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Natural killer cell function is altered during the primary response of aged mice to influenza infection

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1. 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 (Thompson et al., 2003). 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 (de Jong et al., 2006). Similarly, in elderly humans pulmonary complication of viral pneumonia with seasonal influenza infections have also been reported (Falsey and Walsh, 2006; Kashiwagi et al., 1988). Vaccination is the primary strategy to prevent seasonal influenza infections, yet the elderly exhibit impaired antibody responses and remain vulnerable to flu infections (Gardner et al., 2000). 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 (Doherty et al., 2006) 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 (Gardner, 2005) and increased viral titers in their lungs four days post infection, when young mice have decreased titers (Gardner, 2005; Nogusa et al., 2008). 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 (Po et al., 2002). Based on this evidence, we hypothesized that impaired innate immunity in aged mice may contribute to increased susceptibility.

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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 (Denney et al., 2010). During the initial days of influenza infection, NK cells in the lungs become activated and demonstrate high cytotoxic activity, (Nogusa et al., 2008; Stein-Streilein et al., 1983) followed by production of interferon (IFN)- γ , (Guo and Topham, 2010), an important anti-viral cytokine. NK cell depletion results in severe influenza infection (GeurtsvanKessel et al., 2009; Nogusa et al., 2008), higher viral replication (GeurtsvanKessel et al., 2009; Nogusa et al., 2008), reduced cytotoxic responses (Kos and Engleman, 1996; Monteiro et al., 1998), and reduced early IFN- γ production (GeurtsvanKessel et al., 2009; Monteiro et al., 1998). Furthermore, NKp46, an activating receptor on NK cells that recognizes influenza hemagglutinin (Mandelboim et al., 2001) was shown to play a critical role for survival during influenza infection (Gazit et al., 2006). 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 (Ogata et al., 2001). Human studies have shown that healthy elderly have either preserved (Kutza et al., 1996; Kutza and Murasko, 1996; Sansoni et al., 1993; Yamashita et al., 1984), or reduced basal natural cytotoxicity (Facchini et al., 1987; Mariani et al., 1998; Ogata et al., 1997; Solana and Mariani, 2000) compared to healthy young adults. Moreover, after NK cells are stimulated with influenza vaccine (Kutza et al., 1996), 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 (Nogusa et al., 2008). 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.

2. Materials and Methods

2.1 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.

2.2. Cell preparation

Lymphocytes were isolated according previously described protocols (Nogusa et al., 2008; Po et al., 2002) 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.

2.3. 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 (Yokoyama and Kim, 2008). 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-, eFluo647-, or AlexaFluor700- NKp46 (29A1.4), APC-Cy7-, eFluo450-, 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.

2.4. 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, (1µl/ml) and monensin (0.66µl/ml) as well as 8µg/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 1µg/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, 1000IU/ml, (provided by Dr Kim's laboratory) and IL-18, 20ng/mL (MBL, Woburn, MA, USA) for 7 hours.

2.5. 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-Demand™ 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 $\Delta\Delta Ct$ method. All the results were expressed relative to young uninfected mice.

2.6 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. Results

3.1 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 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.

3.2 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 (Nogusa et al., 2008). 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, 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 (Biron et al., 1999), 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.

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 (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.

3.3. 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 (Hayakawa and Smyth, 2006). 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 (Kim et al., 2002). 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 (Chiossone et al., 2009).

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), only the absolute number of the most mature (R3) NK cells was significantly reduced in aged lungs (data not shown). Similarly, in the spleens of uninfected mice, aged NK cells have also more immature (R1) (Figure 5b.iv) and less most-mature (R3) NK cells (Figure 5b.vi). The absolute numbers of both R2 and R3 NK cells were significantly reduced in the spleens of aged mice at baseline (data not shown).

After influenza infection, in the lungs of young mice, the percentages of mature (R3) NK cells reduced, while the intermediate R2 NK cells increased and there was a trend ($p = 0.014$, critical $p = 0.010$, Two way Anova) for the immature R1 to also increase (Figure 5b.i–iii). After influenza infection, aged mice showed an even higher increase of the proportion of immature NK cells (Figure 5b.i) and a larger reduction of the mature (R3) NK cells in their lungs (Figure 5b.iii) compared to young mice. Because of the very low numbers of NK cells in the lungs of aged mice, the absolute number of all 3 subsets was lower compared to young mice, but only the number of the most-mature (R3) NK cells were statistically significant reduced in the lungs of aged mice (data not shown). Interestingly, while there was no effect of the infection in 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.

4. Discussion

We previously showed that influenza infection is more severe in the absence of NK cells (Nogusa et al., 2008). 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 (Zhang et al., 2007). In addition, aging affects migration of mature NK cells in the inflamed tissues during viral infection (Fang et al., 2010). Finally, aging results in a reduction in circulating IL-15 in mice (Quinn et al., 2009), and IL-15 strongly regulates homeostasis of NK cells in the periphery (Cooper et al., 2002). Moreover, aged NK/LAK cells have intrinsic defects in their activation after incubation with IL-15 (Albright et al., 2004). 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 (Guo et al., 2009; Mao et al., 2009), 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 (Weiss et al., 2010) 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 (Fang et al., 2010). 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 (Biron et al., 1999). During influenza infection, NK cells get activated to kill (Guo and Topham, 2010; Nogusa et al., 2008) and produce IFN- γ between 2–3 days p.i. (Guo and Topham, 2010), 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 (Achdout et al., 2010). 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 (Gazit et al., 2006). Regardless, NKp46 engagement results not only in increased NK cell killing but also increased production of cytokines (Mandelboim et al., 2001). 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 (Weiss et al., 2010). However, consistent with others (Weiss et al., 2010), 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 (Biron et al., 1999), and this response is abrogated when NK cells are absent (Monteiro et al., 1998). 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 (Lanier, 2008) 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 (Ortaldo and Young, 2003). Early during influenza infection, stimulatory cytokines for NK cells are produced, such as, type I interferons (IFN α/β), IFN- γ (Weiss et al., 2010), IL-18 (Liu et al., 2004), IL-12 (Monteiro et al., 1998), and IL-15 (Fawaz et al., 1999). Aging results in altered

cytokine milieu and in various models, aged mice had reduced levels of type I IFNs (Jiang et al., 2005), IL-12 (Toapanta and Ross, 2009), IFN- γ (Toapanta and Ross, 2009), and IL-15 (unpublished data, Nogusa) 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 (Monteiro et al., 1998) 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 IL1b, and TNFa as well as chemokines of the Macrophage Inflammatory Protein family (data not shown) but the role of these cytokines in NK cell activation *in vivo* is not well established. We have currently studies underway to examine the differences of the inflammatory cytokine environment in the lungs of young and aged mice with PCR arrays and to identify the role of these cytokines in the activation of NK cells.

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 (Nogusa et al., 2008). 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 (Lanier, 2009) that are shown to be defective in aged T cells (Larbi et al., 2010)].

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 (Lanier, 2008). It has been shown that CD3 ζ chain is down regulated upon high levels of hemagglutinin present (Mao et al., 2010), 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 (Larbi et al., 2010).

Because NK cell is not a homogeneous population but is compromised 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, and Hayakawa et al. (2006) proposed the CD11b/CD27 murine subset classification. 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 (Vahlne et al., 2008). 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 (Krishnaraj and Svanborg, 1992), our data indicate that the most mature NK cells are reduced in aged mice, in confirmation with previous reports in aged mice (Fang et al., 2010). 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. 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 (Kim et al., 2002) 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.

5. 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.

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- Aging results in reduced NK cells in the lungs and spleens
- NK cell function is defective after influenza infection of aged mice
- A specific defect for Interferon- γ production is observed in aged NK cells
- Aged mice have reduced terminally mature NK cells

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).

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).

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).

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).

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

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).

Table 1

Relative expression of mRNA in the lungs of day 2 infected mice, normalized to uninfected young lungs^I

	<i>Young</i>	<i>Aged</i>	<i>t-test</i>
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	$P = 0.802$

^I data are means $\pm$ SEM, n=5 per group