K^b and to provide licensing. Other inhibitory receptors that have some reactivity with self MHC-I have also identified in C57BL/6 mice: Ly49I can bind to H2K^b and CD94/NKG2A interacts with H2D^b peptide presented by Qa-1 [16]. On the other hand, Ly49A has known strong reactivity with H2D^d that is absent from C57BL/6 mice and therefore Ly49A⁺ NK cells are considered unlicensed. However, because of the stochastic way that these

receptors are expressed on NK cells during development, it is possible to get NK cells with different Ly49 receptor combinations. In the following studies, we have characterized the developmental stages of NK cell maturation in the bone marrow and the Ly49 receptor repertoire in aged NK cells, and to our knowledge, this is the first study to provide such information in the context of aging.

After NK cells acquire their Ly49 receptors, immature NK cells undergo functional maturation during a developmental stage that corresponds with an increase of the cell surface density of maturation markers, and a significant expansion of their numbers in the bone marrow. Kim et al. proposed that NK cells acquire function after they express high levels of CD11b and CD43 [14]. During these late developmental stages and after their release to the periphery, a reduction of CD27 and an increase of KLRG1 on NK cell surface is observed, making the CD11b⁺ CD27⁻ NK cells the most differentiated NK cell subset. CD11b⁺ CD27⁻ NK cells generally compose the majority of NK cells circulating in peripheral blood (up to 90%) and in non-lymphoid tissues, they have high potential for cytolytic functions and are the major NK cells producing IFN- γ upon activation.

Our laboratory has previously shown that influenza infection is more severe in the absence of NK cells [13] and that aged mice have reduced NK cells infiltrating in the lungs during the early days of influenza infection [13, 149]. During these studies we also observed that aged NK cells had a specific reduced ability to produce IFN- γ in response to influenza infection, as well as in response to *in vitro* stimulation with YAC-1 cells, cytokines and plate-bound antibodies. These defects were correlated with significantly reduced numbers and percentages of mature, CD11b⁺ CD27⁻ NK cells in aged mice. The following study characterizes in more detail the age-related differences on NK cell phenotype and correlates them with the impaired function

of aged NK cells at basal levels. We show that aged mice have reduced NK cells in most peripheral tissues with exception the bone marrow. Reduction of total NK cells was attributed to a specific reduction of the mature, CD11b⁺ CD27⁻ NK cell subset. Analysis of the developmental stages of NK cells in the bone marrow revealed that aged mice had similar NK cells belonging to the immature stages but reduced NK cells in the terminal maturation stage. We attributed the reduction of mature NK cells to reduced generation of NK cells from the bone marrow, as well as to increased potential for apoptosis in the peripheral tissues. Finally, we show that impaired NK cell function is more related to their immature phenotype rather than their Ly49 receptor repertoire.

4.3 Materials and Methods

Mice

Male, C57BL/6J, young (6 month) and aged (22 month) mice were purchased from National Institute on Aging colony (Charles River Laboratories, Wilmington, MA, USA). Mice were acclimated for at least one week, housed in micro-isolator cages in a Biosafety level 2 room at the Michigan State Research Containment Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International certified facility. All animal procedures were approved by the Michigan State Institutional Animal Care and Use Committee and were in accordance with National Research Council guidelines.

Cell Preparation

Cells isolated from spleen and lungs were prepared according previously published procedures [149] using the GentleMACSTM Dissociator (Miltenyi Biotec Inc, Auburn, CA,

USA) and red blood cells were lysed with ACK lysis buffer. Bone marrow cells were isolated from the femurs and red blood cells were lysed with ACK lysis buffer. Livers were homogenized using the GentleMACS™ Dissociator and lymphocytes were isolated using 37.5% Percoll solution according previously published methods [172]. Blood was collected in heparin tubes, and mononuclear cells were isolated using Ficoll (Sigma-Aldrich, St. Louis, MO, USA) according the manufacturers' instructions. For the lymph nodes, we collected inguinal and brachial lymph nodes. Single cell suspensions of lymph nodes were prepared by forcing through a 70 µm filter and washed with RPMI. Cell counts were performed with a hemocytometer by using trypan blue exclusion dye.

Flow Cytometry

For cell surface staining, one million, and for intracellular staining, two million cells were stained with the appropriate antibodies on ice for 30 min according previously published methods [157]. Before being stained, cells were treated with Fc-Block (antibody to CD16/32) apart from when we stained for CD16 on NK cells. A combination of 7 or 8 of the following monoclonal antibodies conjugated to FITC or Alexa Fluor-488, PE, PE-Cy7, PerCP-Cy5.5, Alexa Fluor-700, APC or efluor-647, APC-Cy7, Pacific Blue or efluor-450 and biotin was used: NK1.1 (PK136), NKp46 (29A1.4), CD3 (145-2C11), CD19 (1D3), CD11b (M1/70), CD27 (LG.3A10), KLRG1 (2F1), CD122 (TMb1), CD49b (DX5), CD43 (S7), NKG2D (CX5), NKG2ACE (20d5), NKG2A (16a11), CD94 (18d3), CD16/32 (93), 2B4 (eBio244F4), Ly49A (JR9), Ly49G2 (4D11), Ly49D (4E5), Ly49C (4LO3311), Ly49I (YLI-90), Ly49H (3D10), CD62L (MEL-14), CD117 (2B8), CD11a (2D7), B220 (RA3-6B2), CD90.2 (53-2.1), CD69 (H1.2F3), CD127 (A7R34), Ly6C (AL-21), CXCR3(CXCR3-173), CCR7 (4B12), CXCR6

(221002), CD160 (13-1601-80), PD-1 (J43), IFN- γ (XMG1.2), Granzyme B (GB12), Bcl-2 (3F11), Bim (Ham 151-149), Ki67 (B56), Annexin V and 7-AAD. Antibodies were purchased from BD Pharmingen (La Jolla, CA, USA), ebiosciences (San Diego, CA, USA), Biolegend (San Diego, CA, USA) or R&D Systems (Minneapolis, MN, USA). Biotinylated antibodies were visualized with Streptavidin PE, PE-Cy7 or APC-Cy7 (BD and ebiosciences). Stained cells were analyzed with a FACS-Canto II or LSR II (BD) and FlowJo software (Treestar, Ashland, OR, USA). The lymphocyte population was gated based on forward and side scatter and NK cells were identified as CD3/CD19 negative and NK1.1/NKp46 positive cells within the lymphocyte gate. Total numbers of NK cells were calculated by extrapolating the percentage of NK cells from the live gate to the total number of cells isolated from each tissue, determined by hemacytometer counts.

In vitro stimulation

Activation with YAC-1 cells, cytokines and plate bound antibodies were performed in RPMI 1640 medium containing 10% heat inactivated FBS. For cytokine activation, 2×10^6 isolated spleen cells were incubated in a 37°C, 5% CO₂ incubator with a combination of IL-2, 1000 IU/ml, (provided by Dr Kim's laboratory) and IL-18, 20 ng/ml (MBL, Woburn, MA, USA) for 7 hours. After 1 hour incubation Brefeldin A (BD) was added at 1 μ l/ml. Upon completion, cells were washed and stained for surface markers and intracellularly for granzyme B and IFN- γ using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit. For stimulation with anti-NK1.1, we coated 12 well plates with anti-NK1.1 monoclonal antibody ascites at a 1:30,000 dilution (provided by Dr. Kim's laboratory) for 80 min at 37°C. We then seeded 2×10^6 spleen

cells, incubated for 7 hours and added brefeldin A 1 hour after initiation of the incubation. For YAC-1 cell stimulation we incubated 2×10^6 spleen cells with 2×10^5 YAC-1 cells (E:T ratio of 10:1) in the presence of Brefeldin A for 4 hours in a 37°C , 5% CO_2 incubator. Subsequently, cells were washed and stained for surface markers and intracellularly for IFN- γ using the BD Cytotfix/Cytoperm Fixation/Permeabilization Solution Kit.

Apoptosis

Annexin V staining was performed on single cell suspensions from freshly isolated spleen and bone marrow cells, without any further activation, using the instructions of the manufacturer (BD Biosciences). We also stained for Annexin V after we stimulated splenic cells for 4 hours with various concentrations of YAC-1 target cells using the same staining kit.

Statistical Analysis

All statistics were generated using Sigma Stat 3.11 (Systat Software Inc, Chicago, IL, USA). Student's t-test was used to compare young and aged NK cells. Two-way ANOVA was used to compare the effect of age and time post infection. Significance level for the two tests was $p < 0.05$ and the post-hoc comparisons were done with the appropriate tests offered by the software.

4.4 Results

Aged mice have fewer NK cells in most peripheral tissues but more in bone marrow

We compared the distribution of NK cells in various tissues of young and aged mice. Figure 6A shows the percentages and Figure 6B the absolute numbers of NK cells. Aged mice

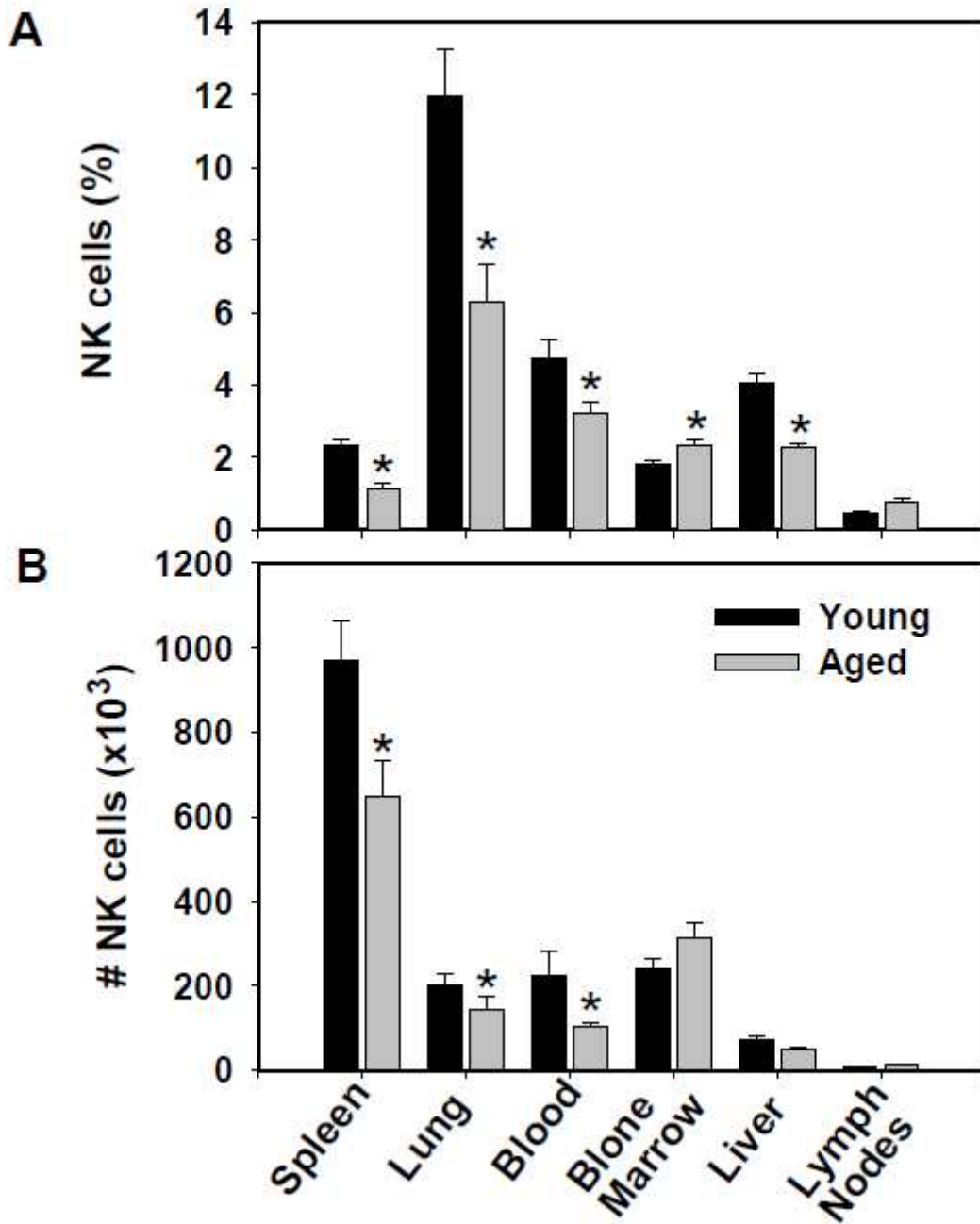


Figure 6. NK cell distribution in various tissues of young and aged mice. NK cells gated as NK1.1^+ , NKp46^+ , $\text{CD3}\epsilon^-$, CD19^- cells. (A) NK cells as percentage of lymphocytes gated based on their FSC/SSC profile. (B) Numbers of NK cells in whole spleen (n=15), lungs (n=15), blood/ml (n=15), bone marrow (n=10), liver (n=5), and lymph nodes (n= 5) per group. Asterisks (*) indicate statistically significant difference between young and aged groups, t-test, $p < 0.05$.

had significantly reduced percentages of NK cells in spleens, lung, blood and slightly lower in the liver. In contrast, aged mice had increased percentage of NK cells in the bone marrow and slightly increased percentages of NK cells in the lymph nodes. The absolute numbers of NK cells confirmed that aged mice had reduced NK cells in the lungs, spleen and blood and maintained NK cells in the bone marrow. So, there is an age-related alteration on the distribution of NK cells in the various tissues at resting conditions, with a preferential reduction of the NK cells in circulation, in spleens and lungs, but not in the bone marrow.

Aged mice have reduced mature $CD11b^{+}CD27^{-}$ NK cells

We then compared the distribution of NK cell subsets in the various tissues of young and aged mice using the CD11b and CD27 classification that represent subpopulations of murine NK cells with distinct functions and differential maturation status [66]. Immature NK cells that are $CD11b^{-}CD27^{+}$ differentiate into $CD11b^{+}CD27^{+}$ and further to $CD11b^{+}CD27^{-}$ that are terminally mature NK cells and represent the majority of NK cells found in circulation [67]. Aged mice have significantly reduced mature, $CD11b^{+}CD27^{-}$ NK cells in all tissues compared to young (Figure 7A). At the same time, aged mice have increased percentage of immature $CD11b^{-}CD27^{+}$ NK cells. To compare if indeed aged mice have more immature NK cells in the periphery or if the percentage of immature NK cells is increased due to a selective reduction of mature NK cells, we compared the numbers of NK cells belonging to each subset in the spleen and the bone marrow. In spleens, is clearly shown that reduced percentages of mature NK cells reflects an actual reduction of the number of $CD11b^{+}CD27^{-}$ NK cells and is not the result of a dilution effect because of the increase of immature NK cells, since young and aged mice had the

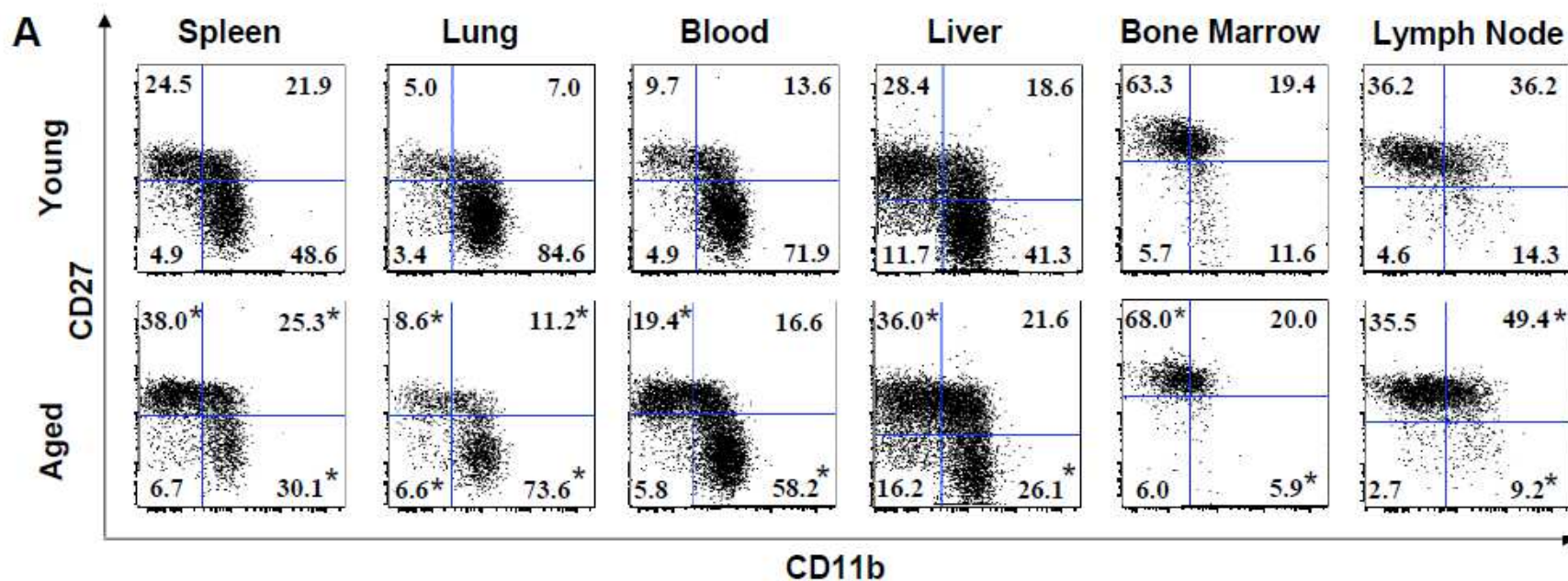


Figure 7. NK cell CD11b/CD27 subset distribution in various tissues from young and aged mice. (A) Flow cytometry dot plots of young and aged mice are representative of the average values for each group. Data represent the average value for each subset in each tissue. Spleen (n=15), lungs (n=15), blood (n=15), bone marrow (n=10), liver (n=5), and lymph nodes (n= 5) per group. Asterisks (*) indicate statistical differences between young and aged groups, t-test, $p < 0.05$. For interpretation of the reference to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

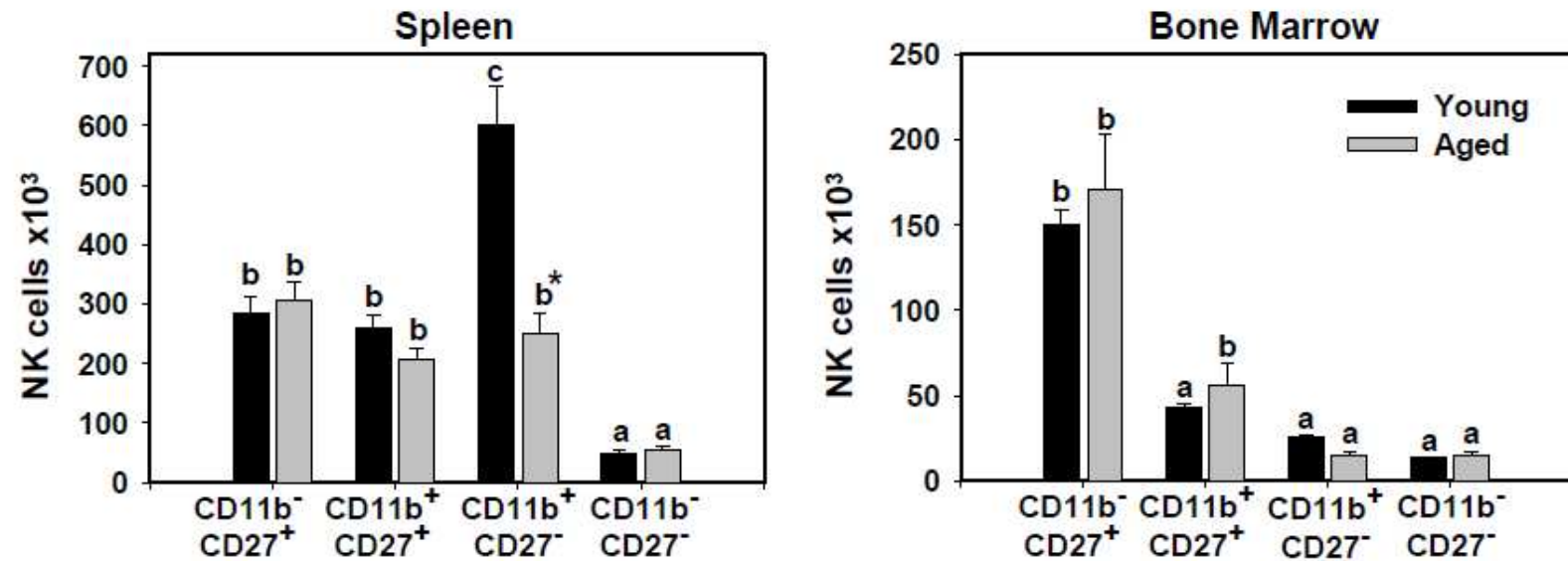
B

Figure 7 (cont'd). (B) Number of NK cells in spleen (n=20) and in bone marrow (n=10). Two Way ANOVA was performed. Different letters indicate statistically significant difference within the same age group, while asterisks (*) indicate statistically significant difference between young and aged.

same numbers of immature NK cells (Figure 7B). In the bone marrow however, there was no statistical difference on the numbers of both immature $CD11b^{-} CD27^{+}$ and mature $CD11b^{+} CD27^{-}$ NK cells although aged mice had slightly more immature NK cells (Figure 7B). Based on these data, we hypothesized that aged NK cells would have reduced function, because they have a significant reduction in their mature NK cell numbers. We also hypothesized that in aged mice survival of NK cells in the peripheral tissues would be decreased and a defect in the maturation of NK cells in the bone marrow would exist.

Aged NK cells have reduced function after activation that is associated with reduced $CD11b^{+} CD27^{-}$ NK cells

To understand the implications of reduced $CD11b^{+} CD27^{-}$ NK cells observed in aged spleens, we stimulated splenocytes of young and aged mice with a combination of cytokines (IL-2 and IL-18) for 7 hours and stained for CD11b/CD27 subsets and granzyme B and IFN- γ . Compared to young, aged NK cells had significantly reduced granzyme B staining and IFN- γ production (Figure 8A). This defect may be intrinsic to the ability of each subset to function, or just a result of reduced mature NK cells in the aged mice. To delineate these possibilities, we gated on each subset and measured their function. Figure 8B, shows that the ability of each subset to produce granzyme B or IFN- γ after stimulation remained approximately the same with advanced age. When we measured the absolute numbers of NK cells that belong to each subset and exhibit a function we clearly see that the reduced NK cell responses are caused from the

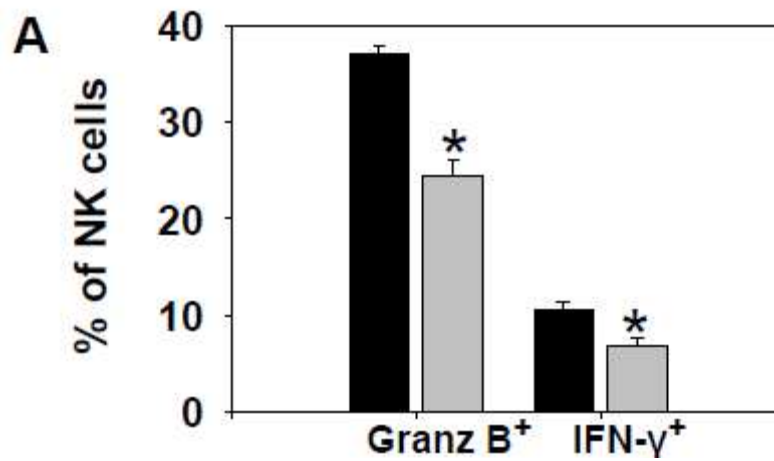


Figure 8. NK cell function after stimulation of freshly isolated splenocytes with IL-2 and IL-18 in young and aged mice (A) Percentages of NK cells positive for granzyme B and IFN- γ , (n=5), experiment repeated twice. Asterisks (*) indicate statistically significant difference, t-test, $p < 0.05$.

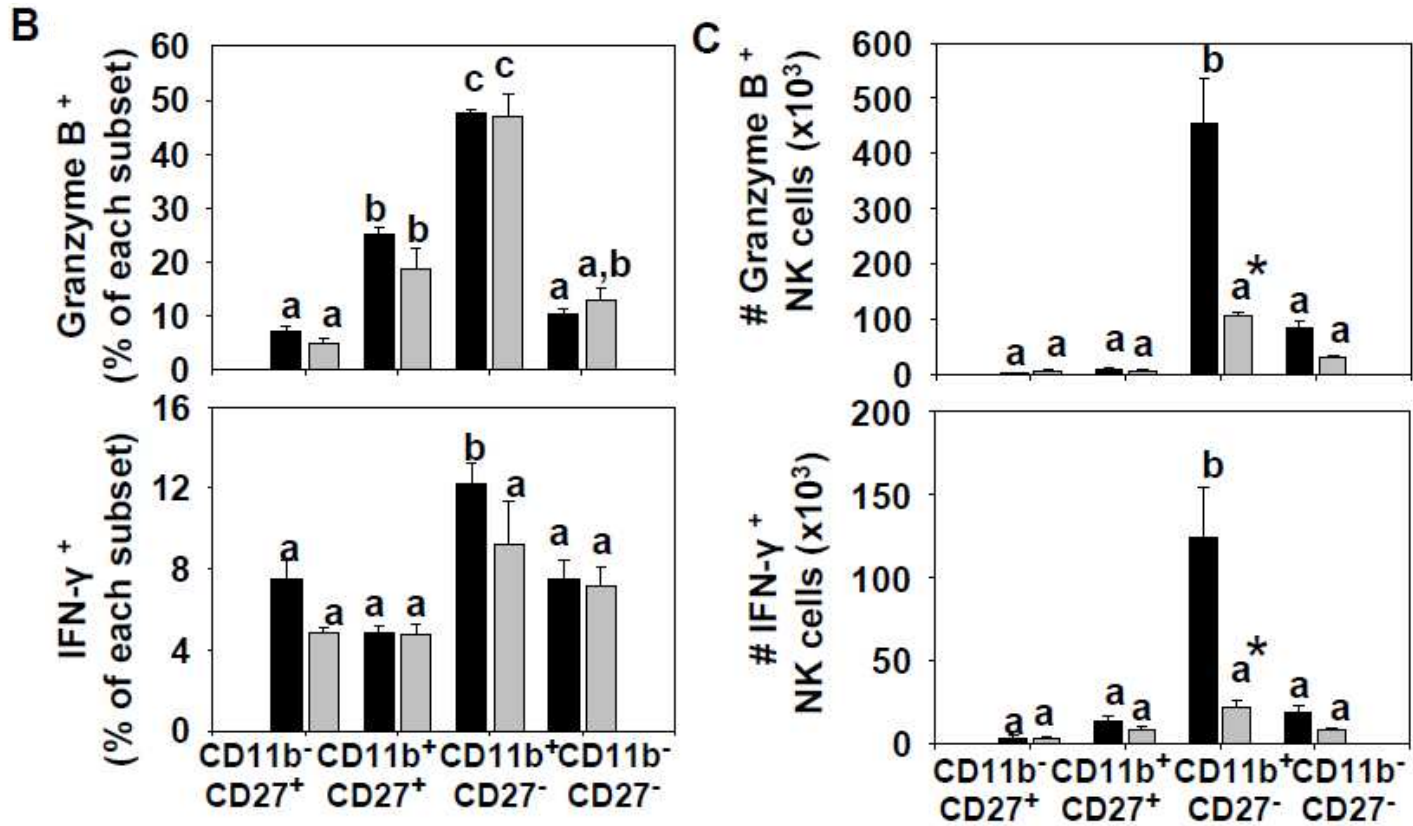


Figure 8 (cont'd). (B) Granzyme B and IFN-γ positive NK cells were analyzed for CD11b/CD27 subset distribution. Counter plots are representative and values are the averages for each group. Asterisks (*) indicate statistically significant differences between young and aged mice, t-test, $p < 0.05$. (C) Numbers of NK cells belonging to each CD11b/CD27 subset that were positive for granzyme B, upper plot, and IFN-γ, lower plot. Two Way ANOVA was performed. Different letters indicate statistically significant difference within the same age group, while asterisks (*) indicate statistical significant difference between young and aged.

reduction of the numbers of $CD11b^{+}CD27^{-}$ NK cells (Figure 8C). Thus, aging results in altered distribution of the functional subsets affecting the overall NK cell responses to stimulatory conditions. Overall the reduced function of aged NK cells is related to the specific absence of $CD11b^{+}CD27^{-}$ NK cells from the periphery.

Aged NK cells accumulate in Stage IV of development and show reduced proliferation in the bone marrow

To examine whether aged mice have a developmental defect that blocks NK cell maturation and therefore the generation of mature NK cells to seed the peripheral tissues, we examined the various developmental stages of NK cells the bone marrow of young and aged mice. Based on the developmental scheme for NK cells proposed by Kim et al. (2002), we analyzed several markers on young and aged NK cells in the bone marrow. We classified cells that were $[CD122^{+}NK1.1^{-}]$ as NK cell progenitors (NKPs) belonging to “*Stage I*” of maturation; $[NK1.1^{+}DX5^{-}]$ cells, as immature NK cells that have acquired the receptors CD94-NKG2 and Ly49s and belong to “*Stage II - III*”; $[NK1.1^{+}, DX5^{+}, CD11b^{-}]$ cells, as immature NK cells that are in the expansion stage and belong to “*Stage IV*”; $[NK1.1^{+}, DX5^{+}, CD11b^{+}]$ cells, as mature cells that have acquired function and belong to “*Stage V*”; and finally, NK cells that were $[CD11b^{+}CD27^{-}]$ as terminally mature NK cells. When we compared these stages of NK cell maturation in the bone marrow of young and aged mice, we observed that approximately the same percentage of NK cells belonged at the early stages of development, *Stages I-IV* but less aged NK cells were moving to the subsequent stage of functional maturation, *Stage V* and after

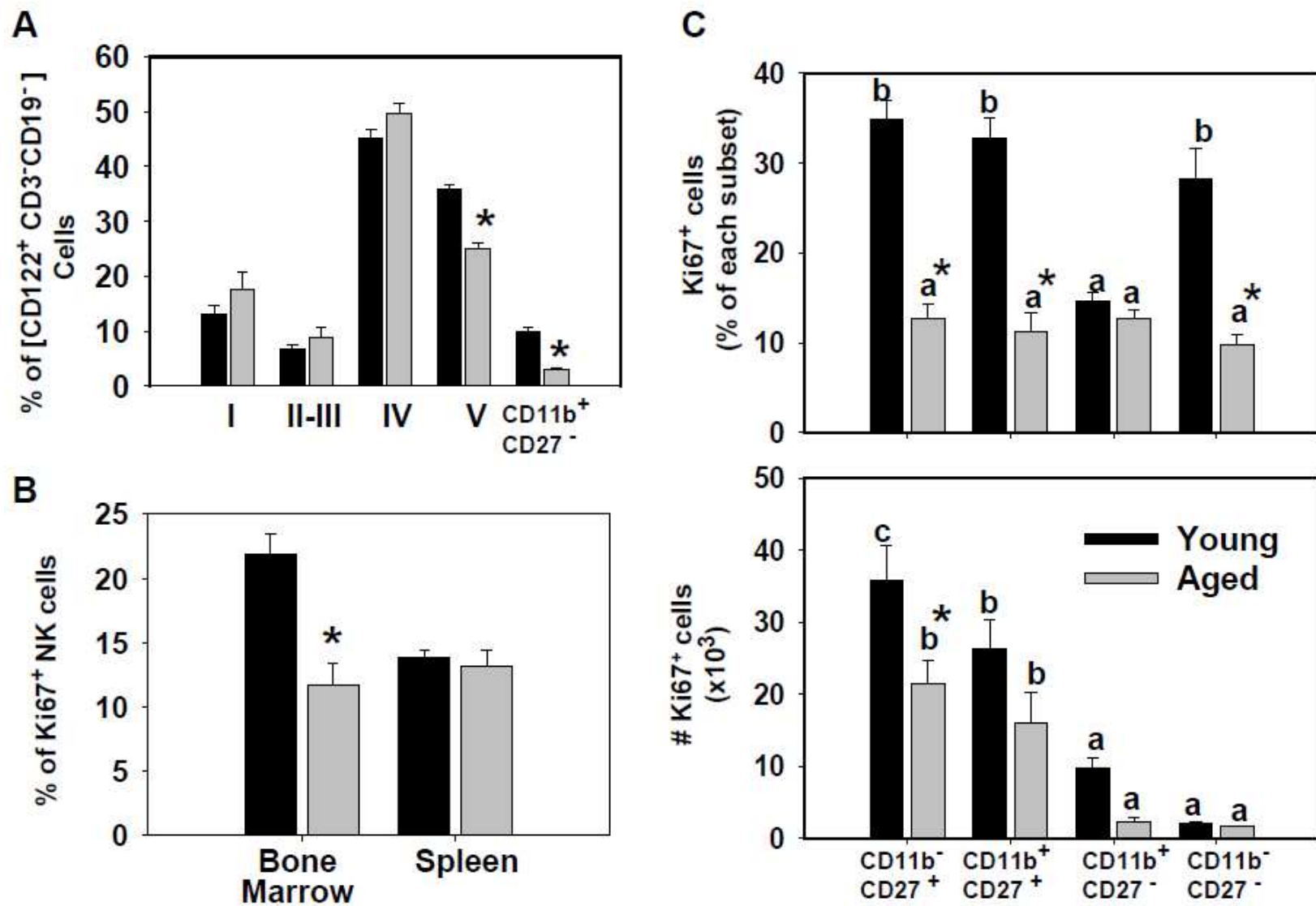


Figure 9. NK cell development and proliferation in young and aged bone marrow.

Figure 9 (cont'd). (A) Percentages of NK cells that belong to each stage of development in the bone marrow, n=5. Asterisks (*) indicate statistically significant difference, t-test, $p < 0.05$. (B) Percentage of NK cells that were Ki67 positive, as an indicator of proliferation, n=5. Asterisks (*) indicate statistically significant difference, t-test, $p < 0.05$. (C) CD11b/CD27 subsets of NK cells were gated and analyzed for Ki67: upper panel percentages, lower panel absolute numbers. Two Way ANOVA was performed. Different letters indicate statistically significant difference within the same age group, while no differences were detected between young and aged, (n=5).

(Figure 9A). Because these last stages of terminal maturation involve expansion of NK cell numbers, we examined whether proliferation of aged NK cells was reduced in the bone marrow using Ki67 as a marker of recently proliferated cells. Indeed, we observed that less NK cells from the bone marrow of aged mice were Ki67 positive (Figure 9B). To examine the possibility that expansion of NK cells in aged bone marrow is normal but Ki67⁺ NK cells are reduced in the aged bone marrow because of increased death, we stained freshly isolated NK cells from the bone marrow with Annexin V antibody and intracellularly with Bcl2/Bim but we didn't observe any differences among young and aged NK cells (data are not shown). Thus, aged NK cells did not undergo higher apoptosis in the bone marrow. We then compared the percentages of each subset that were Ki67⁺ to see whether the same percentage of each subset had undergone proliferation. As it is shown in figure 9C, a smaller percentage of immature CD11b⁻CD27⁺ and CD11b⁺CD27⁺ NK cells had proliferated in the bone marrow of aged mice compared to young, while similar percentages of mature CD11b⁺CD27⁻ NK cells were Ki67⁺, emphasizing the role of proliferation for the maturation of NK cells. Comparisons of the absolute numbers of NK cells from each subset that had proliferated confirmed that less immature NK cells in the bone marrow

of aged mice undergo proliferation and cannot transmit into the mature stage. Overall, these data indicate that there is not a particular block in early development of NK cells and similar percentages of NK cells go through the *Stages I-IV*. However, a problematic transition from *Stage IV* to *Stage V* exists in aged bone marrow, which is related to reduced proliferation of the immature NK cells leading to reduced generation of mature NK cells.

Aged CD11b⁺ CD27⁻ NK cells have reduced potential for survival

The specific reduction of mature CD11b⁺ CD27⁻ NK cells in the peripheral tissues of the aged mice could be a result of reduced survival or increased death. To explore the NK cell apoptotic potential in the periphery, we focused on splenocytes and we stained freshly isolated splenocytes for Annexin V and after incubation with YAC-1 cells, to induce apoptosis. Annexin V staining did not reveal any statistically significant differences among young and aged NK cells (Figure 10A, no target [NT]). However, when NK cells were activated with YAC-1 cells at various effector to target ratio, aged NK cells showed increased Annexin V staining in comparison to young NK cells (Figure 10A). Thus, aged NK cells undergo higher activation induced apoptosis.

To further delineate age- related differences in the survival of young and aged NK cells, we stained freshly isolated splenocytes intracellularly for the antiapoptotic and proapoptotic molecules of the Bcl-2 family. Cell survival in resting conditions is the result of the balance between pro- and antiapoptotic members of the Bcl-2 protein family [173]. Bim, one of the proapoptotic members of the Bcl-2 family binds to the antiapoptotic protein Bcl-2 when apoptosis is initiated resulting in activation of Bax and subsequent apoptosis. Thus, we determined the ratio of the median fluorescence intensities (MFI) of Bcl-2/Bim to assess the

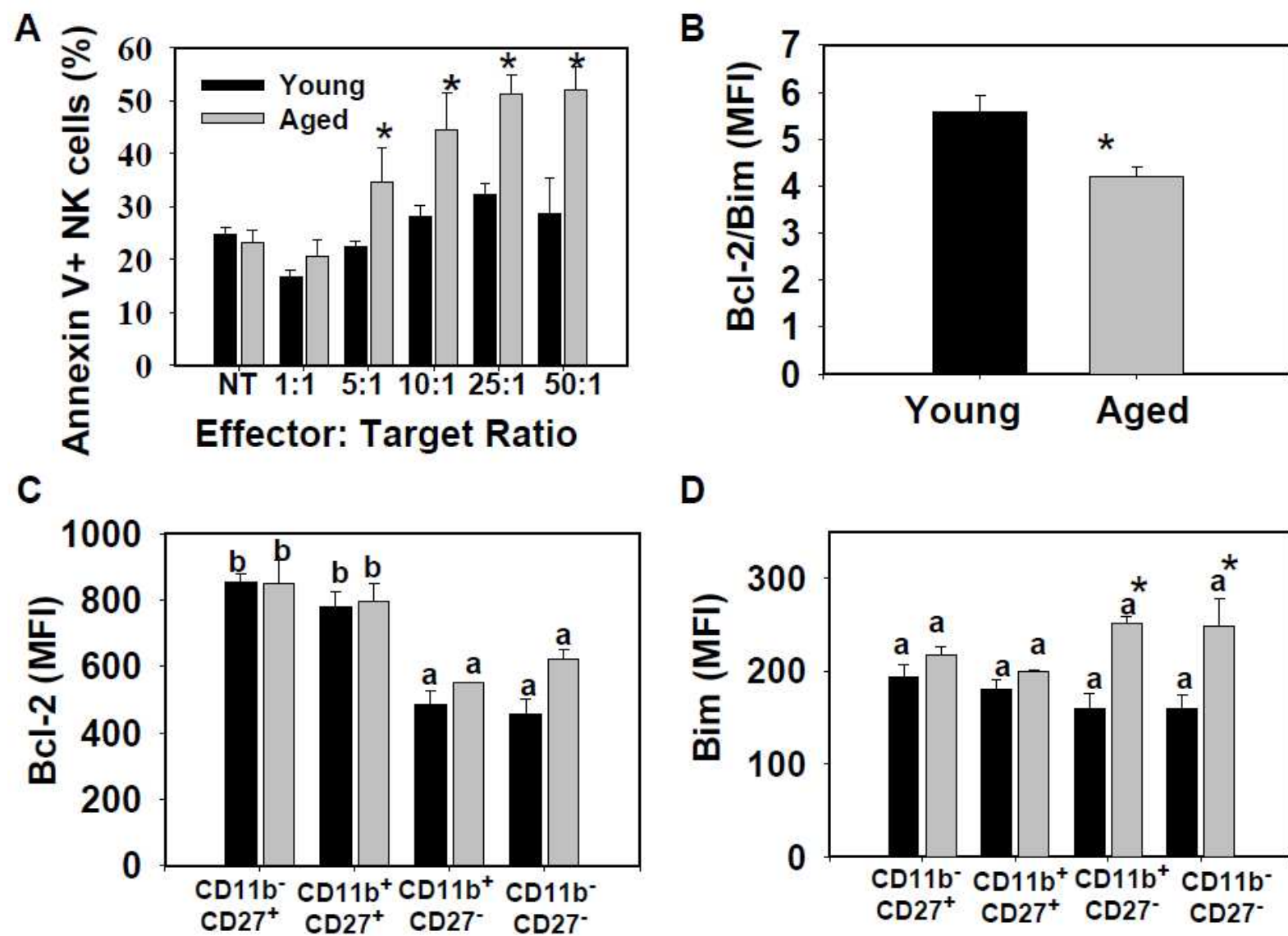


Figure 10. Analysis of the apoptotic potential of resting splenic NK cells from young and aged mice.

Figure 10 (cont'd). (A) Annexin V + NK cells after incubation of splenic single cell suspensions with various concentrations of YAC-1 cells n=5. Asterisks indicate statistical significant difference among the two age groups. t-test, $p < 0.05$. (B) Ratio of Bcl-2/Bim MFI of splenic NK cells. n=5. Asterisks indicate statistical significant difference among the two age groups. t-test, $p < 0.05$. (C) MFI expression of Bcl-2 and (D) of Bim on each CD11b/CD27 subset. n=5. Two Way ANOVA was applied. Different letter indicate statistical significant difference within the same age group, while asterisks indicate statistical significant difference within the same subset.

apoptotic potential of the NK cells. As it is shown in figure 10B, Bcl-2/Bim ratio was significantly reduced in aged NK cells from spleens compared to NK cells from young mouse spleens. Expression of Bcl-2 was similar in both young and aged NK cells, but expression of Bim was increased in aged NK cells (data not shown). We then analyzed each CD11b/CD27 subset for the expression of Bcl-2 and Bim: Bcl-2 expression was reduced in the CD11b⁺ CD27⁻ subset in general, but no age-related differences were detected (Figure 10C). In contrast, Bim expression was increased in the aged NK cells and in particular, among the CD11b⁺ CD27⁻ NK cells (Figure 10D). Thus, aged mice had higher Bim expression in the CD11b⁺ CD27⁻ NK cell subset indicating a reduced potential for survival of this particular subset in aged mice. Overall, in aged mice, the proliferative defects in the bone marrow result in reduced output of mature NK cells, while these cells show further reduced potential for survival in the periphery.

Aging modulates NK cell surface marker expression

Because NK cells are heterogeneous population, we examined several other markers in order to describe aged NK cells better. Among the markers we tested, some characterize immaturity, others activation, some are important for cytotoxicity and others are found mostly on

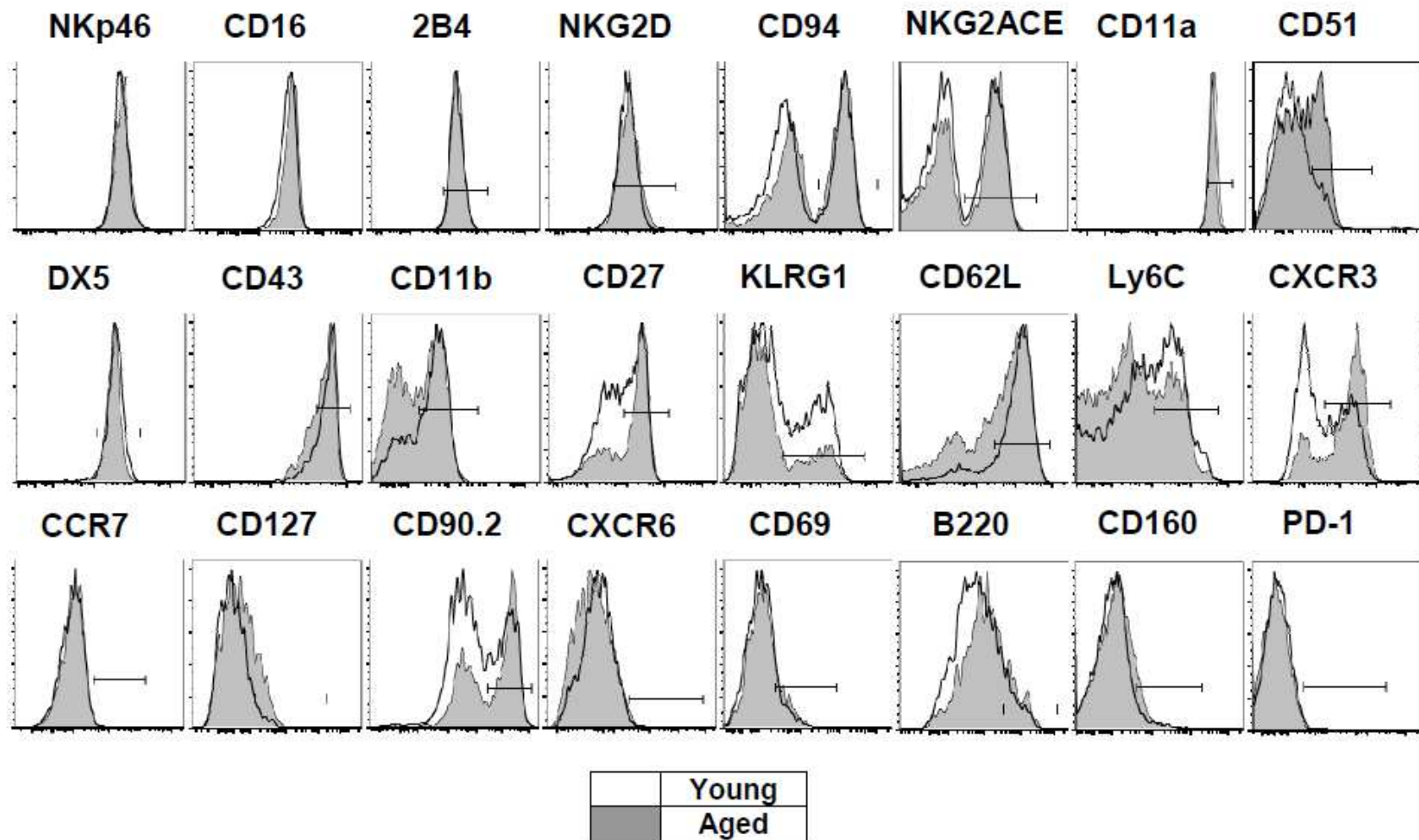


Figure 11. Phenotypic characterization of NK cells from spleens of young and aged mice. Histograms are representative of the average values for each group, $n=5-15$, depending on the marker, but n is equal in young and aged NK cells for each marker. Empty histograms represent NK cells from young mice, while shaded histograms are NK cells from aged mice.

exhausted lymphocytes under conditions of chronic infection, or on NK cells that originate extramedullary (Figure 11). Finally, we also compared the Ly49 receptor repertoire in young and aged NK cells.

NK cells from the spleens of aged mice had similar expression of NKp46, CD16, 2B4, NKG2D, CD94, c-kit, the integrins CD11a and DX5, the chemokine receptors CCR7 and CXCR6, the activation marker CD69 and the inducible costimulatory molecules CD160 and PD-1 that are increased in exhausted lymphocytes (Figure 11). However, aged NK cells had reduced expression of the markers CD11b, KLRG1, CD62L, Ly6C that are in increased levels on mature $CD11b^{+} CD27^{-}$ NK cells. Additionally, aged NK cells had increased expression of NKG2A, CD27, CXCR3, CD127, CD90.2 and B220 markers that are found mainly on immature $CD11b^{-} CD27^{+}$ NK cells, and double positive $CD11b^{+} CD27^{+}$ NK cells. In particular CD127 and CD90.2 are two markers characterizing NK cells that reside in thymus or in the liver respectively. Altogether the phenotypic differences between young and aged NK cells reflect the altered NK cell distribution in the periphery of aged mice and indicate to some developmental defects in NK cells from aged mice.

Because, the acquisition of inhibitory receptors for self MHC-I provides NK cells with licensing to function [15], we set to uncover whether there was a differential acquisition of Ly49s between young and aged NK cells. Young and aged splenic NK cells had indeed an altered distribution of Ly49 NK cell receptors, with more aged NK cells to express Ly49C and Ly49A while less aged NK cells to express the inhibitory Ly49I receptor and the activating Ly49D and Ly49H receptors (Table 2). Because coexpression of multiple inhibitory receptors can affect NK cell ability to get activated and function, we also compared the percentages of NK

Table 2. Ly49 receptor repertoire in splenic NKcells¹

<u>Ly49 Receptor</u>	<u>Young</u>	<u>Aged</u>
Ly49I	56.1 ± 0.7	47.4 ± 1.1*
Ly49C	39.5 ± 0.9	43.2 ± 1.1*
Ly49A	19.9 ± 0.4	22.7 ± 1.1*
Ly49G2	44.4 ± 0.4	43.1 ± 0.5
Ly49D	60.5 ± 1.6	55.4 ± 1.6*
Ly49H	64.5 ± 1.7	57.2 ± 0.7*

¹ Asterisks indicate statistical significant difference, t-test, n=10.

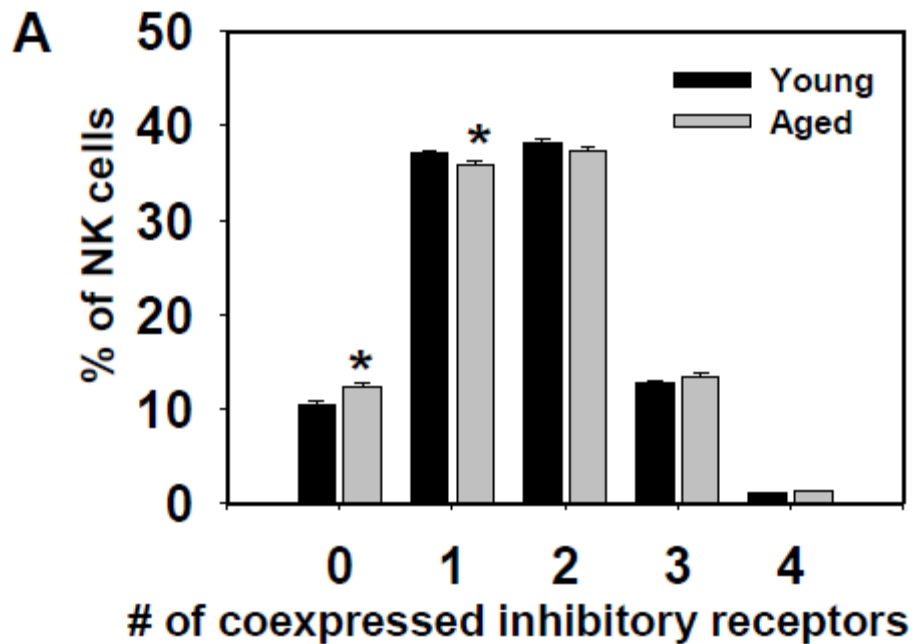


Figure 12. Analysis of Ly49 receptor repertoire on splenic NK cells from young and aged mice. (A) Percentages of NK cells that coexpress on their surface 0, 1, 2, 3 or 4 inhibitory receptors, (n=10). Asterisks (*) indicate statistically significant differences between young and aged, t-test, $p < 0.05$.

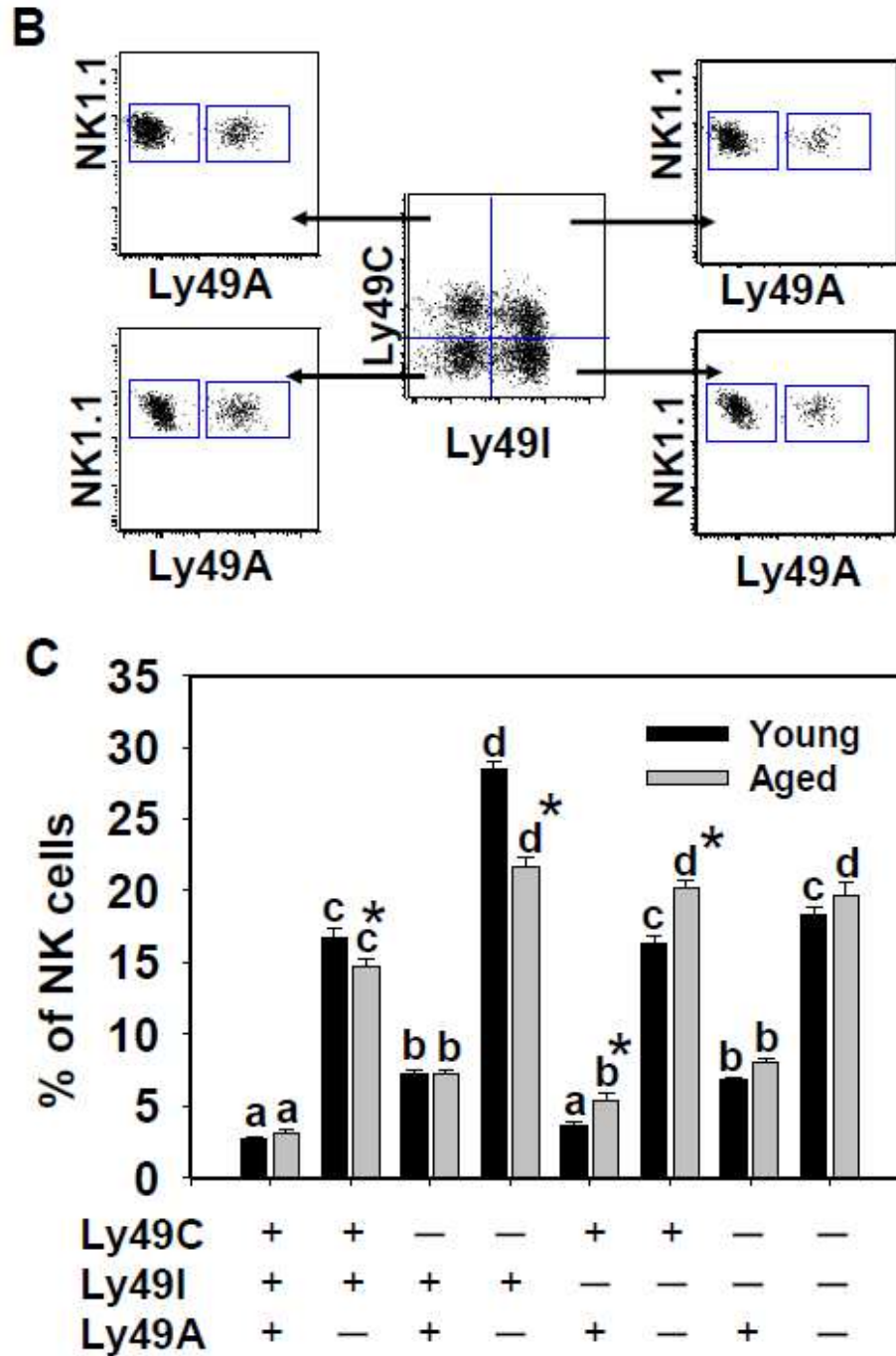


Figure 12 (cont'd). (B) Gating strategy for the analysis of NK cell subsets that express Ly49A, Ly49C and Ly49I inhibitory receptors, key players for NK cell licensing in C57BL/6 mice. (C) Percentages of NK cell subsets defined in C based on their Ly49A, Ly49C, Ly49I receptors (n=10). Two Way ANOVA was performed. Different letters indicate statistical significant difference within the same age group, while asterisks (*) indicate statistically significant difference between young and aged.

cells that co-express one, two, three or four of the inhibitory receptors Ly49A, Ly49C, Ly49I and Ly49G2. As it is shown in figure 12A, the same percentage of NK cells from young or aged NK cells coexpressed two, three or four of any combination of the examined receptors; however, there were less NK cells in aged mice that expressed only one inhibitory receptor and more NK cells that did not express any of the examined receptors. This finding indicates that co-expression of many inhibitory receptors on aged NK cells is not a factor that affects their function. However, the absence of inhibitory receptors may explain partially the functional differences based on the licensing theory.

As explained in the introduction, in C56BL/6 mice, Ly49C and to a smaller degree Ly49I interact with the self MHC class I ligand H2K^b and Ly49A has known strong reactivity with H2D^d that is absent from C57BL/6 mice. Thus, we considered NK cells that express Ly49C and/or Ly49I as licensed and NK cells that express Ly49A and none of the Ly49C/I are unlicensed. Based on the expression of Ly49C, Ly49I and Ly49A, we classified NK cells in eight subsets. Representative flow charts in figure 12B show the gating strategy for the identification of the eight different NK cell subsets expressing none, any one, any pairs, or all of the examined inhibitory receptors. Most of the NK cells were monopositive for Ly49I, or monopositive for Ly49C and a big percentage of NK cells did not had any of the examined receptors. Additionally, among the subsets that coexpressed two receptors, the Ly49C⁺/Ly49I⁺ was more abundant compared to subsets that coexpressed any of the Ly49C/I along with Ly49A or subsets that coexpressed all of those three receptors (Figure 12C). Aged NK cells had increased percentage of Ly49C monopositive and reduced percentage of Ly49I monopositive NK cells (Figure 12C). Notably, aged mice had also reduced percentage of NK cells

coexpressing Ly49C and Ly49I without Ly49A while they had increased percentage of NK cells coexpressing Ly49C and Ly49A (Figure 12C). Altogether, aged NK cells have an altered Ly49 receptor repertoire, with preferential increased expression of Ly49C but decreased expression of Ly49I.

Impaired function of aged NK cells is not related to licensing

To investigate whether the difference in acquisition of Ly49 receptors results in impaired function for the aged NK cells, we examined the eight NK cell subsets defined in figure 12C for their ability to produce IFN- γ upon two stimulation conditions: plate-bound anti-NK1.1 antibody and co-culture with YAC-1 target cells. Both with anti-NK1.1 activation and YAC-1 stimulation, NK cells that expressed Ly49C or Ly49I alone, and Ly49C and Ly49I without Ly49A produced higher IFN- γ (Figure 13A and 13B), verifying the role of these receptors for licensing and indicating the inhibitory role of the Ly49A presence. NK cells that don't have any of these receptors still functioned better than those that had Ly49A, suggesting that other, yet unidentified inhibitory receptors may confer licensing in C57BL/6 mice. Most importantly, we observed that independently of Ly49C or Ly49I acquisition, aged licensed NK cells were producing significantly less IFN- γ than young licensed NK cells. Thus, reduced function of aged NK cells cannot be solely explained by the acquisition of Ly49 receptors, and other intrinsic mechanisms may be responsible.

We then, investigated the maturation status based on the CD11b/CD27 markers on the subsets that are licensed. We excluded NK cells that had expression of Ly49A, because they showed reduced IFN- γ production in Figure 13B. In aged mice, all of these subsets had increased percentages of immature CD11b⁻ CD27⁺ and double positive CD11b⁺ CD27⁺ NK cells and

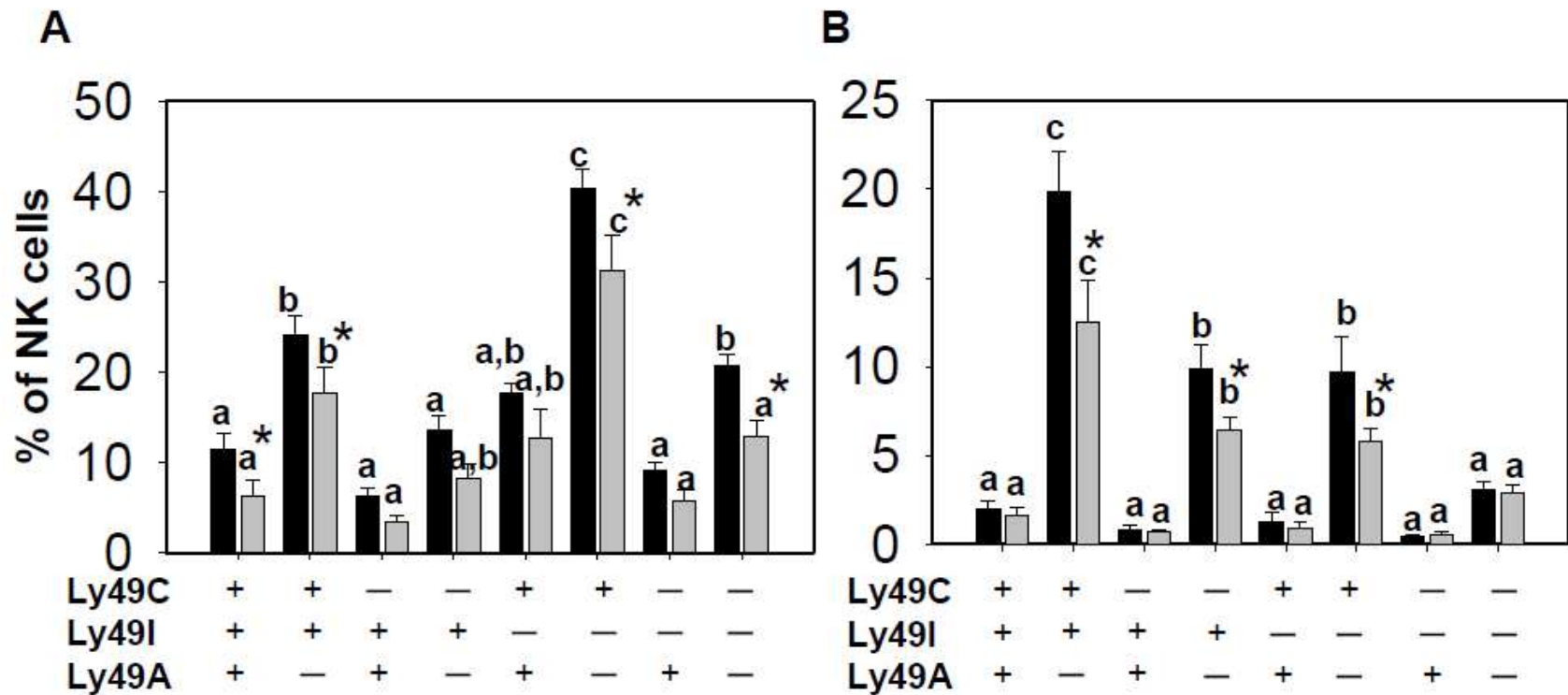
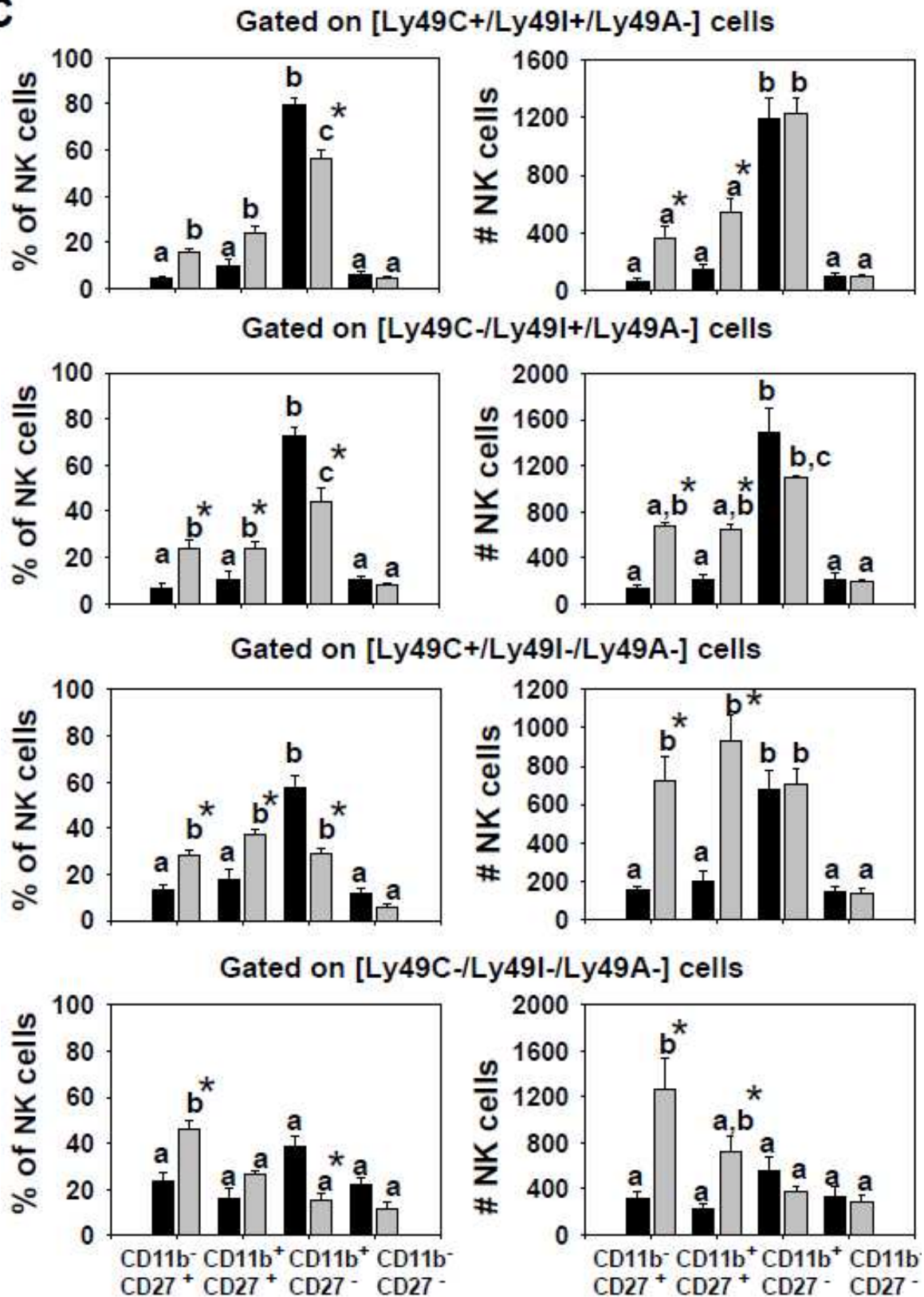


Figure 13. Comparison of IFN- γ production from licensed and unlicensed NK cell subsets in young and aged mice. NK cells were characterized as licensed or unlicensed based on their expression of Ly49C, Ly49I, and Ly49A according Figure 12C. (A) Induction of IFN- γ production by plate bound anti-NK1.1 incubation for 7 hours and (B) induction of IFN- γ production by cocultures with YAC-1 cells for 4 hours (n=5). Two Way ANOVA was performed. Different letters indicate statistically significant differences within the same age group, while asterisks (*) indicate statistically significant differences between young and aged. (C) Distribution of CD11b/CD27 subsets among NK cells characterized as licensed and unlicensed by their expression of Ly49C, Ly49I and Ly49A on their surface (n=10). Two Way ANOVA was performed. Different letters indicate statistically significant differences within the same age group, while asterisks (*) indicate statistically significant differences between young and aged.

Figure 13 (cont'd)

C



reduced percentages of mature $CD11b^{+} CD27^{-}$ NK cells (Figure 13C). To compare if indeed aged mice have increased immature NK cells that are licensed, we compared the absolute numbers of CD11b/CD27 NK cells belonging to the NK cell subsets defined based on their Ly49C/I/A expression (Figure 12C); by doing this, it was revealed that aged mice had indeed more numbers of immature NK cells that were licensed. In contrast, the mature $CD11b^{+} CD27^{-}$ NK cells in both young and aged had similar expression of the inhibitory markers that conferred licensing. Besides, when we co-stained spleen NK cells with more Ly49s and CD11b, we observed that immature $CD11b^{-}$ NK cells from aged mice had increased expression of all examined Ly49s while the expression of Ly49s on mature $CD11b^{+}$ NK cells was either unchanged or reduced (Figure 14). Overall, these data indicate that licensing takes place earlier during development and before the functional maturation of NK cells in the bone marrow, and that in aged mice, although NK cells have acquired the appropriate Ly49s and went through the licensing process normally, they could not functionally mature.

4.5 Discussion

Advancing age is associated with increased incidence of cancers, heart diseases, diabetes, Alzheimer's disease and increased susceptibility to infectious agents that significantly impacts their survival. One factor that contributes to increased susceptibility to these diseases is dysregulation of the immune system, or immunosenescence, as it is called. Studies from our laboratory have indicated that lymphocyte proliferation and cytokine production is reduced, B cell ability to produce antibodies is defective and CD8 T cell cytotoxicity is significantly impaired [9, 168, 174, 175]. Previous studies from our laboratory implied that age-related defects of NK

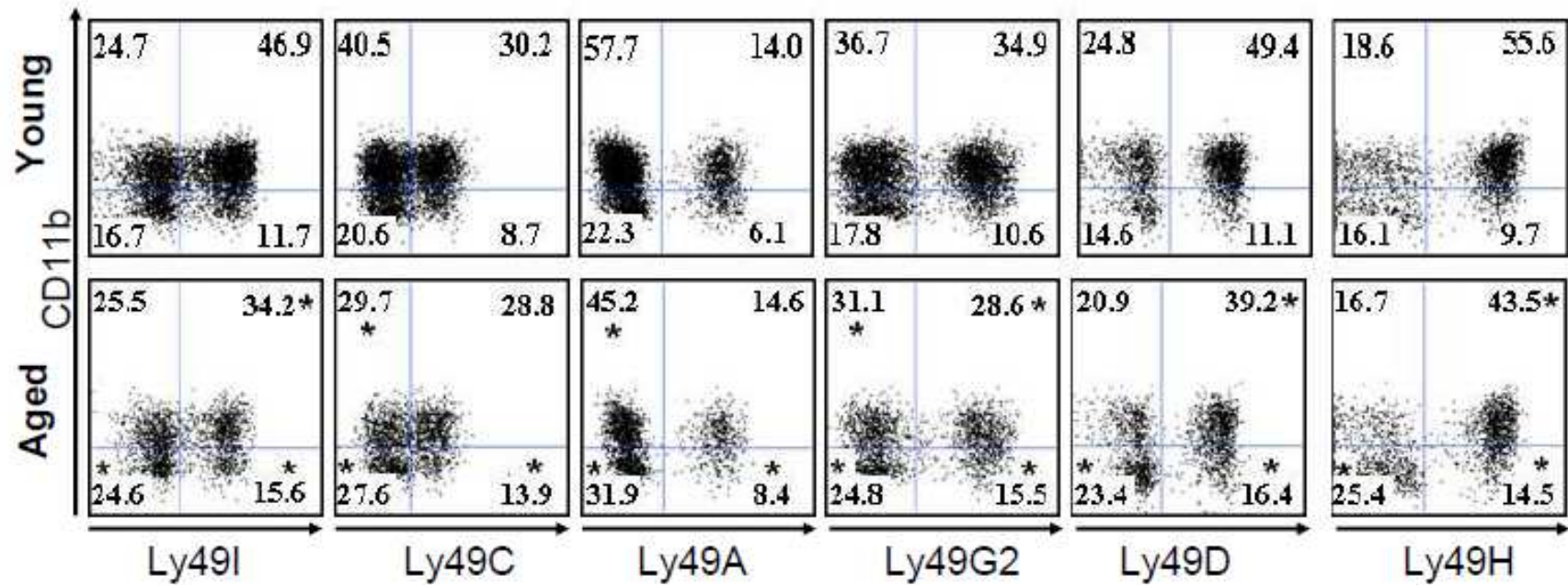


Figure 14. Distribution of Ly49 receptors in immature and mature NK cells. Flow cytometry dot plots of young and aged mice are representative of the average values for each group. Data represent the average value for each subset (n=10). Asterisks (*) indicate statistically significant differences among young and aged groups, t-test, $p < 0.05$.

cell function may also exist similarly to the rest lymphocytes. Aged mice have reduced infiltration of NK cells in their lungs and significant reduction of their activity after influenza infection [11, 13]. Recently, we also showed aged mice have reduced mature NK cells in their spleens, and reduced function after influenza infection [149]. The studies presented here elaborate more on NK cell distribution, maturation and development and present data that enhance our understanding of NK cell biology in advanced age.

In this study we showed that aged mice had reduced NK cells in spleens, lungs, blood and liver, while NK cells accumulated in the bone marrow, and slightly in the lymph nodes. Many studies before this have suggested that aged mice have reduced splenic NK cells [51-53, 149] but not many have investigated the effect of aging on NK cell distribution in other tissues. In some studies, no reduction on spleen NK cells was reported [13, 48-50], but these studies used non-physiological models of aging, or non specific markers and techniques for the identification of NK cells. Finally, in agreement with our results, it was shown before that aged mice had increased frequencies of NK cells in their bone marrow [176]. For humans, it is generally accepted that aging results in maintenance of a high percentage of NK cells in circulation [55-60]. However, human studies are limited to peripheral blood samples and it is not known whether aging affects distribution of NK cells within tissues.

The finding that NK cells are reduced in peripheral tissues with the exception of the lymph nodes while the numbers of NK cells in the bone marrow are higher suggests several possibilities, such as decreased exit from the bone marrow, reduced generation of NK cells, decreased survival in the peripheral tissues and preferential accumulation in certain sites, such as the lymph nodes. In fact it was reported that aged NK cells could not migrate efficiently into the lymph nodes after viral infection [52], indicating that preferential accumulation in lymph nodes

may not deplete NK cells from other peripheral tissues; these studies suggest though, that there are age-related changes in the migration of NK cells. The possibility that aged NK cells are retained in the bone marrow is supported by the findings presented in this study: aged NK cells have higher percentage of NK cells in their bone marrow, but they are primarily immature and therefore may not have the appropriate receptors to migrate to the periphery. The possibility that aged mice cannot generate mature NK cells is also supported by our finding that aged mice had significantly reduced Ki67⁺ NK cells in their bone marrow. However, there is previous evidence that aged NK cells have intrinsic, impaired ability to proliferate in response to IL-2 [55], while *in vivo* labeling experiments in humans [61] and immunoperoxidase labeling techniques in aged mice [51] concluded that total NK cell production rates are impaired with aging. Finally in this study, we present data that support the possibility that aged NK cells cannot survive in peripheral tissues: aged NK cells have reduced Bcl-2/Bim ratio, and specifically the aged mature NK cells have increased expression of Bim, a proapoptotic molecule related to reduced survival. Overall, we have shown that reduced numbers of NK cells in aged mice is the result of many factors, including impaired maturation in the bone marrow, reduced generation of NK cells and decreased survival in the peripheral tissues.

An important finding of this study is that aged mice have reduced percentages of mature, CD11b⁺ CD27⁻ NK cells in all examined tissues indicating that there is a block in the terminal maturation of NK cells in aged mice. In agreement with our results, recently was also reported that aged mice have reduced CD11b⁺ CD27⁻ NK cell numbers in circulation [52]. In further support of this, we also have shown that aged NK cells had higher expression of markers associated with immaturity (NKG2A, CD51, CD27) or extramedullar development (CD127,

CD90.2) and reduced expression of markers associated with maturity (CD11b, CD43, KLRG1, Ly6C). In fact, the decreased percentages of mature NK cells in spleens of aged mice was also reported almost three decades ago, when the lymphokine activated killer cell (LAK) activity was compared in young and aged mice. The majority of cytotoxic precursors in LAK cells from young mice are considered to be mature NK cells and aged mice had almost 4 fold reduced frequency of these precursors determined by limiting dilution assay as the ability of splenocytes to generate natural cytotoxicity in the presence of IL-2 [53]. Additionally, Kawakami et al.(1987) showed that in aged mice, immature CD90.2⁺ (Thy1) cells showed LAK activity while in young mice LAK activity was mostly restricted to mature NK cells [115]. They further elaborated to suggest that freshly isolated splenocytes from aged mice contained significant numbers of inactive pre-NK cells which could differentiate into LAK cells and compensated for the lack of LAK activity from mature NK cells in the spleens of aged mice. The same authors observed that LAK activity from bone marrow was similar in young and aged mice, because bone marrow is comprised mostly of immature NK cells [121]. Thus, it is clear that aged mice have a reduction in their terminally mature NK cells that have the capacity for function.

In contrast to murine studies, studies in humans show that aging brings a shift to NK cell subset diversity, with the accumulation of terminally differentiated NK cells. Findings from murine studies cannot be interpreted directly in humans because the classification NK cell subsets is different in the two species and because human aging is very different than that of laboratory animals that live their life in a minimized disease environment. In humans, NK cells are divided in two main functional subsets: CD56^{bright} CD16⁻ NK cells, known to be potent cytokine producers and found in higher percentages in secondary lymphoid tissues, and CD56^{dim}

CD16⁺ NK cells, with potent cytotoxic capacity. There is evidence suggesting that CD56^{bright} CD16⁻ NK cells can give rise to CD56^{dim} CD16⁺ [76, 77] and recently, it was proposed that CD56^{bright} NK cells become first CD56^{bright} CD16⁺ cells and then CD56^{dim} [79]. In humans, aging results in an overall reduction in the CD56^{bright} NK cells and an increase in CD56^{dim} NK cells in peripheral blood [57, 80-82]. Furthermore, among the CD56^{bright} NK cell subset, more CD56^{bright} NK cells have increased expression of CD16 in the elderly compared to young [79]. Yet, the CD56^{dim} subset is composed of NK cells in various differentiation stages [68, 84, 85] while others showed that they are composed primarily from CD11b⁺ CD27⁻ NK cells [86]. The specific reduction of CD56^{bright} NK cells in blood circulation of elderly could be attributed to altered trafficking into the secondary lymphoid tissues, where they preferentially home, resulting in their depletion from the blood. Yet, no studies have addressed this possibility as well as the CD11b/CD27 subset classification of NK cells in the elderly.

In spite of the clear differences on human and murine NK cells, murine models have been important in understanding the mechanisms of immune cell development, merely because of the advantage that we can study NK cell homeostasis in a variety of tissues that are not accessible in humans. To understand the development of NK cells in aged mice better, we analyzed the developmental stages of NK cells in the bone marrow according to the model proposed by Kim et al. (2002) [14]. Our findings revealed that there was no age-related defect during the early developmental stages when NK cells are still considered immature. Aged mice showed impairment only during the transition from *Stage IV* to *Stage V* where upregulation of CD11b

and CD43 takes place. At this stage, a significant expansion of immature NK cells usually occurs, and we revealed that its level is reduced in aged mice. Thus, we suggest that reduced proliferation in the bone marrow of aged mice affects the terminal maturation of NK cells. In contrast to bone marrow, proliferation of NK cells in the spleen was the same in both young and aged mice, similar to findings from peripheral blood NK cells in young and elderly humans [62]. For humans, it has been proposed that NK cells remain in the bone marrow for a significant time - almost 10 days - after their expansion, before they go to the peripheral blood to mature [61]. Thus, it is possible that bone marrow signals during that period affect NK cell maturation. Actually, the defective support of aged bone marrow stromal cells in the development of other lymphocytes has been observed before [177]. Although impaired proliferation in the bone marrow can partially explain the reduced NK cell maturation, studies on the role of certain transcription factors regulating NK cell development and adoptive transfer experiments could give more insight into the specific mechanisms.

Defects in the bone marrow could result in impaired development of NK cells in aged mice, but the peripheral microenvironment likewise affects their homeostasis. In this study, we provided data that support reduced survival potential of NK cells in the aged spleens. Annexin V staining on freshly isolated NK cells from spleen showed only a trend for increased apoptosis in agreement with human studies, where NK cells from peripheral blood of elderly people had also a non-statistical but higher trend for increased apoptosis as assessed by the TUNEL assay [62]. In contrast, when we incubated splenocytes with YAC-1 target cells, Annexin V staining revealed that aged NK cells had a higher apoptotic potential than young NK cells. Similarly, Plett et al. (2000b) showed that aged NK cells undergo higher apoptosis upon activation with IFN- α/β and YAC-1 cells, related to increased expression of IFN α/β receptor and CD95 in aged

NK cells [120]. A better indication of the potential for apoptosis is the ratio of Bcl-2/Bim, which we showed was significantly reduced in spleen NK cells from aged mice. Although immature NK cells in general have increased expression of the anti-apoptotic Bcl-2 there were no age-related differences in the expression of Bcl-2. However, the increased expression of Bim on the most mature NK cells in the aged mice indicates their susceptibility to apoptotic signals and their reduced survival in the aged microenvironment. Whether this result is intrinsic to aged NK cells or a consequence of the inflammatory microenvironment in aged mice needs to be established. Overall, the reduced survival of mature NK cells in the peripheral tissues of aged mice in combination with the reduced generation of mature NK cells in the bone marrow can explain the reduction of circulating mature NK cells observed in the periphery of aged mice.

As mentioned before, our characterization of the altered NK cell phenotype with aging supported the idea that aged mice have higher expression of markers associated with immaturity, extramedullar development and activation, while they had less markers associated with maturity. Aged mice had increased expression of NKG2A, CD51, CD62L, CXCR3, CD127, CD90.2 and B220 and reduced expression of CD43, CD11b, KLRG1 and Ly6C. Furthermore, aged NK cells did not show differential expression in NKp46, CD16, 2B4, and NKG2D activating receptors that are acquired early during development, nor in CD11a (LFA-1), which plays an important role in the cytotoxic process. Finally, aged NK cells had similar expression of NK1.1, DX5, CD94, CD69 and CCR7. All the above mentioned markers can change upon activation with various cytokines or changes in the populations of other cell types, thus the altered subset distribution of NK cells needs to be examined in correlation with other populations that could affect their homeostasis.

Other important cell surface receptors that regulate NK cell activation are the inhibitory

and activating Ly49 receptors. To our knowledge, the data presented in this study are the first to characterize the Ly49 receptor repertoire in aged mice. We observed that aged mice did not have increased coexpression of more than two inhibitory receptors on the same NK cell, but the percentage of NK cells that do not express any of these receptors was higher, and the percentage of NK cells that expressed only one of them was reduced. The latter may simply reflect the significant reduction of Ly49I observed in the aged NK cells, because aged C57BL/6 mice had fewer NK cells expressing Ly49I, but more NK cells that expressed Ly49C and Ly49A. The differential expression of Ly49C and Ly49I may indicate altered expression of MHC-I molecules in the bone marrow, and therefore altered interactions of these inhibitory receptors with their ligands during development. The increased expression of Ly49A may be explained by defects during NK cell development, because Ly49A is among the first to be expressed, before Ly49C/I, G2 and F [72] and its expression is under the control of a separate transcription factor that doesn't affect the expression of other Ly49s [73]. Finally, we showed that fewer NK cells from aged mice express the activating receptors Ly49D and Ly49H. These receptors are acquired later during development in the bone marrow, after the acquisition of the inhibitory receptors [74]. Altogether, it is possible that the differences observed among young and aged NK cells reflect defective maturation of NK cells in aged mice.

We showed that immature NK cells from aged mice had increased expression of all examined Ly49s, while mature NK cells had either unchanged or reduced expression of Ly49s. Similar results have been shown for NK cells in elderly humans where increased expression of KIRs was observed amongst the CD56^{bright} and not the CD56^{dim} NK cell subset [57]. Absence of MHC-I molecules was shown to result in higher percentages of immature CD11b⁻ CD27⁺ NK cells with increased expression of Ly49C/I [75]. Yet, we do not know whether aging affects

MHC-I expression in the bone marrow and our data point towards a retention of NK cells with a normal Ly49 receptor repertoire in the CD11b negative stage, due to reduced expansion in the bone marrow. The factors regulating upregulation of CD11b are not very well understood, but we propose that signals during the proliferation stage are essential. Finally, we observed that NK cells from aged mice that have acquired Ly49C or Ly49I and lacked Ly49A were not as efficient as their counterparts from young mice to produce IFN- γ upon stimulation with anti-NK1.1 antibody, or co-culture with YAC-1 cells. This defect was associated with increased percentages of immature NK cells that had acquired their Ly49 receptors but were CD11b negative. Thus, we suggest that reduced NK cell function in aged mice is more related to defective terminal maturation rather than the acquisition of the appropriate Ly49 receptors.

What cause the impaired NK cell maturation in aged mice is not known. In this study we suggest that reduced expansion in the aged bone marrow may partly explain the phenomenon. However, immature NK cells are also found in the periphery. Richards et al. (2006) showed tumor growth in the periphery resulted in higher percentages of CD11b⁻ NK cells in the bone marrow and a blockage in the transition of *Stage IV* to *Stage V* of NK cell development similarly to what we observed in aged mice [178]. Additionally, when mice were splenectomized, the percentage of CD11b⁺ CD27⁻ was dramatically reduced in the circulation of these mice suggesting the spleen as an important site for terminal maturation of NK cells [179]. Further studies on the effect of specific cell types in the terminal maturation of NK cells also revealed that NK cell maturation is not dependent on the presence of other lymphocytes or dendritic cells, but rather on cell-to-cell contacts with monocytes residing in the red pulp of the spleen [179] and the presence of neutrophils [180].

Overall, this is the first study to characterize in detail the murine aged NK cells and

provide insight into their developmental defects. Future studies need to investigate the interactions of NK cells with other cell types in aged mice, and to provide mechanistic data for the observed defects.

4.6 Conclusions

Previously we showed that aged mice have less NK cells in their lungs and spleens early after influenza infection, with impaired function and altered phenotype [149]. In this study we extended our characterization of NK cells in uninfected aged mice. We showed that aged mice have reduced NK cells in most peripheral tissues with the exception of the bone marrow, which is attributed to a specific reduction of $CD11b^{+} CD27^{-}$, the terminally mature subset of NK cells. A detailed analysis of the stages of NK cell development in the bone marrow revealed that NK cells go normally through the first stages of development, but because of reduced expansion during terminal maturation, the percentage of mature NK cells ready to traffic to the periphery is significantly reduced. We further suggest that the reduced presence of mature NK cells in peripheral tissues may be related to their reduced potential for survival. Finally, we showed that impaired NK cell function is not related as much to their altered Ly49 receptor repertoire, but rather to their defective terminal maturation, because aged licensed NK cells had intrinsically defective capacity to produce IFN- γ upon activation. Furthermore, aged licensed NK cells were composed of more immature NK cells. Overall we showed that aging affects the numbers, the function and the phenotype of NK cells.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

In the studies presented here, we have analyzed the effect of aging on natural killer cell responses to influenza infection and their homeostasis and development in basal conditions. At the early stages of a pulmonary infection, lung resident immune cells are crucial for the development of a strong antiviral response; activation and trafficking of peripheral immune cells into the lung requires some time. We have showed that aged mice have less NK cells in their lungs (and spleens) before and early after influenza infection, with impaired function and altered phenotype, something that could explain their overall susceptibility to influenza infection. However, our studies have not provided evidence about the reliance of aged mice on strong NK cells responses to fight influenza infection. Future studies need to establish the link between a good NK cell response and resistance to influenza infection in aged mice. These studies can be done with depletion of NK cells from young and aged mice and measuring general survival and susceptibility indicators, as well as the effect of the NK cell absent in the adaptive immunity of aged mice. Another approach will be to administrate agents known to induce NK cell activity in aged mice and measure their susceptibility to infection. Such agents can be either pharmacological, such as PolyI:C and type I IFNs, or neutraceuticals known to enhance NK cell activity, such as the administration of active-hexose correlated compound (AHCC) that we evaluated in the context of influenza vaccination in healthy adults (Appendix B). Finally, it will be interested to adoptively transfer young NK cells in aged NK-depleted mice and evaluate their susceptibility to influenza infection.

In our studies, we observed that aged mice show impaired function depending on the agents used for stimulation: when NK cells stimulated by cell-contact dependent mechanisms with target cells, aged NK cells from both lungs and spleens of infected mice showed reduced degranulation and IFN- γ production. These data indicate that the establishment of a successful immunological synapse with target cells may be impaired in aged mice and *in vitro* studies to examine this defect with isolated NK cells incubated with target cells will provide more insight into the actual mechanisms. We also observed that during influenza infection, spontaneous degranulation was preserved in the aged mice, and only IFN- γ production was severely impaired. Because it is not known how exactly NK cells are activated and function during influenza infection, it is possibly that they get activated by the inflammatory environment and are ready to degranulate, but assaying degranulation alone cannot tell us if they actually are able to kill viral infected cells. Thus, more studies investigating the killing mechanisms of aged NK cells are required.

Moreover, in the influenza infection studies, we observed that aged NK cells showed a constant reduced ability to produce IFN- γ and thus we focused more to characterize this defect. We showed that when the same amount of cytokines or anti-NKp46 antibody was used to stimulate NK cells, aged mice showed reduced IFN- γ production, indicating intrinsic to NK cell defects. In these studies, we only assessed the ability of NK cells to produce only IFN- γ and not other cytokines, or chemokines, it will be interesting to investigate whether aging alters NK cell secretory phenotype and promotes for example the secretion of TNF- α instead of IFN- γ . Furthermore, there is a great need to evaluate the intracellular signaling events that result in reduced cytokine production with the engagement of different activating receptors in aged mice.

Finally, although we attempted to characterize the lung microenvironment by real time PCR and PCR array (Appendix A) and we found no significant differences among young and aged mice, it is important to acknowledge that mRNA expression does not necessarily translates to protein production and presence in the lungs of infected mice. A more thorough investigation of the availability of cytokines in the lung microenvironment of more mice is necessary to give us definite answers about differences in the lung microenvironment. Besides, adoptive transfer experiments, where young NK cells are introduced in aged hosts, or aged NK cells in young hosts, will delineate whether the aged microenvironment or intrinsic defects on the NK cells are the implicating factors for the observed reduced activation of aged NK cells during influenza infection.

One of the most important findings of our work was that aged NK cells consist of less terminally mature $CD11b^{+}CD27^{-}$ NK cells. We started experiments to investigate the homeostasis and distribution of $CD11b/CD27$ subsets in various tissues of uninfected mice. We showed that aged mice have reduced NK cells in most peripheral tissues with exception the bone marrow. Thus, we examined if there is a defective development of NK cells in the bone marrow, or whether mature NK cells were dying due to apoptosis in the periphery. A detailed analysis of the stages of NK cell development in the bone marrow revealed that even though aged NK cells go normally through the first stages of development they showed a block in the terminal maturation stages with accumulation of NK cells that were in the immature stage. Because aged mice showed reduced expansion in the bone marrow, but not increased cell death, we suggested that impaired proliferation might affect the production of mature NK cells in the bone marrow. We further showed that the $CD11b^{+}CD27^{-}$ NK cells in the aged peripheral tissues had reduced survival. The reduction of $CD11b^{+}CD27^{-}$ NK cell subset in the aged mice, can affect their

function, because we showed that these mature NK cells consist the majority of functioning NK cells upon stimulation. These studies were the first to report that aged mice had a maturational defect that could be associated with the observed reduced function. Adoptive transfer experiments in which bone marrow cells from aged or young mice will be transferred to irradiated young or aged host mice would give us more answers about the *in vivo* defects of maturation and proliferation of aged NK progenitor cells, and their homeostasis in peripheral tissues. Furthermore, these experiments will also indicate whether the aged microenvironment can sustain the survival of mature NK cells in the periphery.

Finally, we showed, that in aged mice NK cells that expressed the Ly49 receptors that confer licensing in the C57BL/6 mice had reduced function, which was related to their defective terminal maturation. Licensed NK cells in the aged mice were composed of more immature NK cells. Actually, immature NK cells from aged mice had increased expression of all the Ly49 receptors examined, supporting that there is an accumulation of immature NK cells that have acquired normally their Ly49s but could not transmit to a more mature stage. Functional studies of sorted immature NK cells from young and aged mice, would provide evidence about whether the increase of Ly49s in aged immature NK cells is a compensatory mechanism to increase their functional capacity in the absence of the most mature NK cells, or whether reflects the block in terminal maturation in the bone marrow.

Overall, in the studies presented here, we showed that aging affects the numbers, the function and phenotype of NK cells. We mainly identified that aged mice have a reduced mature phenotype, related to a block in their maturation in the bone marrow and a specific reduced survival potential of the mature NK cells in the periphery. This defective maturation of aged NK cells can have important complications in their functional capacity and their responses during the

early stages of influenza infection, when viral replication takes place and the induction of a strong antiviral immune response to resolve the infection quickly is required.

APPENDICES

APPENDIX A

ASSESSMENT OF THE LUNG MICROENVIRONMENT IN YOUNG AND AGED INFLUENZA INFECTED MICE

Real time PCR

We performed Real Time PCR for various cytokines that are known to affect NK cell activation in the lungs of young and aged infected mice (Table 2). RNA was isolated from lungs collected 2 days post infection with 75 HAU influenza virus from young and aged mice. We observed that there was no statistical significant difference on the mRNA expression of all examined cytokines. Even though, these results may indicate that in both young and aged mice these cytokines are expressed the same, we do not have data to support that their protein levels or their function is the same in aged mice after influenza infection.

Table 3. Relative mRNA expression of various cytokines in the lungs of infected young and aged mice

	Young	Aged	<i>t-test</i>
IFN- α	8.69 \pm 2.5 ¹	6.76 \pm 1.8	<i>p</i> = 0.942
IFN- β	221.83 \pm 64	169.02 \pm 47	<i>p</i> = 0.518
IL-12 p40	1.37 \pm 0.4	0.81 \pm 0.2	<i>p</i> = 0.563
IL-12 p35	2.19 \pm 0.4	4.53 \pm 1.4	<i>p</i> = 0.341
IL-18	0.75 \pm 0.1	0.80 \pm 0.1	<i>p</i> = 0.644
IL-15	1.31 \pm 0.1	1.18 \pm 0.1	<i>p</i> = 0.418
IFN- γ	2.73 \pm 0.8	2.42 \pm 0.9	<i>p</i> = 0.802
TNF- α	14.26 \pm 4.2	18.01 \pm 5.0	<i>p</i> = 0.317

¹ data are means \pm SEM, n=5 per group

PCR array

We further performed a PCR array of mouse inflammatory cytokines and receptors (SABiosciences, PAMM-011C) to get a more complete picture of the lung microenvironment in young and aged mice. The genes included in the PCR array are described in table 3.

Table 4. List of genes tested in the PCR array

Chemokines	CCL1, CCL11, CCL12, CCL17, CCL19, CCL2, CCL20, CCL22, CCL24, CCL25, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CX3CL1, CXCL1, CXCL10, CXCL11, CXCL12 (SDF-1), CXCL13, CXCL15, CXCL4 (PF4), CXCL5, CXCL9, IL-13
Chemokine Receptors	CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR3, CXCR2, XCR1
Cytokines	IFN- γ , IL-10, IL-11, IL-13, IL-15, IL-16, IL-17b, IL-18, IL-1a, IL-1b, IL-1f6, IL-1f8, IL-20, IL-3, IL-4, CD11b (ITGAM), CD18 (ITGB2), LTA, LTB, MIF, AIMP1, SPP1, TGFB1, TNF, CD40lg
Cytokine Receptors	IFN- γ , IL-10R α , IL-10Rb, IL-13, IL-13Ra1, IL-1R1, IL-1R2, IL-2Rb, IL-2Rg, IL-5Ra, IL-6Ra, IL-6st, TNFRsf1a (TNFR1), Tnfrsf1b (TNFR2)
Other genes	ABCF1, BCL-6, CXCR5, C-3, CASP-1, CRP, IL-1R1, CXCR2, TOLLIP

Because this experiment was our first attempt to perform a PCR array, we only used one young and one aged mouse. The criteria used to choose our samples were the following: **(1)** Samples were isolated from lungs collected 2 days post infection. **(2)** Samples were from mice of the same experiment, to account for differences on the magnitude of infection among different experiments. **(3)** Samples were from mice that lost weight during influenza infection, as an indication for a successful infection. **(4)** Samples were from mice that had approximately the same lung viral titers measured by real time PCR to account for the magnitude of the infection. **(5)** Samples gave good RNA yield during RNA isolation and the RNA quality was according the requirements described in the PCR array protocol.

Based on the above criteria, we chose to use the isolated RNA from the lungs of one young and one aged mouse, whose response to influenza infection is described in table 4.

Table 5. Characteristics of the infection in the selected mice

	Viral copies	Weight loss	IFN-γ + lung NK cells
Young mouse	367,530	10.55 %	7.80%
Aged mouse	441,100	7.72 %	1.50% *

Asterisk (*) indicate statistically significant difference

The expression of the genes is shown in the two following scatter plots (Figure 15 and Figure 16). Figure 15 shows the genes that were 1.7 times higher expressed while figure 16 shows the genes that were 1.7 times lowered expressed in the aged compared to young lung. Tables 5-11 list the genes belonging to cytokines, chemokines or their receptors that were more than 1.7 times different in aged lung compared to young lung. A brief description of their function is also shown.

Overall, we observed that in the lung of the chosen aged mouse the expression of proinflammatory cytokines such as IL-1 and TNF- α and the expression of IFN- γ , IL-10 and IL-15 was higher compared to the lung of the chosen young mouse (Table 5). Furthermore, the aged lung microenvironment had higher expression of chemokines of the Macrophage Inflammatory Protein family (Table 7) and reduced expression of some of the chemokines known as eotaxins (Table 8). However, it is not known how these chemokines affect NK cell activation *in vivo*. It was interesting to see high IFN- γ mRNA in the lungs, at the same time that NK cells from that particular mouse were producing very little IFN- γ (Table 4). These data indicate that other than NK cells may contribute to early IFN- γ production in the lungs of aged mice, something that needs to be further evaluated. In the following tables it is reported the expression of genes that were more than 1.7 times different in the aged lung compared to the young, and a short description of the functions of each gene is provided.

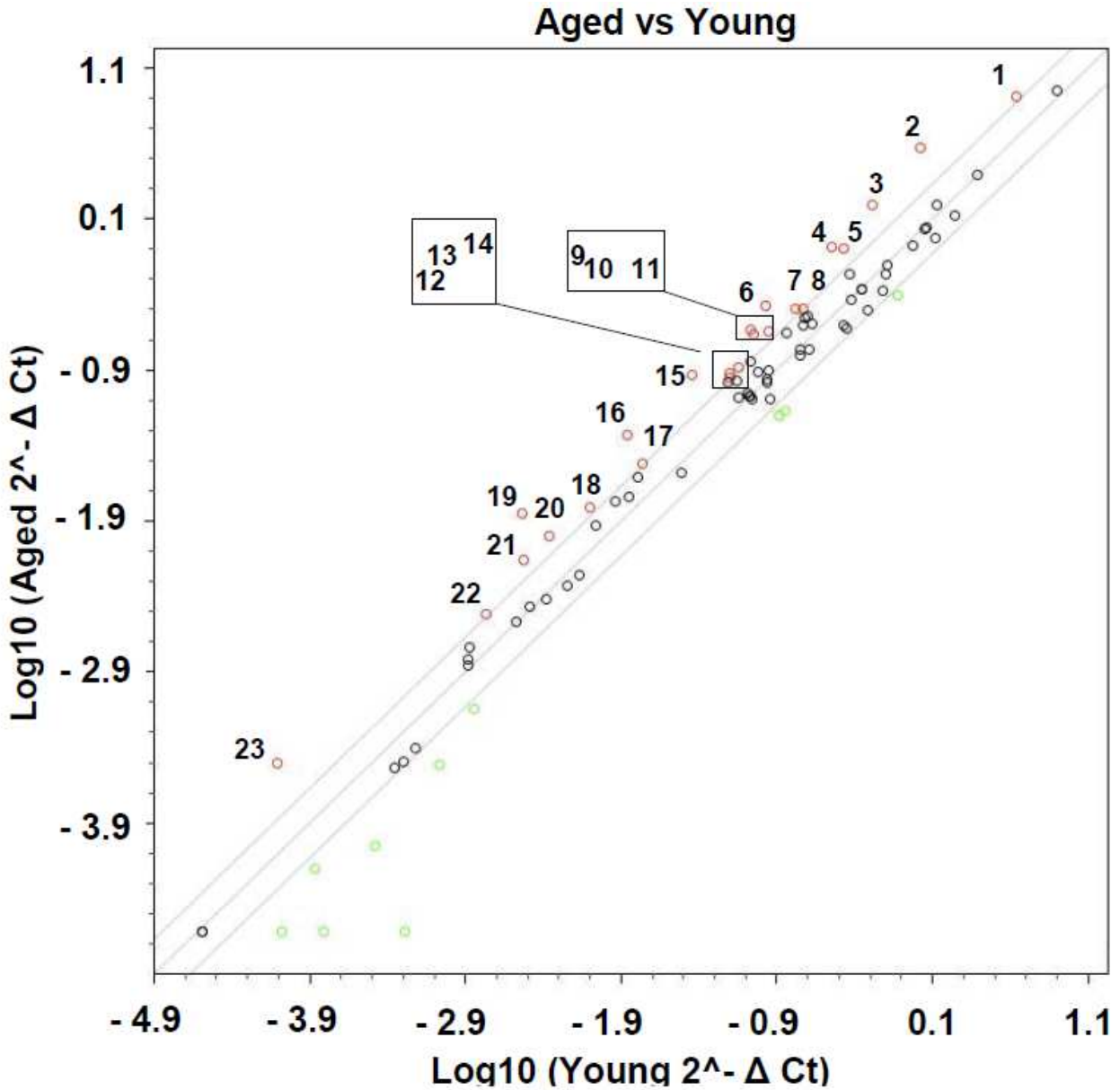


Figure 15. Scatter plot of genes that were upregulated in aged lung in comparison to young lung. (1) CXCL10, (2) IL1 β , (3) IL1r2, (4) CCL4, (5) CCR1, (6) CXCL9, (7) CXCR2, (8) CCL6, (9) CCL3, (10) TNFRsf1b, (11) CXCL9, (12) Casp1, (13) IL-15, (14) TNF, (15) IL10R α , (16) CXCL11, (17) CCL22, (18) IL-10, (19) IFN- γ , (20) CXCR3, (21) CXCR5, (22) CCR8, (23) CCL1.

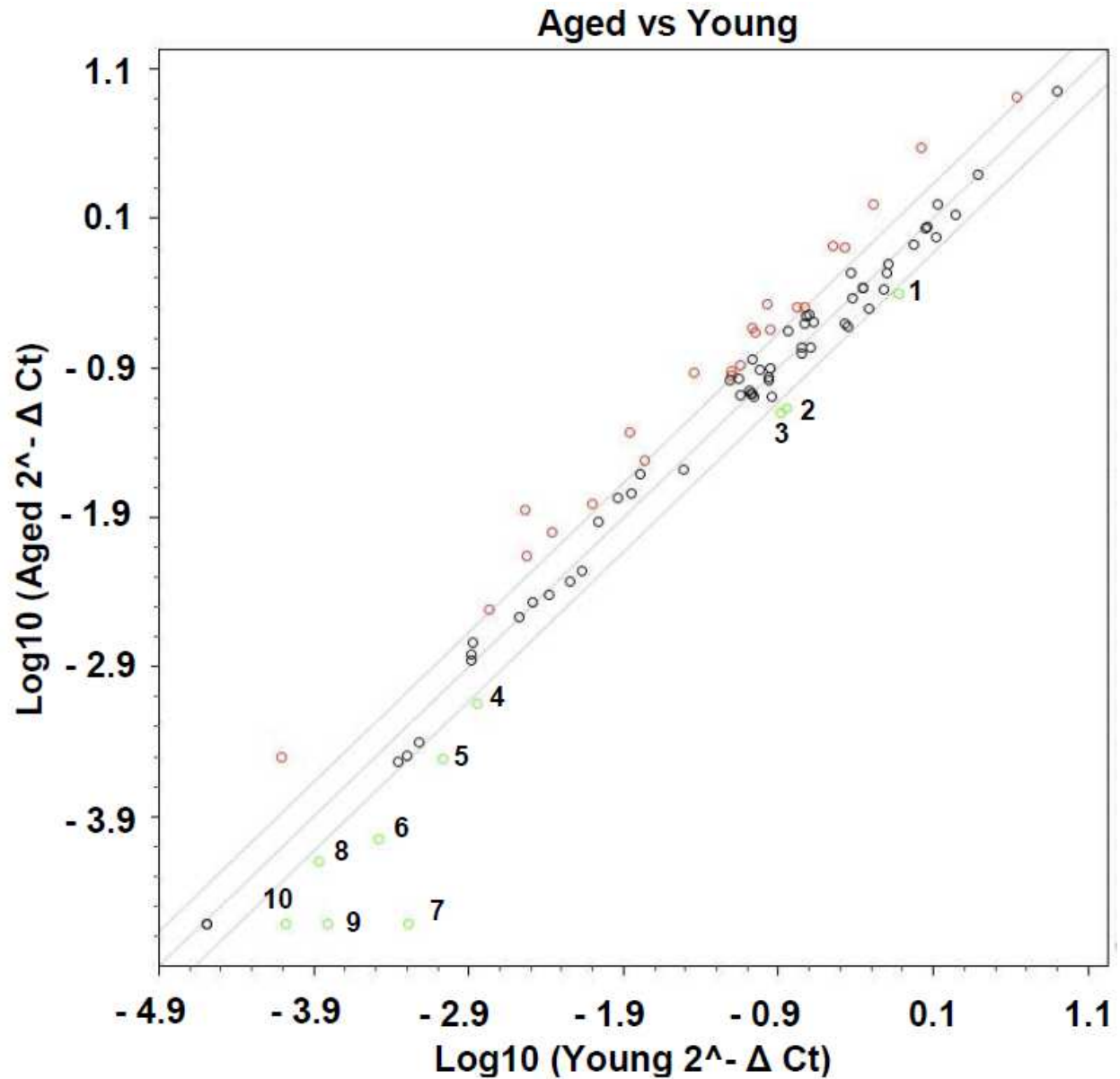


Figure 16. Scatter plot of genes that were downregulated in aged lung in comparison to young lung. (1) CCL20, (2) PF4, (3) CCL11, (4) CCR4, (5) IL13, (6) CCL24, (7) Crp, (8) IL17b, (9) IL20, (10) IL1f8

Table 6. Cytokine genes that were 1.7 fold higher in the aged lung in comparison to young lung¹

Gene	Fold change	Functional significance
IFN- γ	4.8289	Macrophage activation, Induction of MHC-I & -II expression. Inhibits Th2, induces Th1, important in viral infections. Enhances NK cell activation during influenza infection
IL-1 β	3.538	Produced by activated macrophages and monocytes; Pyrogen (causes fever). Activates endothelial cells and T-Cells. Enhances activity of NK cells. Provides a competence signal to allow B-Cell growth.
IL-1 α	3.1018	Known also as catabolin; It is produced by activated macrophages as a proprotein, which is proteolytically processed by Caspase-1; important mediator of the inflammatory response and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
IL-10	1.9659	Produced by Th2 cells. NK cells can also produce it. Inhibits Th1 cytokine synthesis. Down regulates MHC-II expression on APCs. Enhances cytolytic activity on NK cells.
IL-15	1.8912	Important for survival of NK cells, memory T cells. Enhances NK cell activity
TNF	1.8214	Pyrogen. Produced by monocytes, and NK cells. Activates endothelial cells, PMN & macrophages. Increases MHC-I expression. Can cause apoptosis. Cachexia & Shock. Inhibits NK cell function but important for NK cell migration to inflammation sites

¹ No cytokine receptor genes identified to be higher or lower in the aged lung.

Table 7. Cytokine genes that were 1.7 fold lower in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
IL-20	-6.4587	IL-20 is produced by activated keratinocytes and monocytes; it regulates cell growth and differentiation of keratinocytes particularly inflammation associated with the skin; it causes cell expansion of multipotential hematopoietic progenitor cells.
IL-1f8	-3.4595	Member of the IL-1 cytokine family.
IL-13	-2.7983	Mostly produced by Th2 T cells and its effects are similar to those of IL-4; It binds to IL-4R α ; Some NK cells can produce IL-13 in response to IL-2 or PMA plus ionomycin, especially in the absence of IFN- γ ; It induces many features of allergic lung disease.
IL-17b	-2.1377	It acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation; is produced by T-helper cells and is induced by IL-23 which results in destructive tissue damages.

Table 8. Chemokines that were 1.7 fold higher in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
CXCL1	3.3014	Known as IP-9; mostly expressed in leukocytes in pancreas and liver and less in thymus, spleen and lungs. Strongly induced by type II IFNs and weaker by type I IFNs; It binds to CXCR3 more than to CXCL9. It attracts activated T cells.
CCL3	2.6827	Known as MIP-1 α ; produced mostly by macrophages; it is produced during influenza infection; lymphocytes, including NK cells can also produce; recruits and activates PMNs, T cells, B cells and eosinophils; binds to CCR1, CCR5.
CCL4	2.8955	Known as MIP-1 β ; produced mostly by macrophages; it is produced during influenza infection; lymphocytes, including NK cells can also produce; recruits and activates PMNs, T cells, B cells and eosinophils; binds to CCR5
CXCL9	2.0201	Known as MIG; is a T-cell chemoattractant, which is induced by IFN- γ . It is closely related to two other CXC chemokines CXCL10 and CXCL11; binds to CXCR3.
CXCL10	1.8473	Known as IP-10; is secreted by monocytes, endothelial cells and fibroblasts in response to IFN- γ ; It attracts monocytes/macrophages, T cells, NK cells, and dendritic cells; binds to CXCR3. A strong relationship between elevated and sustained levels of the CXCL10 and poor treatment response exists in SARS infected individuals;CXCL10: CXCR3 axis plays a role in the establishment of severe cases of other respiratory illnesses including influenza A and community acquired viral or bacterial pneumonia
CCL22	1.7055	It is secreted by DCs and macrophages, and elicits its effects on its target cells by interacting with cell surface chemokine receptors such as CCR4
CCL6	1.7227	Known as C-10; is expressed in cells from neutrophil and macrophage lineage, and can be induced under conditions suitable for myeloid cell differentiation; it can also be induced in the mouse lung by the cytokine IL-13; its expression is reduced in activated T cell lines

Table 9. Chemokines that were 1.7 fold lower in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
CCL24	-3.8007	Known as eotaxin-2; induces chemotaxis of mostly of eosinophils, and resting T lymphocytes and less of neutrophils; binds to CCR3
CCL11	-2.0729	Known as eotaxin-1; selectively recruits eosinophils; binds to CCR2, CCR3, CCR5; It is increased in the blood from aged mice.
CCL20	-1.8786	Known as MIP3A; can be induced by inflammatory cytokines and downregulated by IL-10; strongly chemotactic for lymphocytes; binds to CCR6;
CXCL4	-2.1246	Known as PF4; released by activated platelets and promotes blood coagulation; it attracts neutrophils, fibroblasts and monocytes; it binds to CXCR3B.

Table 10. Chemokine receptors that were 1.7 fold higher in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
CCR1	2.3506	Regulate leukocyte trafficking in hematopoiesis and in innate and adaptive immunity; its expression correlates with overall survival in the non-germinal center subtype of diffuse large B-cell lymphoma; but it is associated with a shorter survival interval, in follicular lymphoma; is a marker of an immune switch between macrophages and a T cell-dominant response.
CXCR3	2.3049	The CXCL10:CXCR3 axis plays a role in the establishment of severe cases of other respiratory illnesses including influenza A.
CXCR5	2.291	Expressed on a subset of memory CD4 T cells that facilitates B cell activation, rapid antibody production and rapid migration toward the B cell area of lymphoid organ; its ligand is CXCL13.
CXCR2	1.9052	Known as IL8-Rb; facilitates neutrophil migration to inflamed

tissues; is expressed on PMN cells of myeloid origin.

CCR8	1.7638	Expressed on activated T cells and monocytes and in response to CCL1 helps vascular chemotaxis and participate in the response of the vessel wall to injury.
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Table 11. Chemokine receptors that were 1.7 fold lower in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
CCR4	-1.9800	A receptor for CCL17 and CCL22 produced by DCs and macrophages; is expressed on subsets of T cells, activated T cells, TH2 cells, and T reg cells; It has also been found on platelets, NK cells, macrophages, and DCs

Table 12. Other genes that were altered by 1.7 fold in the aged lung in comparison to young lung

Gene	Fold change	Functional significance
Casp1	1.7644	Caspase 1 is a central component of inflammasome that promotes processing and secretion of IL-1b and IL-18 that help recruit inflammatory cells to the sites of infection. It is part of the NLRP3 Inflammasome.
CRP	-21.5353	C-reactive protein; its protein levels are used as marker of inflammation; is elevated in blood of elderly and consists an indicator of risk for atherosclerotic disease; the regulation of the protein levels in relation to its mRNA levels is not known.

APPENDIX B

A PILOT STUDY TO EVALUATE THE EFFECTS OF SHORT-TERM SUPPLEMENTATION WITH ACTIVE HEXOSE CORRELATED COMPOUND (AHCC) ON THE IMMUNE RESPONSES TO INFLUENZA VACCINATION IN HEALTHY ADULTS

Abstract

A feasible approach to stimulate the immune responses of humans to vaccination has been to administer bioactive nutritional supplements near or at the time of immunization. Particular for influenza infection, one nutraceutical, Active Hexose Correlated Compound (AHCC), a mushroom extract, has been shown to protect mice against lethal primary influenza infection. Moreover, when AHCC was administered before immunization, protection from lethal avian flu infection mice was improved compared to those immunized alone. In this study, we hypothesized that AHCC will also improve the immune responses of healthy individuals to influenza vaccine. A randomized controlled study was performed with 30 healthy adults to evaluate the effects of AHCC supplementation on the immune response to the 2010 seasonal influenza vaccine. The AHCC group was supplemented with AHCC (3,000 mg/day) on the day of vaccination throughout three weeks post-vaccination while a control group received only the vaccine. Blood was drawn before immunization and three weeks post immunization. Flow cytometric analysis of lymphocyte subpopulations revealed that AHCC supplementation increased T-cells, and CD8 T-cells post-vaccination compared to control, especially among the elderly. Analysis antibody production after vaccination revealed that AHCC supplementation did not improve significantly protective antibody titers to influenza vaccine. Overall, our study showed that AHCC supplementation improved lymphocyte percentages but did not change antibody production. Future studies are required to determine the kinetics of AHCC supplementation to improve the overall response to influenza vaccination in healthy populations.

Introduction

Influenza infection is a major public health threat worldwide resulting in 36,000 deaths and over 200,000 hospitalizations each year [150]. While vaccination is effective in protecting 70-90% of young individuals from influenza disease, it is only about 45% effective in immunocompromised populations, such as in cancer patients and the elderly [168]. Thus, there is a growing interest in identifying alternative strategies that may improve the immune response to influenza vaccination to reduce morbidity and mortality from infection and its secondary complications during influenza season. One easy approach could be to administer bioactive nutritional supplements along with immunization to maintain or stimulate the immune responses in an effort to reduce the severity of infection in the event that vaccination is not effective.

Active Hexose Correlated Compound, (AHCC) is a dietary supplement prepared from Basidiomycetes mushrooms, largely comprised of α -1,4-glucans [181]. Animal studies have demonstrated that AHCC increases survival during acute infections such as influenza virus [182-184], avian flu [183], West Nile virus [185], and opportunistic infections [186-189]. Specifically, our laboratory has shown that AHCC supplementation-even in small doses- increased survival of young mice against influenza infection [182, 184]. Thus, there is clear evidence that AHCC has the capacity to improve or maintain immune function to primary virus infections, including influenza, in animal models.

Importantly, studies in human populations have demonstrated that AHCC can improve immune parameters in immunocompromised populations, such as the elderly [190] and cancer patients, with minimal adverse consequences [191]. Supplementation with AHCC increases natural killer (NK) cell cytotoxicity [192], number and function of dendritic cells (DCs) [193], and the functions of CD4 and CD8 T cells [190]. However, no studies have examined if short-

term supplementation with AHCC may improve the immune response after influenza vaccination in healthy individuals. Such studies are essential given the ineffectiveness of current influenza vaccination strategies in human populations

In the current study, we examined whether or not supplementation with AHCC could improve the efficiency of seasonal influenza vaccination in a pilot study of healthy individuals. Our hypothesis was that short term supplementation with AHCC would result in altered lymphocyte phenotype and increase the protective antibody titers against the influenza vaccine. Our data indicated that AHCC supplementation resulted in a higher increase in T cell and CD8 T cells, especially among the elderly, but there was no effect on the antibody titers after influenza vaccination. This suggests that short-term AHCC supplementation may be a therapeutic strategy to improve the cell-mediated immune response of older people after influenza vaccination.

Methods and materials

Subjects

All protocols were approved by the Institutional Review Board of Michigan State University. The distribution of study subjects by age and gender is illustrated in Table 12. A total of 30 healthy individuals initially enrolled in the study with 29 of the subjects completing the study, indicating a 97% retention rate. All participants gave written informed consent before enrollment into the study. Subjects also provided information about their general health status at enrollment and again at the end of the study. Subjects with diseases associated with immune disorders or who were taking medications known to alter immune function were excluded from the study.

All subjects were vaccinated in October and November of 2010 with the recommended commercially available subvirion trivalent influenza vaccine Flushield (Wyeth Laboratories, PA, USA). Pre-vaccination blood samples were collected on the day of vaccination, while post-vaccination samples were obtained three weeks after immunization.

Supplementation with AHCC

This study was a randomized controlled trial in which subjects were randomly separated into two groups. The AHCC group was supplemented with AHCC (3gr / day), for three weeks beginning on the day of vaccination. The control group were vaccinated but did not receive the supplement.

Sample preparation

Pre and post-vaccination plasma and serum samples were drawn from all subjects. At both time points, blood samples were drawn from all individuals between 06:00 and 08:00 h after an overnight fast (12 h from previous meal). Serum and plasma were processed and stored at -80°C until analysis for antibody and cytokines. Peripheral blood mononuclear cells (PBMC) were isolated from plasma with histopaque 1770 (Sigma-Aldrich, MO, USA) according the manufacturer's protocol.

Flow cytometry

Immune phenotyping of PBMCs was performed using previously published protocols [194]. Briefly, 1×10^6 PBMCs were stained with the appropriate antibodies and four-color flow cytometric analysis was employed to identify lymphocyte populations. All fluorochrome-

conjugated monoclonal antibodies used to detect lymphocyte populations were purchased from Pharmingen, (CA, USA), and the following monoclonal antibodies were used for staining: FITC CD8, PE CD56, CD4, PerCP-Cy5.5 CD3, CD16, CD45RA, and APC NKp46, CD19. Samples were acquired on the Accuri C6 (BD Accuri Cytometers, MI, USA) and analyzed using FlowJo software (Tree Star, OR, USA). Lymphocytes were gated using FSC vs SSC and cell populations identified as following: $[CD3^{+}]$ cells = T cells, $[CD19^{+}]$ cells = B cells, $[CD56^{+}, CD3^{-}]$ cells = NK cells, $[CD56^{+}, CD3^{+}]$ cells = NKT cells

Antibody Titers

Serum samples were sent to Enzo Clinical Diagnostic Laboratories (New York, USA) for analysis of antibody production against the influenza A and B strains in the 2010 vaccine. Titers were determined using the standard Hemagglutinin Inhibition Assay (HI test) and values were reported using geometric mean of serum anti-HA antibody titers. Antibody titers ≥ 40 post-vaccination were considered protective and a four-fold rise in specific antibody titers post-vaccination was considered a seropositive response to the current vaccine.

Cytokine Analysis

Custom Bio-Plex Pro Assay (Biorad, CA, USA) was utilized to assess the following cytokines: IL-2, IL-4, IL-6, IL-10 and IFN- γ . Briefly, undiluted plasma samples were thawed on ice and assessed according to manufacturer's instructions and via Luminex 100 technology (Luminex, TX, USA).

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 4 (Graph Pad Software, CA, USA). Paired t-test was performed to identify statistical significance before and after vaccination in each group, and t-test was used to identify changes between the two treatments. Statistical significance was set at $p < 0.05$.

Results

Demographic characteristics of participants

A total of 30 healthy individuals were recruited for the study and were randomly assigned to receive a daily supplement of AHCC or were assigned to the control group, who did not receive any intervention, prior to vaccination. Table 12 shows the demographics of the population. There were 14 individuals in the AHCC group (mean age 60.8 years) and 15 individuals in the control group (mean age 57.8 years). There was no statistical difference between the ages of the two groups. There was only one drop-out in the study from AHCC group, indicating 97% compliance for the overall study. The ratio of males to females was similar in the control group (7:8); however, the ratio of males to females was skewed in the AHCC group (9:5). However, there were no differences in mean age based upon gender.

Table 13. Demographic characteristics of study participants

All subjects	Control	AHCC
Number of participants ¹	15	14
Age (yr)	57.8± 5.3	60.8± 4.0
Gender (Male:Female)	7:8	9:5

¹ 30 participants were recruited and randomized. 29 individuals completed the study

Effect of AHCC on lymphocyte populations 3 weeks after influenza vaccination

We assessed by flow cytometry whether or not short-term supplementation with AHCC altered the phenotype of lymphocyte populations in response to vaccination. Figure 17 shows the percentages of B cells, T cells, cytotoxic CD8 T cells and helper CD4 T cells in the peripheral blood of participants pre- and post-vaccination. Figure 16 shows the percentages of NKT cells and NK cells. It is notable that AHCC group started with significant less T cells (Figure 17C), CD8 T cells (Figure 17D) and NKT cells (Figure 18A) at baseline. In order to control for this difference among groups at baseline, we compared the fold-change between the two treatment groups from pre- to post- vaccination. There was no change in the percentages of B cells (Figure 17B) but AHCC resulted in a significant increase of T cells (Figure 17D). Among T cell subsets, AHCC resulted in significant increase of CD8 T cells (Figure 17F), but not in CD4 T cells (Figure 17H). Although, AHCC did not result in higher levels of these cells in peripheral blood, it facilitated increased expansion of T cells and particular CD8 T cells that brought the population levels at approximately the same levels in both groups post vaccination as indicated in figure 17C, and 17E. There were not statistical significant effects of AHCC supplementation on NKT cells (Figure 18A, B) and NK cells (Figure 18C, D) at 3 weeks post vaccination.

Because the age of the study participants was not homogeneous, in order to examine the effect of AHCC supplementation taking into consideration their age we separated our participants into two, equal in number, groups: “young” (< 60 years old) and “aged” (>60 years old). The baseline difference of reduced initial T cells, CD8 T cells and NKT cells, in the AHCC group was independent of age and existed in both young and aged group of the AHCC treatment (data not shown). But most importantly, only for the aged group supplemented with AHCC it

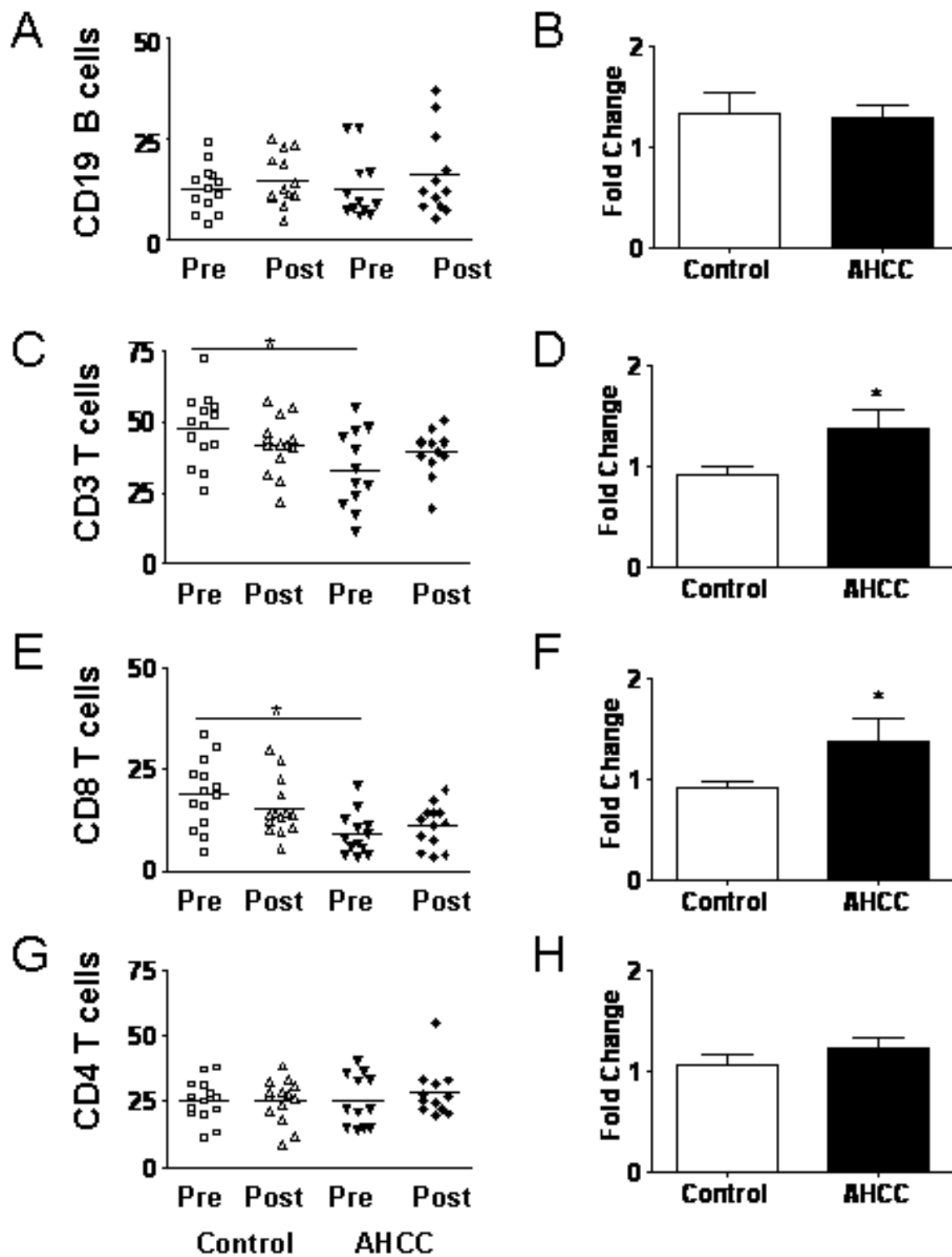


Figure 17. Analysis of peripheral blood mononuclear cells pre and post treatment

Figure 17 (cont'd). Percentages (A,C,E,G) and fold change over 3 weeks of treatment (B,D,F,H). Open bullets and bars represent the control group. Closed bullets and bars represent the AHCC group. Asterisk denotes $p<0.05$. (A) Individual responses of B cells gated as $CD3^{+}CD19^{-}$. (B) Fold change of B cells. (C) Individual responses of T cells gated as $CD3^{+}$. (D) Fold change of T cells. (E) Individual responses of CD8 T cells gated as $CD3^{+}CD8^{+}$. (F) Fold change of CD8 T cells. (G) Individual responses of CD4 T cells gated as $CD3^{+}CD4^{+}$. (H) Fold change of CD4 T cells

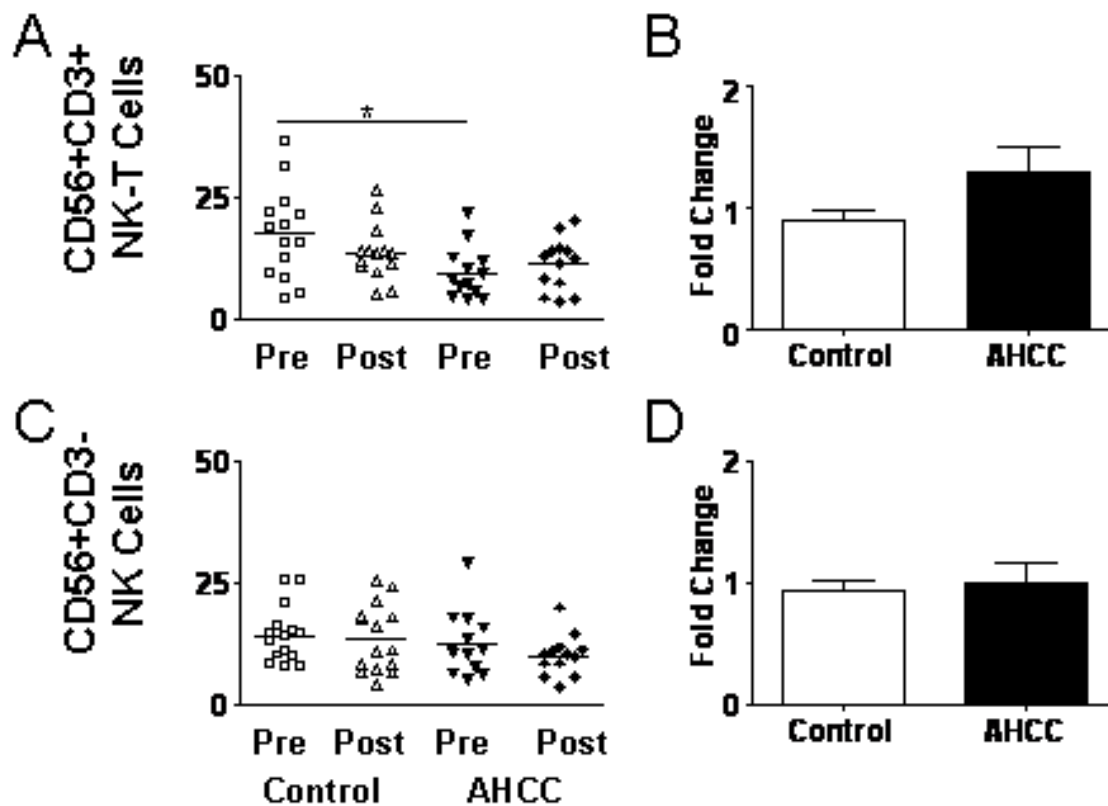


Figure 18. Analysis of NK cells and NKT cells pre and post treatment. Percentages (A,C) and fold change over 3 weeks of treatment (B,D). Open bullets and bars represent the control group. Closed bullets and bars represent the AHCC group. No significant results were found. (A) Individual responses of NK-T cells gated as CD3+ CD56+. (B) Fold change of NK-T cells. (C) Individual responses of NK cells gated as CD3-CD56+. (D) Fold change of NK cells.

was observed the statistical significant fold-increase of the percentages of CD8 T cells and CD56⁺ T cells (data not shown). Thus, AHCC effect on lymphocyte populations was mostly observed among elderly individuals.

Effect of AHCC on serum anti- influenza A and B antibody levels

To evaluate the effect of AHCC on vaccine efficiency, we measured the anti- influenza A and B antibody titers in the serum 3 weeks post vaccination. Figure 19 indicates that antibody titers to influenza A and B increased pre- to post-vaccination for both the AHCC and control groups; however, these differences were not statistically significant. We further analyzed the percentage of individuals who achieved titers ≥ 40 and had a four-fold rise post-vaccination to each of the strains of influenza. Table 13 indicates that an equal percentage of individuals in the AHCC and control groups achieved titers ≥ 40 to Influenza A post-vaccination. In addition, the response rate indicated by a 4-fold rise post-vaccination, to Influenza A was poor for both the AHCC and control groups.

When we separated our participants to “young” and “aged”, we observed that only the young had a response to the vaccine, while aged individuals, independently of supplementation, did not exhibit increased titers (data not shown). Among the “young”, we observed that only young participants from the AHCC group achieved more than 4 fold increase from baseline in anti- influenza B titers (data not shown). Furthermore, 6 out of 7 young participants supplemented with AHCC achieved a titer ≥ 40 for influenza A and 3 out of 7 for influenza B, which was higher than what was observed among young controls (4 out of 6 for influenza A and 1 out of 6 for influenza B). These data could indicate that young participants may benefit more from short-term supplementation with AHCC but we did not observe statistical significant

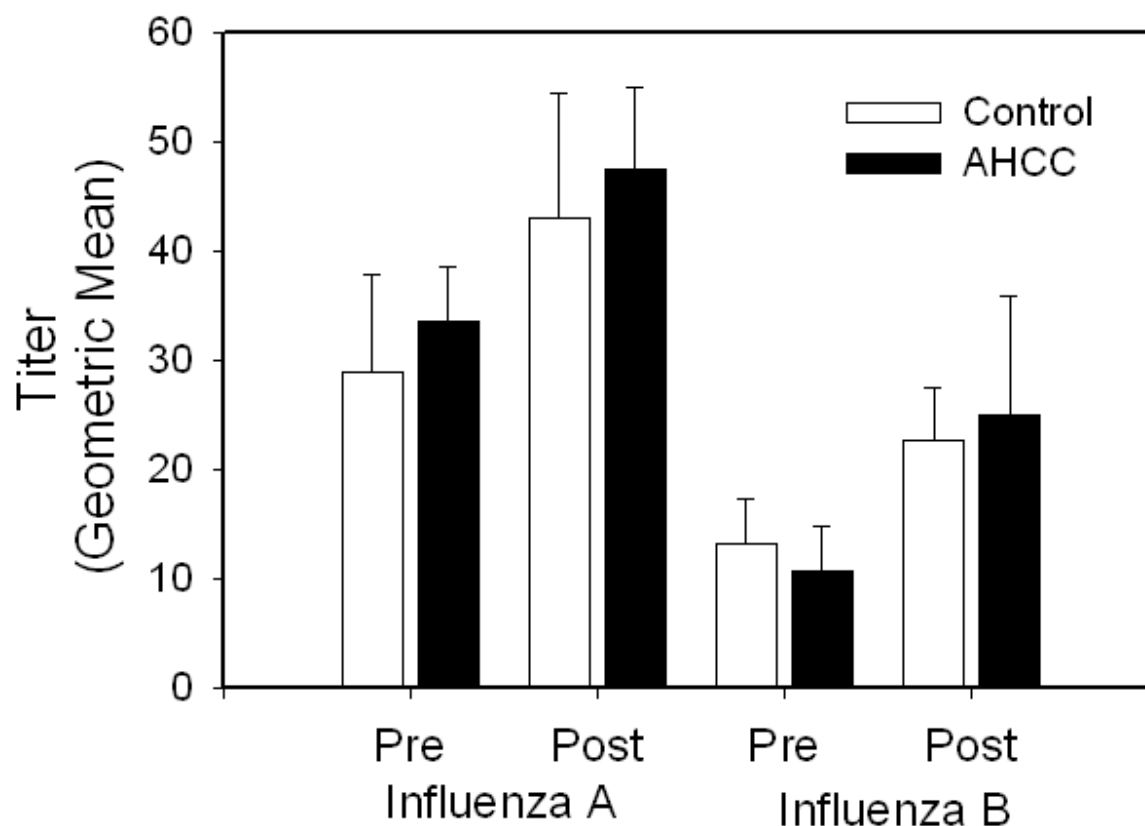


Figure 19. Geometric mean of anti-Influenza A and anti-Influenza B plasma antibody titers pre and post treatment. No significant results found

Table 14. Plasma antibody titers of healthy vaccinated adults in control and AHCC groups¹

		Percentage of participants with:			
		Titer > 40		4 Fold Rise	
Influenza A	Control	57.1%	8(14) ²	14.3%	2(14)
	AHCC	57.1%	8(14)	0.0%	0(14)
Influenza B	Control	14.3%	2(14)	14.3%	2(14)
	AHCC	21.4%	3(14)	21.4%	3(14)

¹No statistical significant differences were found.

²Number of participants (total participants in the group)

differences due to the low numbers of participants.

Effect of AHCC on plasma cytokine levels after influenza vaccination

To have a better picture of the immunomodulatory effects of AHCC during flu vaccination, we also performed a bioplex pro assay for the following cytokines: IL-2, IL-4, IL-6, IL-10 and IFN- γ . The cytokines were chosen to differentiate effects of AHCC on Th1/ Th2 responses (IL-2, IFN- γ / IL-4) and to indicate inflammation (IL-6) and the function of regulatory T cells (IL-10). We used frozen plasma for our assays. Table 13 summarizes the results. We obtained data for IL-4, IL-6, IL-10 and IFN- γ while IL-2 was not detected. We did not observe statistical significant differences due to the low numbers of detectable samples. Only for IL-6 and IL-10, the majority of the samples had a detectable value, but there was not significant difference in inflammation or T-reg activity with vaccination or with AHCC supplementation. It was interesting that IFN- γ was elevated in the control group compared to AHCC, but it was not significant.

Table 15. Plasma cytokine levels of healthy vaccinated adults in control and AHCC groups¹

	Control (pg/ml)		AHCC (pg/ml)	
	Pre	Post	Pre	Post
IL-2	ND	ND	ND	ND
IFN-γ	550.4 \pm 224.7	595.4 \pm 271.0	260.2 \pm 39.3	191.7 \pm 56.1
IL-4	3.6 \pm 1.8	6.1 \pm 1.8	2.2 \pm 0.0	2.2 \pm 0.0
IL-6	15.6 \pm 4.3	18.7 \pm 6.5	28.2 \pm 5.2	15.6 \pm 3.9
IL-10	7.3 \pm 2.3	7.7 \pm 1.1	17.8 \pm 8.8	7.7 \pm 1.4

¹No significant differences were found.

Discussion

This pilot study evaluated the effect of short-term supplementation with AHCC on the immune response to influenza vaccination. Flow cytometric analysis of the phenotypic changes on the lymphocyte subpopulations revealed that AHCC supplementation increased T-cells, and CD8 T-cells post-vaccination compared to the controls. This could indicate that short-term AHCC supplementation may be therapeutic strategy to improve the cell-mediated immune response after influenza vaccination. Previously published studies also suggest that AHCC may be beneficial to immunocompromised individuals, such as cancer patients [192, 195-197]. Analysis of the produced antibodies after vaccination revealed that AHCC supplementation in this study setting was not efficient to improve significantly the protective antibody titers to influenza vaccine. Finally, analysis of cytokine profile did not reveal any significant inflammatory effect of the AHCC. Collectively, these data indicate no adverse effects of AHCC supplementation and suggest that increase numbers of participants and a longer duration of supplementation may yield more positive results.

A trivalent split vaccine like the flu vaccine used in this study is expected to promote high antibody responses in comparison to live attenuated vaccines that induce a more cytotoxic memory response [198]. Thus, B cells and CD4 T cells are expected to proliferate and contribute to antibody production. Indeed, vaccination resulted in elevated percentages of B cells measured three weeks post vaccination, but there was not a significant effect of AHCC, since no differences observed on the final levels of B cells, and on the relative fold changes among the two groups. Similarly, vaccination did not result in significant changes on the percentages of CD4 T cells, and there was not a significant effect of AHCC. Overall, we cannot exclude the possibility that activation of B and CD4 T cells was different among the two groups, because we

did not follow the kinetics of activation, neither we characterized antigen specific cellular responses. Yet, if there was a particular effect of AHCC one could assume that it could be detected by measuring the levels of antibodies.

Vaccination resulted in seroprotection (titers ≥ 40) for up to approximately 60% of the participants independently from the treatment but resulted in limited four-fold rises. However, there was not a significant effect of AHCC supplementation on antibody titers, although the geometric mean of antibody titers was increased compared to baseline for both influenza A and B. When we considered the age of the participants we observed that almost half of the participants were more than 60 years old, which could compromise significantly the power of the study to examine the effectiveness of AHCC on the vaccine. Our results showed that older people had very small changes on their titers pre to post vaccination and almost none achieved a four- fold rise, confirming previous published studies describing low immune responses to flu vaccine among the elderly [168]. According to our data, AHCC supplementation may result to higher responses in antibody titers among the young participants; however, the low responsiveness to the vaccine and the small number of young participants in the study is a limiting factor to make certain conclusions. Nevertheless, in a murine model of West Nile viral infection, it was shown that AHCC supplementation resulted in elevated titers of antibodies [185]. Although, these data reinforce the idea that AHCC supplementation enhances the immune responses to infectious agents in naïve healthy hosts, future age- and immune status- controlled studies are required to examine the effect of AHCC in enhancing immune responses to vaccination.

Many animal studies support that AHCC enhances survival from several primary infections [182-189], a protective effect attributed to AHCC actions on DCs, CD8 T cells and

NK cells. The bioactive compound of AHCC is acetylated α -glucans but the exact mechanism of action is not known. Yet, β -glucans, another component of mushroom extracts, have received a special interest for their adjuvant effects in vaccination, because they are recognized by innate immune cell receptors and activate immune responses [199, 200]. Thus, it is possibly that AHCC also works through activating the innate immunity. In our study, we observed that AHCC supplementation resulted in significant higher expansion of T-cells, CD8 T-cells and CD56 T cells over the baseline compared to the control, but not of the NK cells. Although we did not see any change on NK cell percentages pre and post vaccination, there are several reports indicating that AHCC supplementation enhances NK cell cytotoxicity [182, 184, 192, 196, 201] and that it increased the number of DCs and especially myeloid DCs in humans [193]. Studies for the effect of AHCC on both NK cell and DC numbers, subsets, activation and function at earlier time points post vaccination would be useful in understanding the role of AHCC on the innate immune system.

It is clear from the increased fold changes of T cells pre to post vaccination that any immunomodulatory role of AHCC is probably associated with the activation of the Th1 pathway. In the absence of viral insult, supplementation of healthy adults with AHCC for 4 weeks does not alter the percentages of T cells [184, 193], but it increases the number of myeloid DCs [193] that present antigens to promote activation and proliferation of antigen specific T cells. In this study, we did not examine DCs but the expansion of CD8 T cells could indicate increased activation and proliferation. Furthermore, additional studies support that AHCC supplementation enhances antigen specific activation and proliferation of T cells [196] and production of IFN- γ and TNF- α after incubation with mitogens or monoclonal antibodies [190]. It is of particular interest that the observed expansion of CD8 T cells and CD56⁺ T cells pre to post vaccination was specifically

concentrated among the aged participants. Because aged individuals produce less protective antibodies during vaccination they may be more benefited by AHCC through an enhancement of their cytotoxic arm of immunity. Yet, the increased fold change in the populations observed particularly for the AHCC supplemented elderly, could merely be a result from delayed kinetics in activation, proliferation and contraction of those particular cells. Therefore, analysis of the kinetics of the various subpopulations, measuring the development of specific memory T cell and the function of these cells would give valuable data to support administration of AHCC supplements along with immunization to maintain or stimulate the immune responses in an effort to reduce the severity of infection in the elderly.

Overall, there is little evidence that orally ingested compounds can show measurable effects in the *in vivo* immune response in humans. AHCC has been shown to protect mice from primary infections, but a protective effect in humans from influenza infection is very difficult to prove. Studies to show a preventive role of AHCC against common colds, and influenza infections will require very big numbers of participants, and recording the occurrence and severity of infections. Vaccination studies can only give certain information about the effect of AHCC on immune stimulation to influenza antigens in humans and the choice of the sampling time limits the findings to few observations. With the current design, we have not examined immune cell activation and function, and the changes in the subpopulations of lymphocytes. We detected antibodies, as an endpoint of B cell function but the level of pre-existing antibodies may have impeded a significant increase in titers, and confounded the effect of AHCC in the immune response to influenza. Based on our data we could suggest that AHCC may have beneficial effects in increasing the antibody titers in naïve, young participants, but this would have to be confirmed with further animal or human studies. Unfortunately, there was not additional benefit

for the elderly as far as antibody production, but they had increased T and CD8 T cells, which could indicate that AHCC enhanced a Th1 response, giving hope that the elderly could have a certain advantage from AHCC supplementation in prevention of primary influenza infections. It is essential that further studies should be performed to examine the specific effect of AHCC on the immunity to influenza, so that current claims about its preventive nature for viral infections to be supported by mechanistic data.

Conclusions

In this study, we evaluated the effect of short term supplementation with AHCC on the immune responses to influenza vaccination. Flow cytometric analysis of the phenotypic changes on the lymphocyte subpopulations revealed that AHCC supplementation resulted in some changes among the lymphocytes. Most notable effect was that AHCC supplemented individuals showed a significant higher increase of T-cells, and CD8 T cells over the baseline compared to the control, and that this increase was mostly shown among the elderly supplemented with AHCC. Minor effects were observed for other lymphocyte populations, including a slightly increased proportion of CD56^{bright} NK cells among elderly individuals. Analysis of the produced antibodies after vaccination revealed that AHCC supplementation slightly enhanced younger participants' antibody titers, but it had minimum effect in the antibody production from elderly people. Finally, analysis of cytokine profile did not reveal significant effect of the AHCC. Overall, our study suggests that AHCC may cause some alterations of cellular homeostasis and possibly function of cytotoxic lymphocytes, especially in the elderly, and more studies are required to evaluate the effects of AHCC as a preventive regiment for influenza infections.

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