Experimental Gerontology 104 (2018) 9–16

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Genetic influence on splenic natural killer cell frequencies and maturation among aged mice

S.A. Bumgardner[a], Y. Zhou[a], Z. Jiang[b], E.J. Coe[a], C.L. Yakaitis[a], Y. Xiao[a], R. Pazdro[a,*]

[a] Department of Foods and Nutrition, University of Georgia, 305 Sanford Drive, Athens, GA, United States
[b] Department of Statistics, University of Georgia, 101 Cedar Street, Athens, GA, United States

ARTICLE INFO

Section Editor: Richard Aspinall

Keywords:
Natural killer cells
Longevity
Genetics

ABSTRACT

Natural killer (NK) cells are cytotoxic innate lymphocytes that are integral to host defenses against viruses and neoplastic cells. Aging causes phenotypic and functional impairment of NK cells, which diminishes innate immune surveillance, yet the factors that determine the aged NK cell phenotype have not been completely defined. For instance, the genetic basis of the aged NK cell phenotype has not been established, but if determined, could highlight important genetic regulators of NK cells later in life. In this study, we estimated the heritability of splenic NK cell frequencies in aged mice from 15 classical and four wild-derived inbred strains. Our data suggest that frequencies of total ($NKp46^+CD3^-$) NK and mature ($NKp46^+CD3^-CD11b^+CD27^-$) NK cells were highly heritable at old age, and that total NK cell frequencies were independent predictors of median strain life spans. Strains with divergent phenotypes were compared to young-adult controls, and trends of age-related NK cell phenotypic alterations were confirmed. Finally, *in silico* mapping techniques revealed candidate genes associated with the aged NK cell phenotype. To our knowledge, these results are the first to demonstrate the genetic basis of the aged NK cell phenotype and will inform future mechanistic studies of NK cell dysfunction during aging.

1. Introduction

Natural killer (NK) cells are innate lymphoid cells on the forefront of controlling viral infections and eliminating senescent, nutrient-deprived, and malignant cells (Chan et al., 2014; Tarazona et al., 2017). NK cells identify target cells through a diverse spectrum of both activating and inhibitory receptors, whose combined engagement dictates NK cell target cell activation and killing (Bryceson et al., 2006; Bryceson et al., 2011; Vivier et al., 2008). Following activation, NK cells execute effector functions through secretion of cytotoxic granules, engagement of death receptors, and production of immuno-modulating cytokines and chemokines (prototypically interferon (IFN)-γ), all of which serve to bridge and regulate innate and antigen-specific immune responses (Chan et al., 2014; Yokoyama and Plougastel, 2003).

NK cells represent a heterogeneous population whose effector functions correlate with maturational stage (Chiossone et al., 2009; Shehata et al., 2015). Both human and murine NK cells undergo an ordered maturation process that primarily originates in the bone marrow—with hematopoietic stem cells and the common lymphoid progenitor lineage—and is completed in bone marrow and secondary lymphoid tissues (Freud et al., 2014; Yu et al., 2013). Murine NK cells proceed through a four-stage maturation model based upon distinct genetic profiles and surface expression of the integrin, CD11b, and the tumor necrosis factor (TNF) receptor family member, CD27 (Chiossone et al., 2009; Fu et al., 2011). NK cell maturation starts with double-negative (DN, $CD11b^{low/-}CD27^{low/-}$) cells, which progressively differentiate into immature ($CD11b^{low/-}CD27^{high/+}$), transitional ($CD11b^{high/+}CD27^{high/+}$), and mature ($CD11b^{high/+}CD27^{low/-}$) NK cells with enhanced cytotoxic effector functions (Chiossone et al., 2009).

Defects in NK cell maturation and function increase susceptibility to viral and intracellular bacterial infections and have been associated with numerous diseases, including several auto-immune diseases (Fogel et al., 2013; Orange, 2013). Functional and phenotypic impairments in circulating NK cells are also a prominent feature of aging (Beli et al., 2011; Hazeldine and Lord, 2013). Age-related NK cell dysfunction is characterized by decreased expression of certain activating receptors, impaired killing due to altered cell signaling, and diminished migration to sites of inflammation (Campos et al., 2014; Hazeldine and Lord, 2013; Lopez-Botet et al., 2014; Tarazona et al., 2017). In aged mice, reduced NK cell cytotoxicity is associated with fewer phenotypically mature NK cells in both the bone marrow and peripheral tissues

* Corresponding author.
E-mail addresses: sara.bumgardner@uga.edu (S.A. Bumgardner), zzydcat@g.ucla.edu (Y. Zhou), zjiang@uga.edu (Z. Jiang), ericacoe@uga.edu (E.J. Coe), claireyak@uga.edu (C.L. Yakaitis), yanfang.xiao@emory.edu (Y. Xiao), rpazdro@uga.edu (R. Pazdro).

https://doi.org/10.1016/j.exger.2018.01.010
Received 27 October 2017; Received in revised form 5 January 2018; Accepted 8 January 2018
Available online 03 February 2018
0531-5565/

(Hazeldine and Lord, 2013; Nair et al., 2015; Shehata et al., 2015). These NK cell defects appear to be attributable, in part, to external factors: altered bone marrow stromal cues and the non-hematopoietic environment at old age (Chiossone et al., 2009; Nair et al., 2015; Shehata et al., 2015).

Despite recent progress, factors that underlie NK cell maturational deficits in old individuals have not been completely defined. Understanding those factors would have significant effects, notably enhancing knowledge of the aging process and underscoring possible approaches to increase NK cell functions in the elderly. Our central hypothesis predicted that genetic variation is a crucial determinant of the aged NK cell phenotype, and more specifically, that NK cell maturation markers will stratify across aged mice from different genetic backgrounds (Petkova et al., 2008; Sellers et al., 2012). To test our hypothesis, we surveyed splenic NK cell frequencies and maturation across a panel of 19 inbred strains, estimated the heritability of those NK cell phenotypes, and used an *in silico* genome-wide association (GWA) mapping approach to identify novel genes that may be involved in the regulation of the aged NK cell phenotype. Overall, we expect these results to support new mechanistic efforts to understand the regulation of NK cells throughout the life span and how specific molecular pathways affect the onset of age-related diseases.

2. Materials and methods

2.1. Mice

Female mice from the following inbred strains were purchased from The Jackson Laboratory (Bar Harbor, ME, USA): 129S1/SvlmJ (JAX® 002448, 129S1), 129X1/SvJ (JAX® 000691,129X1), A/J (JAX® 000646, A), BALB/cByJ (JAX® 001026, BALB), BTBRT^{+} $ltpr3^{tf}$/J (JAX® 002282, BTBR), C3H/HeJ (JAX® 000659, C3H), C57BL/10J (JAX® 000665, B10), C57BL/6J (JAX® 000664, B6), C57BLKS/J (JAX® 00662, BLKS), C57L/J (JAX® 000668, C57L), CAST/EiJ (JAX® 000928, CAST), DBA/1J (JAX® 000670, D1), DBA/2J (JAX® 000671, D2), FVB/NJ (JAX® 001800, FVB), NZW/LacJ (JAX® 001058, NZW), PWK/PhJ (JAX® 003715, PWK), SWR/J (JAX® 000689, SWR), and WSB/EiJ (JAX® 001145, WSB). POHN/Deh (POHN) mice were obtained as a generous gift from Dr. David Harrison at The Jackson Laboratory. POHN mice were imported to the Pazdro mouse room at the University of Georgia and maintained through brother-sister matings. Housing conditions were specific pathogen free (SPF) and in accordance with the Association for Assessment and Accreditation for Laboratory Animal Care and the guidelines for Institutional Animal Care and Use Committee of the University of Georgia. In all experiments, young adult mice were used at 2–4 months of age and aged mice were used at 18–20 months of age, except for POHN/Deh, which were 21 months of age at the time of sacrifice.

2.2. Cell preparation and flow cytometry

Spleens were harvested and single cell suspensions of lymphocytes were derived. Briefly, spleens were manually dissociated through a 70 μm filter with a syringe plunger and red blood cells were lysed with ammonium chloride potassium (ACK) lysing buffer (Gibco, ThermoFisher Scientific, Waltham, MA, USA). Freshly-isolated splenocytes were suspended in Flow Cytometry Staining Buffer (eBioScience, San Diego, CA, USA), Fc receptor blocked (anti-mouse CD16/32, eBioScience), and stained with anti-CD3-eFluor450 (clone 500A2), anti-CD19-PE-Cy7 (clone 1D3), anti-NKp46-PE (clone 29A1.4), anti-CD27-APC (clone LG.7F9), and anti-CD11b-FITC (clone M1/70) (all eBioScience). Samples from each individual were also live/dead stained with Live/Dead Fixable Yellow Dead Stain Kit (Life Technologies, ThermoFisher Scientific), and shown to be > 99% viable (data not shown). Fluorescence compensations were performed using UltraComp eBeads (eBioScience) along with unstained and fluorescence-minus-one splenocyte controls. All data were collected on Beckman Coulter HyperCyAn flow cytometer and analyzed with FlowJo (version 10.1, Ashland, OR, USA).

2.3. Statistics

All statistical analyses were performed in R or GraphPad Prism 7.01 with statistical significance set at $p < .05$. Values for each variable were transformed to better satisfy the assumptions for analysis of variance (ANOVA). Total splenic NK cells frequencies were first arcsine of square root transformed. Frequencies of mature, transitional, immature, and DN NK cells were square root transformed. The following variables were Log10 transformed: all CD11b MFI values and CD27 MFI for mature and transitional NK cells. To evaluate the assumption of normality of variances, Q-Q plots were evaluated; and to verify the assumption of equality of group variances was met, the standard deviations of each group were evaluated and a Brown-Forsythe Levene-type test was performed for each transformed variable. While the Brown-Forsythe test was only significant for immature NK cell CD11b MFI ($p = .0076$, Table S1), the assumption of equality of variance for all variables, except for the CD27 MFI variable, was not met based upon the assessment of the fold differences of the group standard deviations. We assessed the significance of strain at predicting each response variable using ANOVA. The coefficient of determination, R^2, or the proportion of variability within the phenotype explained by the model with strain as the explanatory factor, was reported as an estimate of the narrow-sense heritability. The comparison of young versus aged NK cell data was performed with an ANOVA linear model and a Sidak's multiple comparison adjustment applied posttest. Correlations were tested using a two-tailed Pearson r correlation test.

An *in silico* approach was used to identify single nucleotide polymorphisms (SNPs) and candidate genes associated with each NK cell phenotype at old age. Transformed data were imported into the Efficient Mixed Model Association (EMMA) system (http://mouse.cs.ucla.edu/emma), which uses a linear mixed model algorithm designed to limit rates of false positive discoveries by correcting for model population structure and genetic similarity among strains (Kang et al., 2008). The approach has several strengths, such as reduced false positives and greater power than previous methods. Frequencies of total splenic, mature, transitional, immature, and DN NK cells were imported on a per-animal level (N = 3–6 per strain; N = 95 total) and analysis performed using a panel of 132,000 SNPs. All p values were transformed using $-\log_{10}(p \text{ value})$ in the scan plots score. A corrected statistical significance was set at $p < 1.0 \times 10^{-5}$ (Chen et al., 2012) for this study to avoid too conservative type I error control and facilitate identification of SNPs for hypothesis generation (Kang et al., 2008). Output from the EMMA server was converted to Build 38 by Ensemble Assembly Converter. Genes and loci were identified in the Mouse Genome Informatics website (informatics.jax.org).

3. Results

3.1. Mouse strains vary in splenic NK cell frequencies and maturation patterns at old age

Surface expression of CD11b and CD27 are important for deciphering the maturation stage of conventional circulating and tissue-resident splenic NK cells (Chiossone et al., 2009; Sojka et al., 2014). We measured the expression of these markers in splenic NK cells isolated from 19 inbred strains of mice at old age, representing a high degree of genetic diversity, to determine the extent to which the maturation process is governed by genetic background (Fig. 1). Total splenic NK cells were defined as the percentage of splenic lymphocytes that were $CD3^{-}CD19^{-}NKp46^{+}$ and that group was further divided into mature ($CD11b^{+/high}CD27^{-/low}$), transitional ($CD11b^{+/high}CD27^{+/high}$), immature ($CD11b^{-/low}CD27^{+/high}$), and double-negative (DN, $CD11b^{-/}$