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Natural killer cell development and maturation in aged mice

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ABSTRACT

The effect of aging on natural killer cell homeostasis is not well studied in humans or in animal models. We compared natural killer (NK) cells from young and aged mice to investigate age-related defects in NK cell distribution and development. Our findings indicate aged mice have reduced NK cells in most peripheral tissues, but not in bone marrow. Reduction of NK cells in periphery was attributed to a reduction of the most mature CD11b$^+$ CD27$^-$ NK cells. Apoptosis was not found to explain this specific reduction of mature NK cells. Analysis of NK cell development in bone marrow revealed that aged NK cells progress normally through early stages of development, but a smaller percentage of aged NK cells achieved terminal maturation. Less mature NK cells in aged bone marrow correlated with reduced proliferation of immature NK cells. We propose that advanced age impairs bone marrow maturation of NK cells, possibly affecting homeostasis of NK cells in peripheral tissues. These alterations in NK cell maturational status have critical consequences for NK cell function in advanced age: reduction of the mature circulating NK cells in peripheral tissues of aged mice affects their overall capacity to patrol and eliminate cancerous and viral infected cells.

1. Introduction

Studies on immunosenescence have primarily focused on the impairment of adaptive immunity in part because of the reduced responsiveness of elderly people to vaccination (Gardner et al., 2001). It is well accepted that lymphocytes of adaptive immunity exhibit reduced function and altered composition with aging, but less is known about the lymphocytes of innate immunity, natural killer (NK) cells. NK cells are known as innate cells based on their spontaneous killing of tumor cells and their antiviral properties. The increased incidence of infectious diseases and cancer among the elderly, suggests NK cell responses are impaired in advanced ages. Because NK cells consist of various subsets with different functions, reduced function with advanced age may be the result of altered homeostasis. To date, there is an incomplete understanding of how aging affects NK cell homeostasis. In this study we examined NK cell phenotype, tissue distribution and development in a model of naturally aged C57BL/6J mice.

Our current understanding of NK cell development is that NK cells are produced in the bone marrow and seed the peripheral tissues during their last stages of maturation. Although immature NK cells can be found in liver, thymus, spleen and lymph nodes, the bone marrow is considered the primary site for NK cell development (Di Santo, 2008; Yokoyama et al., 2004). In the bone marrow, NK cell precursors (NKPs) undergo several stages of differentiation that can be tracked by the coordinated expression of cell surface markers (Kim et al., 2002). Immature NK cells that have acquired Ly49 receptors undergo functional maturation during a developmental stage that corresponds with an increase expression of maturation markers, and a significant expansion of their numbers in the bone marrow (Kim et al., 2002). It is proposed that NK cells acquire function after they express high levels of CD11b and CD43 (Kim et al., 2002). 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$^-$ KLRG1$^+$ NK cells the most differentiated NK cell subset (Huntington et al., 2007). CD11b$^+$ CD27$^-$ NK cells generally compose the majority of NK cells circulating in peripheral blood (up to 90%) and in non-lymphoid tissues. This NK cell subset is the major producer of IFN-γ and cytotoxic function upon activation (Di Santo, 2008; Yokoyama et al., 2004).

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Our laboratory has previously shown that influenza infection is more severe in the absence of NK cells (Nogusa et al., 2008) and that aged mice have reduced NK cells infiltrating in the lungs during the early days of influenza infection (Beli et al., 2011; Nogusa et al., 2008). We also have shown that aged NK cells had reduced ability to produce IFN-γ in response to influenza infection and to various stimulants in vitro which was correlated with significantly reduced numbers and percentages of mature, $CD11b^+$ $CD27^-$ NK cells in aged mice (Beli et al., 2011). In this manuscript, we show that aged mice have reduced NK cells in most peripheral tissues but not in the bone marrow. Reduction of total NK cells is 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 early stages of development but reduced NK cells in the terminal maturation stage, suggesting a block in their terminal maturation. We attribute the reduction of mature circulation of NK cells to reduced proliferation of NK cells in the bone marrow, as evidence for increased death in the peripheral tissues was not observed.

2. Materials and methods

2.1. Mice

Male, C57BL/6J, young adult (6 month – from now on referred as young) 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.

2.2. Cell preparation

Cells isolated from spleen and lungs were prepared according previously published procedures (Beli et al., 2011) with minor modifications. Collected lungs were dissociated using the GentleMACS™ Dissociator (Miltenyi Biotec Inc, Auburn, CA, USA). The homogenates were incubated at 37 °C for 1 h in a 5% CO_2 incubator with digestion buffer [1 mg/mL Collagenase D (Sigma–Aldrich St. Louis, MO, USA) and 80 Kuntz units/mL (Roche, Indianapolis, IN, USA) in RPMI-1640 media (Sigma–Aldrich) with 5% FBS (Atlanta Biologicals, Flowery Branch, GA, USA)]. The digested tissue was grinded through a 40 μm cell strainer and single cell suspensions washed 2 times with 5% FBS-RPMI media. Red blood cell lysis was performed with ACK lysis buffer. Spleens were grounded with a Dounce homogenizer, washed and directly lysed with ACK lysis buffer to remove red blood cells. Bone marrow cells were flushed from femurs according previously published methods (Hayakawa et al., 2010) and red blood cells were lysed directly with ACK lysis buffer. Livers were homogenized using the GentleMACS™ Dissociator and lymphocytes were isolated using 37.5% Percoll solution according to previously published methods (Hayakawa et al., 2010). Blood was collected in heparin tubes, and mononuclear cells were isolated using 1083 Histopaque (Sigma–Aldrich) according to manufacturer 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-1640 media. The number of cells isolated from each tissue was determined by counting live cells using a hematocytometer and trypan blue staining.

2.3. 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 to previously published methods (Yokoyama and Kim, 2008). Before cell surface staining, cells were treated with Fc-Block (antibody to CD16/32) except during staining 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), CD62L (MEL-14), CD11a (2D7), B220 (RA3-6B2), CD90.2 (53–2.1), CD69 (H1.2F3), CD127 (A7R34), Ly6 C (AL-21), CXCR3 (CXCR3-173), CCR7 (4B12), CXCR6 (221002), CD160 (13–1601-80), PD-1 (J43), Ki67 (B56), Bim (Ham 151–149), Bcl-2 (3F11), Caspase-3 (C92-605), 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). Streptavidin PE, PE-Cy7, or, APC-Cy7 (BD and ebiosciences) were used as secondary reagents for biotinylated antibodies. For intracellular staining, cells were first surface-stained followed by fixation/permeabilization using BD Bioscience Fix/Perm and Perm/Wash buffer according to manufacturer's instructions. Subsequently, cells were incubated with antibodies for intracellular markers, washed, and analyzed by flow cytometry. Data were acquired with FACS-Canto II or LSR II (BD Biosciences) and analyzed with 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 multiplying the percentage of NK cells from the live cell gate by the total number of cells determined by hematocytometer counts for each tissue.

2.4. Determination of apoptosis

Freshly isolated single cell suspension of spleen and bone marrow cells were directly stained intracellularly with Bcl-2, Bim and caspace-3 according to the intracellular protocol described above. For annexin V staining we incubated splenocytes and bone marrow cells (2×10^7 cells/mL) at 37 °C for 10 h in a 5% CO_2 incubator in serum free media. Cells were collected and stained for cell surface markers and Annexin V according to manufacturer's instructions (BD Biosciences).

2.5. BrdU incorporation assay

Mice were injected i.p. every 12 h with 100 μL of 1 mg/mL BrdU for a total of 96 h. Mice were euthanized, tissues processed, and cells were surfaced stained as described above. Subsequently, cells were fixed, permeabilized, and stained with anti-BrdU according to manufacture instructions (BD Biosciences).

2.6. Statistical analysis

All statistics were generated using Prism 6 (GraphPad Software Inc, La Jolla, CA, USA). Two-tailed student's *t*-test was used to compare adult and aged NK cells with a significance level of $p < 0.05$, Welch's correction was used on data with unequal variance which is specified in figure legends.

3. Results

3.1. NK cell phenotype is altered in aged mice

Initially we examined a plethora of markers associated with NK cells to characterize the phenotype of aged NK cells at resting conditions (Fig. 1). Resting NK cells from the spleens of aged mice had similar expression of NKp46, CD16, 2B4, NKG2D, CD94, integrins CD11a and DX5, chemokine receptors CCR7 and CXCR6, activation marker CD69 and exhaustion/senescent molecules CD160 and PD-1. However, aged NK cells had reduced expression of markers associated with mature NK cells including CD43, CD11b, KLRG1, CD62L, and Ly6C and they had increased expression of markers found mainly on immature NK cells, such as NKG2AEC, CD27, CXCR3, CD127, CD90.2 and B220. Altogether, our phenotypic characterization indicated that in aged mice NK cells some maturational defects.

3.2. Aged mice have reduced total and mature NK cells in most peripheral tissues

Based on previous data indicating reduced NK cells in aged mice during influenza infection (Beli et al., 2011), we determined whether the distribution of NK cells in various tissues differs between adult and aged mice at resting conditions. Aged mice had significantly reduced percentage of NK cells in spleen, liver, lung, and blood (Fig. 2A) and total numbers in spleen, liver and blood (Fig. 2B). When we examined the distribution of CD11b/CD27 NK cells, we observed that aging resulted in a reduction of the relative frequency of terminal mature, $CD11b^+$ $CD27^-$ NK cells in most of the peripheral tissues (Fig. 2C). Reduced relative frequency of mature NK cells appeared to be due to a selective reduction of the absolute number of mature NK cells and not due to an increase of the immature NK cells (Fig. 2D). We further examined NK cells for the distribution of KLRG1/CD27 subsets, another set of markers used to characterize NK subsets based on their maturation status.