Universidad de Córdoba
Departamento de Biología Celular, Fisiología e Inmunología
Instituto Maimónides de Investigación Biomédica de Córdoba

Subpoblaciones de Células NK: Cambios con la Inmunosenescencia

Subpopulations of NK Cells:

Changes with Immunosenescence

Nelson López Sejas

Córdoba 2019

TITULO: *SUBPOBLACIONES DE CÉLULAS NK: CAMBIOS CON LA INMUNOSENESCENCIA*

AUTOR: *Nelson López Sejas*

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Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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Departamento de Biología Celular, Fisiología e Inmunología

Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC)

Tesis para optar al grado de Doctor (Ph.D.)

Por:

Nelson López Sejas

Director:

Dr. Rafael Solana Lara

Córdoba 2019

TÍTULO DE LA TESIS:

Subpoblaciones de Células NK: Cambios con la Inmunosenescencia

DOCTORANDO: Nelson Rodolfo López Sejas

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

D. Nelson López Sejas presenta un trabajo original en el que ha analizado el efecto del envejecimiento, la infección por citomegalovirus y el impacto de la leucemia mieloide crónica, sobre subpoblaciones de células NK. Este trabajo ha sido realizado bajo mi dirección y la de Carmen Campos Fernández, que participó como codirectora del mismo desde principios de 2015 hasta finales de 2018, fecha en la que presentó su renuncia a continuar con la codirección de la tesis al haber obtenido por oposición una plaza de Profesora Técnica de Formación Profesional que la obligó a desplazarse fuera de Córdoba imposibilitándola para mantener la dedicación que requiere esta codirección.

Durante el tiempo de la realización de la Tesis doctoral el doctorando ha adquirido conocimientos técnico-científicos que le permiten elaborar hipótesis, diseñar la metodología y loa estudios necesarios para contrastarlas, analizar críticamente los resultados y discutirlos en base a la información científica disponible y obtener las conclusiones adecuadas que le ha permitido la integración de análisis inmunológicos, fenotípicos y funcionales y profundizar en las bases de la inmunosenescencia NK en diferentes situaciones clínicas. Asimismo la formación adquirida le ha permitido la elaboración de presentaciones en congresos nacionales e internacionales y la elaboración de manuscritos científicos que han sido publicados en revistas con prestigio en el área de Inmunología y Envejecimiento como son *Biogerontology* y *Frontiers in Immunology (2 publicaciones),* lo que ha permitido la presentación de esta Tesis por compendio de artículos.

Asimismo la Tesis se presenta con mención internacional, ya que el doctorando ha realizado una estancia predoctoral de cinco meses en el Laboratorio del Instituto de Inmunología de la Facultad de Medicina en la Universidad de Coimbra, Portugal, bajo la supervisión del Prof. Manuel Santos-Rosa, Catedrático de Inmunología de esa Universidad.

Considero que la actividad investigadora desarrollada le ha capacitado para el diseño y desarrollo de un estudio de investigación original, y su posterior difusión a la comunidad científica internacional.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 15 de Febrero de 2019

Firma del director

Fdo.: Rafael Solana Lara

Información Adicional

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II. **Effect of CMV and aging on the differential expression of CD300a, CD161, T-bet, and Eomes on NK cell subsets. Nelson López-Sejas**, Carmen Campos, Fakhri Hassouneh, Beatriz Sanchez-Correa, Raquel Tarazona, Alejandra Pera and Rafael Solana. Frontiers in Immunology. Nov. 07; 2016, DOI: 10.3389/fimmu.2016.00476.

III. **Effect of age on NK cell compartment in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors.** Paulo Rodrigues-Santos*, **Nelson López-Sejas*** , Jani Sofia Almeida, Lenka Ruzicková, Patricia Couceiro, Vera Alves, Carmen Campos , Corona Alonso, Raquel Tarazona, Paulo Freitas-Tavares , Rafael Solana* and Manuel Santos-Rosa*. Frontiers in Immunology. Nov. 08; 2018, DOI: 10.3389/fimmu.2018.02587

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1. Resumen

La inmunosenescencia se define como un deterioro progresivo de la función inmune asociada a la edad, este estado produce cambios que dan lugar a una remodelación del sistema inmunológico que afecta a la inmunidad innata y adaptativa. Sin embargo, además del envejecimiento “per se” como un factor inductor de estos cambios, las células del sistema inmune sufren un tipo de inmunosenescencia prematura en diversas situaciones de activación crónica del sistema inmune, como infecciones virales, enfermedades autoinmunes y cáncer. En este trabajo analizamos el efecto de la edad, la infección por citomegalovirus (CMV) y el impacto de la leucemia mieloide crónica (LMC) sobre la frecuencia, fenotipo y función de subpoblaciones de células NK.

Nuestros resultados confirman la disminución de la subpoblación de células NK CD56^{bright} asociada al envejecimiento y el incremento de la subpoblación CD56^{-}CD16^{+}, así como un incremento de la expresión de CD57 en las subpoblaciones de células NK CD56^{dim}CD16^{+} y CD56^{-}CD16^{+}. En individuos jóvenes, la seropositividad a CMV no afecta la distribución de las subpoblaciones NK CD56^{bright}, CD56^{dim}CD16^{+} y CD56^{-}CD16^{+}, mientras que se asocia a un incremento de la expresión de CD57 en las subpoblaciones de células NK CD56^{dim}CD16^{+} y CD56^{-}CD16^{+}. El estudio de otros receptores NK en estas subpoblaciones celulares muestra una menor expresión de DNAM-1 en células NK CD56^{dim} relacionada con el envejecimiento en individuos CMV seropositivos. Asimismo, la comparación de la expresión de receptores, en subpoblaciones NK de individuos jóvenes según la infección por CMV, muestra un aumento de NKp46 en la subpoblación NK CD56^{bright} y una disminución de la expresión de NKp30 en células NK CD56^{dim}. El descenso de la expresión de NKp30 asociado a la infección por CMV y el descenso de la expresión de DNAM-1 asociado a la edad se observan especialmente en las subpoblaciones de células que coexpresan CD57^{+}. La expresión de NKp46 y NKp30 es menor en células NK CD56^{dim}CD57^{+} que en las células CD56^{dim}CD57^{-}, mientras que la expresión de DNAM-1 es mayor en células NK CD57^{+}. La activación in vitro de células NK por IL-2 aumenta la expresión de NKp46 y NKp30 tanto en la subpoblación CD56^{dim}CD57^{+} como en la CD56^{dim}CD57^{-}.

Por otro lado, hemos estudiado la expresión de los receptores inhibidores CD300a y CD161 en células NK en relación con la edad y el seroestatus CMV. El receptor inhibidor CD300a es expresado por la mayoría de las células NK, si bien las células NK CD56^{bright} expresan niveles más altos de CD300a que las células NK CD56^{dim}. La edad se asocia con un aumento en la expresión de CD300a. La expresión de CD161 en células NK CD56^{dim} se encuentra disminuida en jóvenes CMV seropositivos en comparación con jóvenes CMV seronegativos. En las células NK CD56^{dim}, la seropositividad al CMV se asocia a un mayor porcentaje de células CD57^{+}CD300a^{+} y una reducción en el porcentaje de células CD161^{+}CD300a^{+}.

El estudio de los factores de transcripción T-bet y Eomes en células NK en relación con la edad y la seropositividad al CMV, muestra una disminución de T-bet^{hi} en células NK CD56^{dim}CD57^{+} de individuos jóvenes CMV seropositivos, mientras que la expresión de Eomes aumentó con la seropositividad al CMV en CD56^{bright} de individuos de mediana edad y en CD56^{dim}CD57$^{+/-}$ de individuos jóvenes. La expresión de Eomes disminuyó con el envejecimiento en todos los subconjuntos de células NK de los tres grupos de edad.

Por último, hemos analizado la expresión de receptores y marcadores de activación y de diferenciación en las subpoblaciones de células NK CD56^{bright} y CD56^{dim} en pacientes con leucemia mieloide crónica (LMC) tratados con inhibidores de tirosina kinasas (tyrosine kinase inhibitors, TKI). Mientras que encontramos diferencias significativas en el fenotipo y la función

de las células NK entre individuos sanos de mediana edad y ancianos, las células NK de pacientes con LMC tratados con TKI no muestran diferencias significativas relacionadas con la edad en la mayoría de los parámetros estudiados, lo que indica que la edad no es una limitación de la recuperación de células NK después del tratamiento con TKI. Nuestros resultados también revelan diferencias en la expresión de receptores NK, marcadores de activación y ensayos funcionales (expresión de CD107a e IFN-γ en células NK estimuladas con la línea celular K562) en células NK de pacientes con LMC tratados con TKI en comparación con controles sanos de la misma edad.

En conclusión, tomados en conjunto, estos resultados, indican que la edad y la infección por CMV inducen cambios significativos en la expresión de receptores NK, sugerimos varios enfoques como la determinación del CMV o terapias inmunomoduladoras que pueden facilitar el estudio y manejo clínico, especialmente en individuos de edad avanzada. Además, hemos identificado que en pacientes con LMC tratados con TKI, la edad no es una limitación para la recuperación de los receptores de células NK, lo que permite considerar la posibilidad de potenciar la actividad citotóxica de estas células en futuros tratamientos, especialmente en pacientes de mayor edad, para conseguir una remisión más efectiva de la enfermedad después del cese del tratamiento con TKI.

Summary

Immunosenescence is defined as a progressive deterioration of the immune function associated with age, this state produces changes that result in a remodeling of the immune system that affects the innate and adaptive immunity. However, in addition to aging "per se" as a factor that induces these changes, the cells of the immune system suffer a type of premature immunosenescence in various situations of chronic activation of the immune system, such as viral infections, autoimmune diseases and cancer. In this paper we analyze the effect of age, cytomegalovirus (CMV) infection and the impact of chronic myeloid leukemia (CML) on the frequency, phenotype and function of NK cell subpopulations.

Our results confirm the decrease of the subpopulation of $CD56^{bright}$ NK cells associated with aging and the increase of the $CD56^{-}CD16^{+}$ subpopulation, as well as an increase in the expression of CD57 in the subpopulations of NK $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ cells. In young individuals, seropositivity to CMV does not affect the distribution of the NK $CD56^{bright}$, $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ subpopulations, whereas it is associated with an increase in CD57 expression in the subpopulations of $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ NK cells. The study of other NK receptors in these cellular subpopulations shows a lower expression of DNAM-1 in CD56dim NK cells related to aging in CMV seropositive individuals. Besides, the comparison of activation receptors expression in NK subpopulations of young individuals shows an increase of NKp46 in the NK $CD56^{bright}$ subpopulation and a decrease in the expression of NKp30 in $CD56^{dim}$ NK cells. The decrease in NKp30 expression was associated with CMV infection only and the decreases in age-associated expression of DNAM-1 were observed especially in subpopulations of cells co-expressing CD57. The expression of NKp46 and NKp30 were lower in NK $CD56^{dim}CD57^{+}$ cells than in $CD56^{dim}CD57^{-}$ cells, while the expression of DNAM-1 is higher in $CD57^{+}$ NK cells. The in-vitro activation of NK cells by IL-2 increases the expression of NKp46 and NKp30 in both subpopulation $CD56^{dim}CD57^{+}$ and the $CD56^{dim}CD57^{-}$.

On the other hand, we have studied the expression of inhibitory receptors CD300a and CD161 in NK cells in relation to age and CMV seroestatus. The CD300a is expressed by most NK cells, showing higher levels of expression in $CD56^{bright}$ NK cells than NK $CD56^{dim}$ cells. Age is associated with an increase in CD300a expression. The expression of CD161 in NK $CD56^{dim}$ cells is decreased in young seropositive CMV compared to young seronegative CMV. In NK $CD56^{dim}$ cells, CMV seropositivity is associated with a higher percentage of $CD57^{+}CD300a^{+}$ cells and a reduction in the percentage of $CD161^{+}CD300a^{+}$ cells.

The study of T-bet and Eomes transcription factors in NK cells in relation to age and seropositivity to CMV showed a decrease in $T\text{-}bet^{hi}$ in NK $CD56^{dim}CD57^{+}$ cells of young seropositive CMV individuals, while the expression of Eomes increased with CMV seropositivity in $CD56^{bright}$ of middle-aged individuals and in $CD56^{dim}CD57^{+/-}$ of young individuals. The expression of Eomes decreased with aging in all subsets of NK cells of the three age groups.

Finally, we have analyzed the expression of receptors and markers of activation and differentiation in the subpopulations of $CD56^{bright}$ NK cells and $CD56^{dim}$ in patients with chronic myeloid leukemia (CML) treated with tyrosine kinase inhibitors (TKI). While we found significant differences in the phenotype and function of NK cells between healthy

middle-aged and elderly individuals, NK cells from patients with CML treated with TKI do not show significant age-related differences in most of the parameters studied, which indicates that age is not a limitation of NK cell recovery after treatment with TKI. Our results also reveal differences in the expression of NK receptors, activation markers and functional assays (expression of CD107a and IFN-γ in NK cells stimulated with the K562 cell line) in NK cells from patients with CML treated with TKI compared with healthy controls of the same age.

In conclusion, taken together, these results indicate that age and CMV infection induce significant changes in the expression of NK receptors. We suggest several approaches such as the determination of CMV or immunomodulatory therapies that can facilitate the study and the clinical management, especially in elderly individuals. In addition, we have identified that in patients with CML treated with TKI, age is not a limitation for the recovery of NK cell receptors, which allows us to consider the possibility of enhancing the cytotoxic activity of these cells in future treatments, especially in older patients, to achieve a more effective remission of the disease after cessation of treatment with TKI.

2. Introducción General

Han transcurrido más de 40 años desde el descubrimiento por parte de Keissling y colaboradores de un grupo de linfocitos granulares grandes (LGL por sus siglas en Inglés) con la capacidad de lisar células tumorales de forma natural y sin sensibilización previa, estos linfocitos fueron denominados células Natural Killer (NK) [1]. El mecanismo utilizado por las células NK para reconocer directamente células tumorales o células infectadas por virus permaneció sin clarificar hasta que años más tarde Kärre y colaboradores formularon la hipótesis del "missing-self" o reconocimiento de "la ausencia de lo propio", sobre la base de observaciones en células NK que hacían diana contra células tumorales de linfoma murino con una expresión reducida o ausente de las moléculas MHC de clase I [2]. Con el paso de los años el estudio de la biología de las células NK ha ido en constante evolución, y numerosos trabajos sobre la caracterización de su fenotipo, la comprensión del complejo sistema de receptores a través de los cuales median sus capacidades secretoras, el efecto de la edad y la infección sobre su capacidad funcional y hasta su prometedor papel en la inmunoterapia adoptiva contra el cáncer, han arrojado una complejidad que rivaliza con sus contrapartes del sistema inmune adaptativo, estas características hacen de las células NK componentes cruciales del sistema inmune y un objeto constante de estudio.

2.1 Citometría de flujo

La citometría de flujo (CF) como la conocemos hoy, fue introducida a principios de la década de los 70 y con el tiempo ha supuesto un gran avance en la caracterización celular, por la posibilidad de estudiar más de un parámetro de manera simultánea, lo que conocemos como "análisis multiparamétrico" [3, 4]. La CF es una técnica que consiste en hacer pasar células en un flujo envolvente a través de un haz de luz (laser) a gran velocidad, la luz dispersada y la luz emitida por los fluorocromos utilizados para el marcaje celular es amplificada y reconvertida en impulsos eléctricos que son almacenados en forma digitalizada en el citómetro [5]. Básicamente son tres los componentes de un citómetro; el sistema de fluidos que conduce a las células a través del rayo láser hasta su posterior clasificación y recogida mediante conductos en cubos de desecho; la parte óptica que consta del laser, filtros ópticos con diferentes longitudes de paso de banda, paso largo (long pass, LP) o paso corto (short pass, SP) y espejos que conducen la luz emitida y filtrada a los

detectores; y la parte electrónica con los detectores o tubos fotomultiplicadores (PMT) que amplifican las señales de luz emitidas por las células tras el paso por el laser y las convierten en señales eléctricas que son grabadas en forma digital en el citómetro hasta su posterior análisis a través de un software especializado [6, 7]. El uso de fluorocromos es la característica distintiva de la CF, son moléculas capaces de absorber la luz a una determinada longitud de onda y emitirla en otra, lo que se conoce como el espectro de absorción/emisión de un fluorocromo, que como explicamos anteriormente es recogida por la parte óptica-electrónica del citómetro y almacenada en su sistema [7].

El descubrimiento casi simultáneo de las células NK por R. Keissling y de los anticuerpos monoclonales por G. Kohler y C. Milstein en 1975, [1, 8] allanó el camino para la caracterización mediante citometría del inmunofenotipado de las células del sistema inmune, permitiendo la identificación de subpoblaciones celulares en una muestra incluso si esta es pequeña, y ayudó en la realización de ensayos funcionales en células citolíticas, (NK, T CD8) mediante la interacción entre células efectoras y células blanco marcadas con anticuerpos, [9-11] estas características y otras como su utilización para confirmar diagnósticos en enfermedades, análisis del ADN, ciclo y cinética celular o producción de citoquinas, hacen de la citometría de flujo una herramienta poderosa al servicio del investigador.

2.2 Sistema Inmune Humano

Dos “líneas de defensa” forman parte del sistema inmune humano. La inmunidad innata, más primitiva en términos evolutivos, [12] es la primera que actúa en defensa del huésped contra patógenos invasores con un reclutamiento rápido a los sitios de infección o daño tisular, primariamente forman parte de esta, barreras físicas, químicas, proteínas solubles, moléculas antibacterianas, monocitos, macrófagos, eosinófilos, neutrófilos, células dendríticas y células NK [13]. La segunda línea de defensa, la inmunidad adaptativa, establece su respuesta en la expresión de receptores específicos de antígeno en su superficie, codificados por reordenamiento génico, haciéndola mucho más específica y especializada contra los patógenos siendo sus componentes principales los linfocitos T y B. [14, 15]

2.3 Biología de las células NK: Conceptos básicos

Las células NK humanas comprenden entre un 10 a 15% del total de linfocitos mononucleares de sangre periférica, son componentes del sistema inmune innato que pueden interaccionar con células infectadas por virus, células tumorales o células dañadas. Tras el reconocimiento de estas células y su posterior activación las células NK ejercen potentes efectos citotóxicos y de producción de citoquinas [13]. Su vida media es alrededor de dos semanas y su importancia en la inmunidad antiviral se demostró con estudios realizados en individuos con deficiencias o fallos funcionales de células NK. Estos pacientes son más susceptibles a padecer infecciones virales por Citomegalovirus (CMV), Virus del Herpes simple (HSV), Epstein-Bar Virus (EBV), y Varicela Zoster Virus (VZV) [16-18].

2.3.1 Origen y desarrollo de las células NK

Las células NK se originan y maduran a partir de un precursor linfoide común a los linfocitos T y B en la médula ósea que expresa el marcador CD34, [19, 20] están presentes en otros órganos linfoides secundarios, (amígdalas, ganglios linfáticos, bazo) donde pueden completar su maduración. El estímulo de la IL-15 es fundamental para que las células NK precursoras, lleven a cabo su diferenciación, maduración y supervivencia, durante el desarrollo adquieren receptores propios de una célula precursora NK, IL-7R, CD45RA, CD10, CD117 y CD122 (cadena β del receptor de IL-2 e IL-15), en estadios finales de su diferenciación, pierden CD34 y expresan progresivamente receptores funcionales como CD56, FCγRIII (CD16), NKp30, NKG2D, CD94/NKG2A [20, 21]. La expresión diferencial de estos y otros receptores NK, como CD27 y CD11b, permiten definir etapas de su desarrollo y distintas subpoblaciones con diferente capacidad funcional, con perfiles predominantemente inmunoregulador o citotóxico [22, 23].

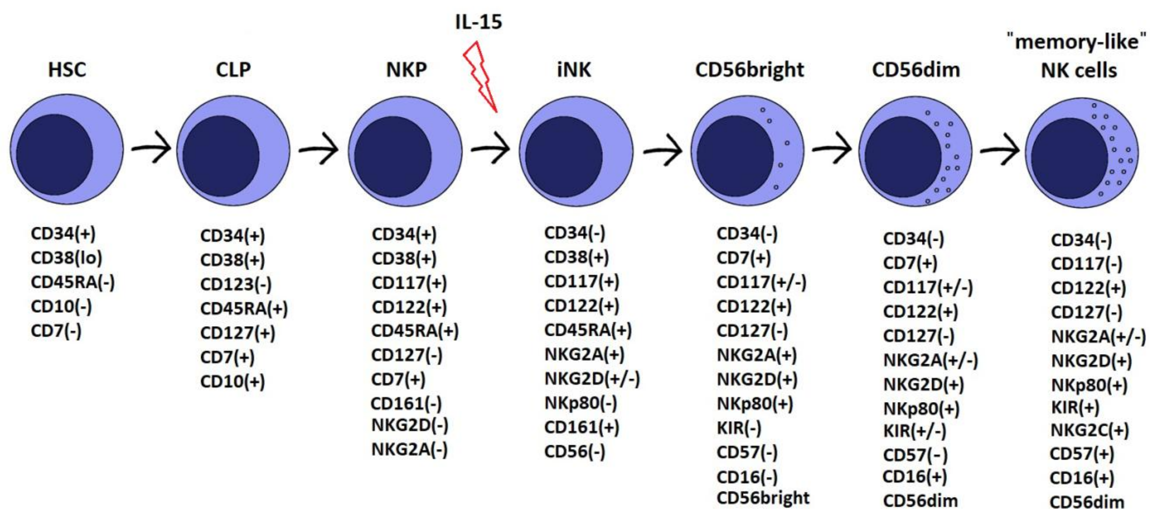

Figura 1. Esquema del desarrollo de las células NK. Las células NK maduran a partir de un progenitor linfoide común (common lymphoid progenitor, CLP) CD34+ derivado de células madre hematopoyéticas (hematopoietic stem cells, HSC), continuando su desarrollo a células NK precursoras (natural killer cell precursor, NKP) CD117+, CD122+, en esta etapa el estímulo de la IL-15 establece un punto de no retorno pasando a ser células NK inmaduras (immature NK cells, iNK) CD34-, NKG2D+. En etapas finales adquieren la expresión CD56+, NKp80+, CD16+, en subpoblaciones CD56(bright/dim) y se distingue una etapa de "diferenciación terminal" caracterizada por la expresión de CD56dim, CD16+, CD57+, NKG2C+ identificada como células NK similar a memoria (memory-like, NK cells).

2.3.2 Fenotipo y Función

Las células NK son linfocitos de la inmunidad innata (innate lymphoid cells, ILC) que se caracterizan por no tener reordenados los genes que codifican el TcR o los Ac y por la expresión de las moléculas CD56 y CD16 en su superficie. Se identifican como linfocitos $CD3^-CD56^+$ y según la intensidad de la expresión de CD56 distinguen dos subpoblaciones claramente definidas, $CD56^{bright}$ y $CD56^{dim}$. Las células NK $CD56^{bright}$ representan alrededor de un 10% del total de las células NK en sangre periférica, presentan un perfil inmunorregulador, con una alta producción de quimioquinas y citoquinas como IFN-γ, TNFa, IL-10, y GM-CSF en respuesta a la estimulación con IL-2, IL-12, IL-15, IL-18, y expresan los receptores de quimioquinas CCR7 y CD62L que facilitan su migración hacia los órganos linfáticos secundarios [(24-27)]. Las células NK $CD56^{dim}$, alrededor de un 90%, representan un perfil más citotóxico, se ha postulado que representan un estadio más maduro que las $CD56^{bright}$ en el desarrollo de las células NK, presentan una alta expresión de la molécula CD16 que interviene en la citotoxicidad dependiente de anticuerpos, (antibody dependent cell cytotoxicity, ADCC), pueden secretar citoquinas y quimioquinas como IFN-γ, CCL3 y CCL4 y expresar receptores de quimioquinas como CXCR1, CXCR2 y CX3CR1 que les confieren la cualidad de migrar a tejidos inflamados, además son más granulares a causa de

los gránulos de perforina y granzima en su interior con los que ejercen un potente efecto citotóxico al contacto con la célula diana [24, 28, 29]. Se ha identificado un tercer grupo de células NK, células $CD56^{-}CD16^{+}$, que aparecen expandidas en individuos ancianos sanos, pacientes infectados con el virus VIH-1 o el de la hepatitis C, esta expansión se ha asociado con una infección crónica, altas cargas virales, altos niveles de receptores NK inhibitorios y bajos niveles de receptores NK activadores, [30-32] su función no está del todo clara, aunque se definen como células disfuncionales con una baja función replicativa y citotóxica, [33, 34] estudios en pacientes con VIH-1, han arrojado una mejora considerable en la citotoxicidad, expresión de receptores NK, secreción de citoquinas, y el restablecimiento de la expresión CD56, cuando se disminuye la replicación del VIH-1 con una terapia antirretroviral efectiva [35, 36].

2.3.3 Receptores NK

El reconocimiento de células dañadas, infectadas por virus o que han sufrido una transformación tumoral se realiza a través de receptores expresados por las células NK y que reconocen sus ligandos en las células diana. Se distinguen varias familias de receptores NK que pueden actuar como receptores activadores o inhibidores de la función de estas células: receptores activadores de citotoxicidad natural NCR, (NKp30, NKp44, NKp46), el correceptor de activación DNAM-1, receptores activadores/inhibidores KIR tipo inmunoglobulina (KIR2DL1/5, KIR3DL1/2, KIR2DS1/5, KIR3DS1), receptores activadores/inhibidores tipo lectina derivados del heterodimero CD94-NKG2 (NKG2C, NKG2E, NKG2A) y el homodímero NKG2D, y el receptor activador tipo lectina II NKp80, otros receptores como LIR, KLRG-1, y 2B4,[37, 38]. De acuerdo la hipótesis del “missing self” formulada por Karré en los años 80, los receptores inhibidores KIR y CD94/NKG2A, que reconocen moléculas de histocompatibilidad de clase I (MHC-I) expresadas normalmente en células sanas, transmiten señales inhibitorias que frenan la citotoxicidad NK. Las células infectadas por virus o células tumorales pueden presentar un descenso o alteración en la expresión de estas moléculas MHC-I siendo susceptibles a ser reconocidas y eliminadas por las células NK. [2, 24]

Tabla 1. Receptores de las células NK

Receptor	Función	Ligando
Receptor Lectina Tipo-C		
CD94/NKG2A	Inhibidor	HLA-E
CD94/NKG2C	Activador	HLA-E
CD94/NKG2E	Activador	HLA-E
NKG2D	Activador	MICA/B, ULBP1–6
KLRG-1	Inhibidor	E-, R-, y R-Cadherinas
Receptor Lectina Tipo-C II		
NKp80	Activador	AICL
Receptor KIR tipo Inmunoglobulina		
KIR2DL 1/5	Inhibidor	HLA-C, grupo I-II
KIR3DL 1/2	Inhibidor	HLA-BW4, HLA-A-3, A11
KIR2DS 1/5	Activador	HLA-C, grupo I-II
KIR3DS 1	Activador	HLA-BW4
Receptor de Citotoxicidad Natural (NCR)		
NKp30	Activador	B7-H6, BAT3 ,pp65 (HCMV)
NKp44	Activador	Viral HA, NKp44L, PCNA,HSPG
NKp46	Activador	HSPG, heparin, VM, HNs, HAs
Correceptor de activación		
DNAM-1	Activador	Nectin-2(CD112), PVR(CD155)
CD16	Activador	IgG
CD69	Activador	Gal-1
2B4	Activador	CD48
Otros receptores		
CD300	Inhibidor	PS, PE
CD161	Inhibidor	LLT1
CEACAM-1	Inhibidor	CEACAM-1
LIR-1	Inhibidor	HLA-G, UL18?

La función de las células NK depende del balance de señales de activación e inhibición, de forma que si las señales de activación prevalecen las células NK ejercen citotoxicidad mediante gránulos de perforinas y granzimas y secretan quimioquinas y citoquinas al interaccionar con las células diana. [39, 40]

Otra función de las células NK es participar en el equilibrio inmune, eliminando células dendríticas (CD) inmaduras y favoreciendo su maduración mediante la interacción (cross-talk) entre células NK y CD que permite la posterior sinapsis entre CD maduras y linfocitos T CD4 y CD8, estableciendo así un nexo con la inmunidad adaptativa [41, 42].

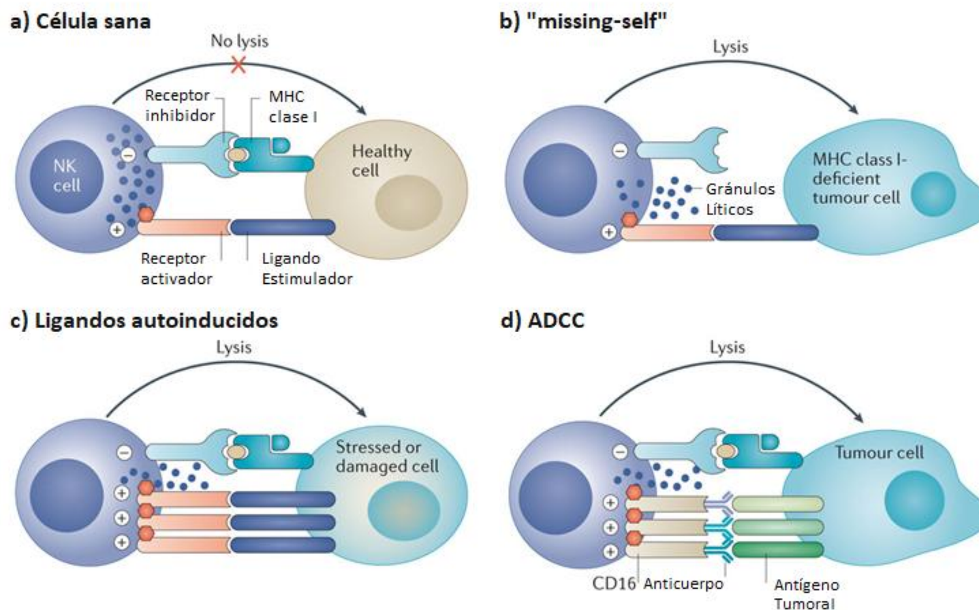

Figura 2. Balance Activación-Inhibición en las células NK. a) Las señales de activación e inhibición en equilibrio de células sanas, inhiben el ataque de las células NK tras su reconocimiento. b) La expresión reducida o carente del complejo mayor de histocompatibilidad Clase I (major histocompatibility complex, MHC-I) conocida como "missing-self" en células tumorales o infectadas, es reconocida por la células NK que activan sus mecanismos efectores de ataque. c) Células estresadas o dañadas expresan ligandos de receptores activadores que superan las señales inhibidores, lo que les convierten en blancos de las células NK. d) Células tumorales expresan antígenos que se acoplan a anticuerpos que son suceptibles al reconocimiento y citotoxicidad celular dependiente de anticuerpos (antibody dependent cellular cytotoxicity, ADCC) por las células NK. (Adaptado de Morvan MG. et. al. 2016).

2.4 Receptores CD300a y CD161

Existen otros receptores inhibidores NK que interactúan con ligandos distintos a MHC-I. CD300a es una proteína transmembrana que se expresa en todas las célula NK, presenta motivos ITIMs intracelulares que le confieren características inhibidoras. Los ligandos de CD300a son fosfatidil-serina (PS) y fosfatidil-etanolamina (PE), presentes en células apoptóticas, células infectadas por virus y células tumorales. Las células apoptóticas y tumorales que expresan PS inhiben la fagocitosis por macrófagos y la citotoxicidad de las células NK. Estas interacciones receptor-ligando y su papel clave en varias patologías como alergias, procesos autoinmunes o cáncer, han hecho que CD300a sea propuesto como un importante regulador de la función inmune [43-46]. El receptor inhibidor CD161 (NKR-P1A) se expresa en células NK y reconoce al ligando tipo lectina (LLT1) que no se expresa en células en reposo, pero está sobreexpresado en leucocitos activados, células pulmonares infectadas por VSR, o en activación por citoquinas, su expresión en células NK se reduce en la infección por Citomegalovirus (CMV), por el estimulo con IL-2 o el bloqueo con

anticuerpos específicos anti-CD161, que en un ensayo con tejido cartilaginoso dio como resultado una mayor eliminación de condrocitos por células NK, sin embargo el estimulo con IL-12 aumenta su expresión de forma selectiva ya que otros receptores NK como CD16 o KIR no se vieron afectados, se conocen pocos estudios que exploran la función de este marcador en la regulación del sistema inmune con el envejecimiento[47-49].

2.5 Inmunosenescencia

El envejecimiento del sistema inmune está relacionado con un estado de desregulación asociado a la edad, que se caracteriza por una disminución de la inmunidad, una menor respuesta a las vacunas y una predisposición del organismo a padecer enfermedades infecciosas, procesos autoinmunes y cáncer, [50-55]este fenómeno es conocido como inmunosenescencia y los cambios que produce afectan principalmente a la respuesta inmune adaptativa, sin embargo las evidencias revelan que la respuesta inmune innata también se ve afectada [51, 56-59]. Aunque la relación envejecimiento cronológico-inmunosenescencia es comúnmente aceptada, no es la única, existen muchos estudios sobre enfermedades que someten a las células inmunológicas a un estimulo antigénico crónico y que son responsables de conducirlas hacia un tipo de "Inmunosenescencia prematura", tales como infecciones virales (CMV), enfermedades autoinmunes (artritis reumatoide, psoriasis) y cáncer (LMA, LMC) [60-64]. Finalmente factores como el estilo de vida (tabaquismo, alcoholismo, estrés, etc.) y la nutrición (deficiencias en micronutrientes, PUFA´s, etc.) juegan un rol importante en el progreso de la inmunosenescencia [65, 66].

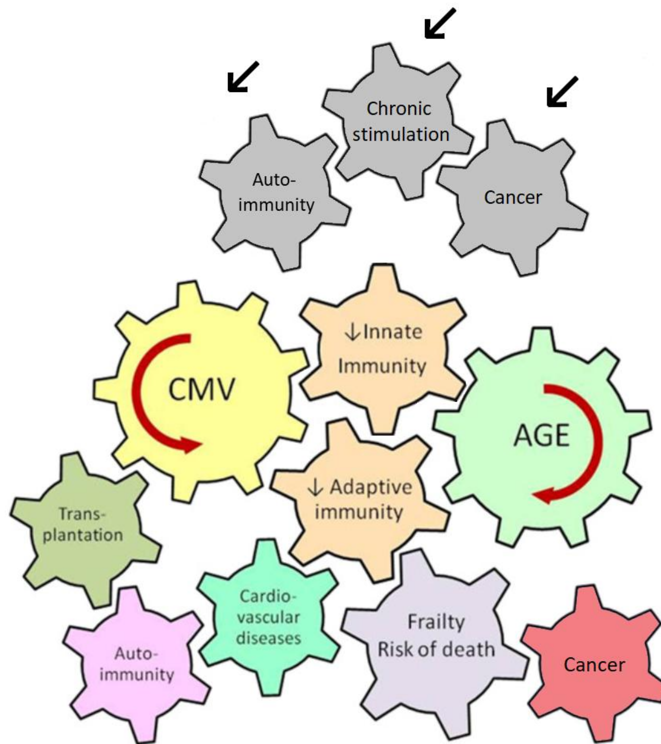

Figura 3. La edad y la infección por citomegalovirus (CMV) inducen cambios significativos en la inmunidad innata y adaptativa, fenómeno conocido como Inmunosenescencia, que contribuye al aumento de la fragilidad y riesgo de padecer enfermedades en individuos de edad avanzada. Infecciones, procesos autoinmunes y cáncer (parte superior de figura) pueden provocar un tipo de "Inmunosenescencia precoz" en etapas más tempranas de la vida. (Adaptado de Solana et. al. Immunity & Aeiging 2012).

2.5.1 Efecto de la edad sobre las células NK

El envejecimiento produce cambios en el compartimento de las células NK que afecta a su frecuencia, fenotipo y función [58, 59]. En ancianos saludables el número de células NK tiende a incrementarse, sin embargo este aumento se relaciona con una disminución de células $CD56^{bright}$ más inmaduras y una expansión de células $CD56^{dim}$ con una mayor diferenciación, la citotoxicidad global en las células NK no se ve afectada, pero sí la citotoxicidad por célula observada en ensayos con células eritroleucémicas humanas de la línea K562 [59, 67, 68]. La expresión de los receptores activadores, NKp30, NKp46 y DNAM-1 se encuentran disminuidos así como otros receptores que median lisis como CD69 y KLRG-1, no se han descrito cambios en los receptores activadores NKG2D y CD16, por el contrario hay discrepancias en cuanto a los niveles de los receptores KIR inhibidores (en aumento) y el receptor inhibidor NKGA (disminuido) descritos en diferentes estudios, otros trabajos sin embargo no reportan diferencias [58, 59, 69-72]. La secreción de citoquinas y quimioquinas tras estimulación o por el contacto con células diana se encuentra relativamente conservada en las células NK con la edad, aunque se ha descrito que los niveles observados son menores

en ancianos comparados con individuos jóvenes, estose explica por la disminución de las células $CD56^{bright}$ (fenotipo más inmunorregulador) en individuos de edad avanzada[58].

2.5.2 Expresión de CD57 en células NK

El marcador CD57 llamado también HNK-1/Leu-7 o L2, se identificó por vez primera en células NK de ratón, en un principio se creyó que era un marcador exclusivo de células NK, con el tiempo fue identificado también en linfocitos T [73, 74]. En células NK, CD57 representa un marcador de maduración terminal, a partir de células inmaduras $CD56^{bright}$ a $CD56^{dim}CD57^{-}$ y de estas a $CD56^{dim}CD57^{+}CD16^{+}$, esta última subpoblación es considerada como terminalmente diferenciada y definida por un fenotipo maduro con alta expresión de KIR, mayores capacidades citotóxicas (a través CD16), baja respuesta a estimulación por citoquinas (IL-2 e IL-15), menor producción de IFN-γ (por IL-12 e IL-18) y baja proliferación. En resumen, esta adquisición de CD57 por parte de las células $CD56^{dim}$ refleja una progresión hacia la maduración de células NK altamente citotóxicas, postulando a las células $CD57^{+}$ como células NK de larga vida [75-77].

2.5.3 Efecto del CMV sobre las células NK

El citomegalovirus Humano (HCMV) es un vírusβ-herpesvirus (HHV5) que afecta a toda la población humana con una prevalencia que varía según la edad y las condiciones socio-económicas y geográficas[78]. Después de la infección, el CMV permanece en un estado de latencia y puede crear reservorios en células madre hematopoyéticas (haematopoietic stem cells, HSCs), células de linaje mieloide monocitos/macrófagos, células dendríticas, en células endoteliales y posiblemente vasos linfáticos[79]. La infección por CMV se relaciona con un deterioro del sistema inmune, afectando tanto a la inmunidad innata como a la inmunidad adaptativa, este desbalance apunta al virus como una poderosa fuerza motriz que lleva a las células inmunes a la inmunosenescencia[80, 81]. El CMV tiene la capacidad de remodelar el repertorio de las células NK como se demostró en sujetos CMV seropositivos que presentaron una disminución de los receptores NKp30, NKp46 y CD161. En donantes jóvenes CMV^{+} comparados con sus pares negativos, se observó un aumento de células $KLRG1^{-}/CD57^{+}$ similar a individuos de edad avanzada, con la infección ocurre una expansión de células NK $CD57^{+}$, descrita anteriormente en el envejecimiento, estas células expresan

NKG2C un receptor activador ligado a la respuesta contra el CMV que reconoce el antígeno leucocitario humano HLA-E, por tanto estas células $NKG2C^{+}CD57^{+}$ están consideradas células de larga vida o “similar a memoria NK”, en estudios con sujetos CMV^{+} de diferentes edades no se encontraron diferencias en la expresión de CD57, lo que sugiere que las células $CD57^{+}$ expandidas en el envejecimiento en realidad están asociadas a la infección por CMV [69, 77, 82-84].

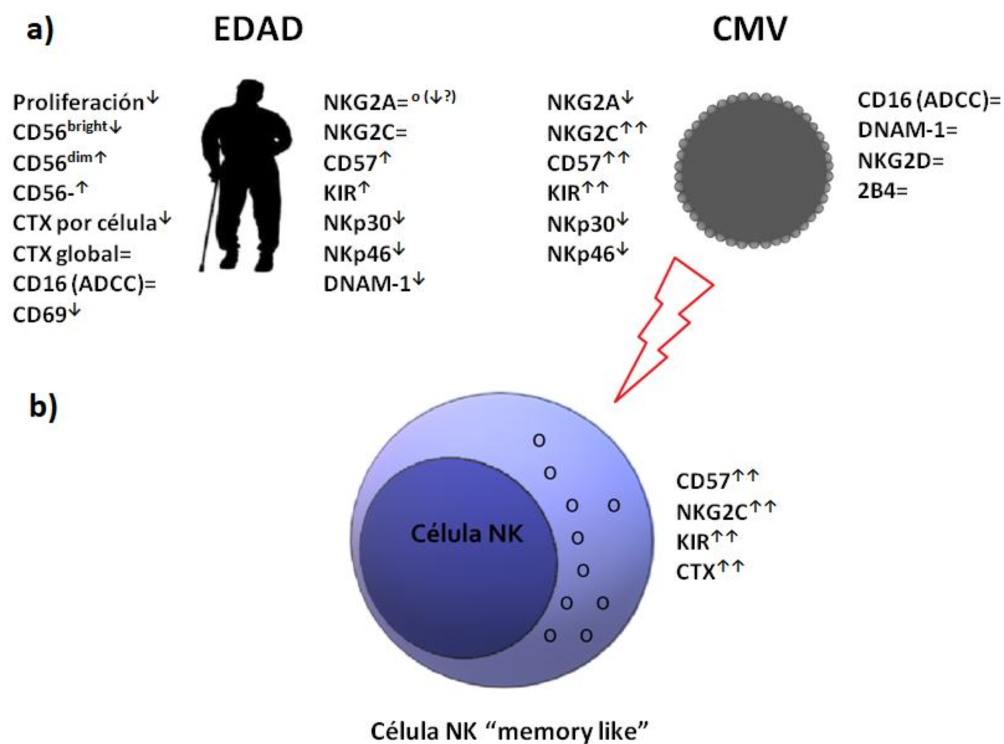

Figura 4. Efecto de la edad y el CMV sobre las células NK. a) Cambios en los receptores NK con la edad y con la infección por CMV. b) Célula NK de larga vida (memory-like).

2.6 Factores de transcripción Eomes y T-bet

Los factores de transcripción de T-box, Eomesodermin (Eomes) y T-bet, están implicados en importantes procesos del desarrollo en células de organismos vertebrados. En linfocitos T coordinan la diferenciación de células T CD8 a efectoras de memoria y por lo tanto son considerados reguladores maestros de su función [85-87]. Eomes y T-bet se expresan en todas las células NK maduras y por sus niveles de expresión se ha sugerido que pueden regular mutuamente su expresión. En las primeras fases del desarrollo de las células NK la expresión de Eomes en sangre periférica se encuentra elevada y la de T-bet disminuida.

Durante el proceso de maduración la expresión de Eomes decrece y T-bet incrementa. Este control en las etapas de desarrollo y maduración de las células NK se puede observar en células $CD56^{bright}$ de sangre periférica donde Eomes expresa niveles más altos, mientras que en $CD56^{dim}57^{+}$ es T-bet el que aparece sobreexpresado, lo que sugiere que tanto Eomes como T-bet regulan las funciones inmunorreguladoras (producción de IFN-γ), mientras que T-bet solo estaría implicado en la regulación de la función citotóxica [88-90]. Las células NK carentes de Eomes y T-bet pierden gradualmente los marcadores del linaje NK y sufren una regresión a un fenotipo más inmaduro, presumiblemente por la pérdida de expresión de CD122 (cadena β del receptor de IL-2 e IL-15) importante para el desarrollo de las funciones NK. Por otra parte los ratones $T\text{-}bet^{negativos}$ infectados con citomegalovirus muestran una reducción significativa en la citotoxicidad, aunque la carga viral se mantiene sin cambios, demostrando que las células NK $T\text{-}bet^{negativas}$ podrían ser suficientes para controlar la replicación viral. Similares resultados se obtuvieron con los patógenos Listeria Monocytogenes y Toxoplasma Gondii.[88, 89, 91, 92] Dado que tanto la edad como la infección por CMV se asocia a cambios en el fenotipo y función de las células NK es de interés el estudio de la expresión de Eomes y T-bet en células NK en relación con el envejecimiento y el seroestatus para CMV.

2.7 Leucemia Mieloide Crónica (LMC)

La Leucemia Mieloide Crónica (LMC) es un trastorno mieloproliferativo de las células madre hematopoyéticas, ocasionada por una translocación reciproca de los cromosomas 9 y 22 que dan lugar al cromosoma Filadelfia (Ph) y al oncogén BCR-ABL que codifica una proteína tirosina kinasa con una actividad aberrante, responsable de la proliferación de las células mieloides en la médula ósea. Esta yuxtaposición del gen ABL del cromosoma 9 sobre la región de corte BCR en el cromosoma 22, es generalmente aceptada como el evento iniciador de la LMC [93-95]. Aproximadamente la mitad de los casos diagnosticados con LMC son personas mayores de 65 años. La edad es un factor de mal pronóstico para la supervivencia a LMC y la respuesta a los tratamientos y la esperanza de vida es menor en pacientes mayores con LMC que en pacientes más jóvenes[96-101]. La introducción de inhibidores de la tirosina kinasa (tyrosine kinase inhibitors, TKI) como el Imatinib (Glivec®), fármaco de primera generación, y otros más potentes de segunda y tercera generación como Dasatinib (Sprycel™) y Ponatinib (Iclusig®), ha supuesto un gran avance en el

tratamiento, mejorado el pronóstico y elevando la tasa de supervivencia para enfermos con LMC. Con Imatinib la supervivencia a 8 años aumentó de un 6% en 1975 a un 87% desde el 2001. Además, es posible hablar de remisión de la enfermedad en un 50 a 60 % de pacientes que han dejado la medicación después de 5 a 10 años de tratamiento en los que se ha demostrado una respuesta citogenética (RCC) y molecular completa (RMC) [102-107].

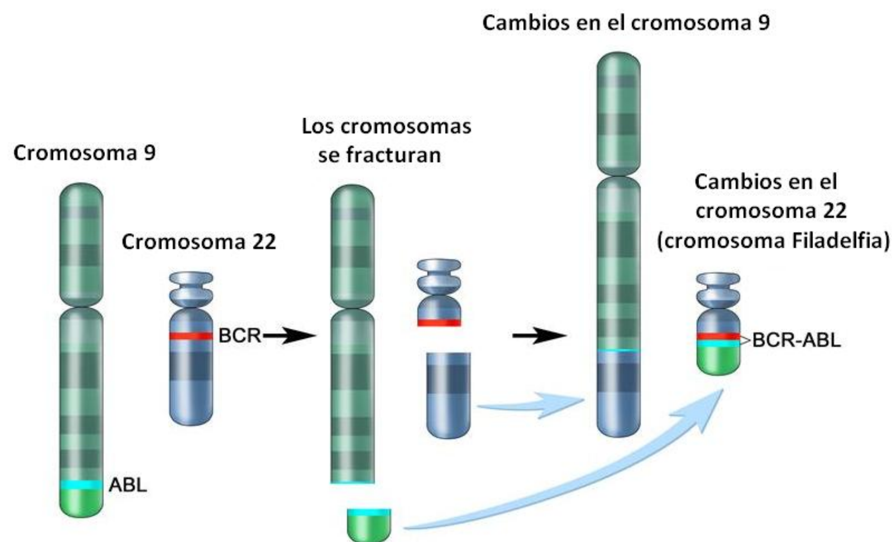

Figura 5. En células madre hematopoyéticas, la región distal del cromosoma 9 (ABL) se fusiona a la región proximal del cromosoma 22 (BCR), conformando el cromosoma "Filadelfia" y un nuevo gen de fusión BCR-ABL que codifica una oncoproteína del mismo nombre con una alta actividad proliferativa, este evento inicia la leucemia mieloide crónica. (Adaptado de Terese Winslow, 2007).

2.7.1 Células NK y LMC

Las células NK participan en el control de células tumorales en diferentes neoplasias hematológicas incluida la LMC, a través de receptores que pueden reconocer diversas oncoproteínas presentes en las células tumorales del huésped. Esta sobreexposición de las células NK a los ligandos tumorales puede ocasionar alteraciones similares a las que se observan en células NK con un fenotipo inmunosenescente [80, 108-111]. Varios estudios han encontrado una disminución en la frecuencia y función de las células NK con un deterioro funcional progresivo durante todas las fases de la enfermedad y niveles de expresión disminuidos de receptores como NKG2A, NKG2C, NKp30, NKp46 y KIR en pacientes con LMC al momento del diagnóstico,[64, 112-116] también se ha descrito una disminución del receptor activador NKG2D que media respuestas NK anti-CML a través de los ligandos MICA/B cuando son comparados con controles sanos, la sobreexposición a estos ligandos

disminuye la expresión de NKG2D, contribuyendo de esta manera a la evasión de las células tumorales de la vigilancia inmune por las células NK,[110, 117, 118]. Recientemente se ha informado sobre el impacto de los TKI sobre los receptores de las células NK, encontrando que la expresión de los receptores de activación se restablecen a niveles normales en comparación con su bajo nivel al momento del diagnóstico[115, 116].

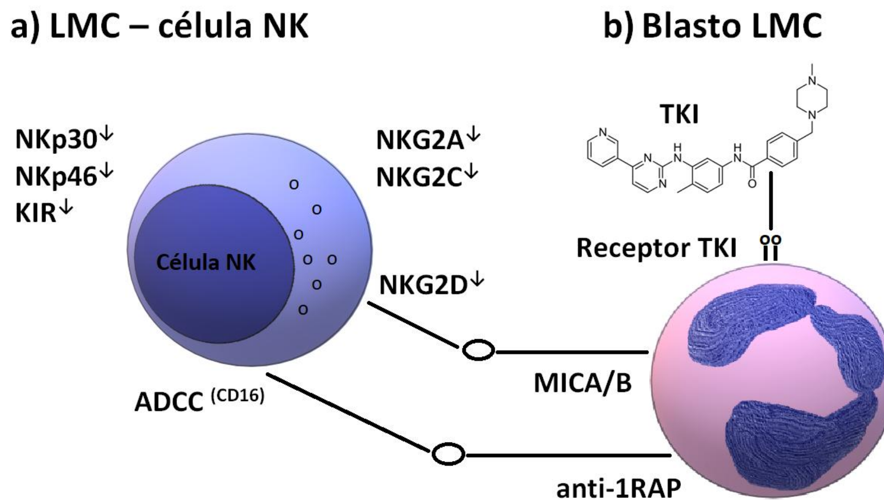

Figura 6. Receptores NK y LMC. a) Descenso de la expresión de receptores NK tras su interacción con células de la LMC. b) Ligandos de LMC para células NK, y dominio extracelular del receptor para tyrosine kinase inhibitors, TKI.

En la presente tesis doctoral presentamos los resultados, reflejados en la publicación de tres artículos de investigación originales, con el objetivo de profundizar en los cambios que se producen en las células NK con la edad y la infección por CMV y en pacientes con LMC tratados con TKI.

3. Hipótesis y Objetivos

El envejecimiento, la infección por CMV y la Leucemia Mieloide Crónica, se asocian a cambios en el fenotipo y función de las células NK.

Los objetivos derivados de la Tesis Doctoral son:

Objetivo 1

Investigar el efecto de la infección latente por CMV y del envejecimiento sobre la expresión de los receptores activadores NKp46, NKp30 y DNAM-1 en células NK en reposo y activadas con IL-2 definidas por la expresión CD56, CD16 y CD57. Abordamos este objetivo en el artículo: "**Expression of NKp30, NKp46 and DNAM-1 activating receptors on resting and IL-2 activated NK cells from healthy donors according to CMV-serostatus and age**". Biogerontology. 2015, 16:671–683doi: 10.1007/s10522-015-9581-0

Objetivo 2

Analizar el efecto de la edad y la seropositividad por CMV sobre la expresión de los receptores inhibitorios CD300a y CD161 y de los factores de transcripción Eomes y T-bet en las subpoblaciones de células NK $CD56^{bright}$ y $CD56^{dim}$ definidas por la expresión del marcador CD57. Abordamos este objetivo en el artículo: **"Effect of CMV and aging on the differential expression of CD300a, CD161, T-bet, and Eomes on NK cell subsets".** Frontiers in Immunology. Nov. 07; 2016, https://doi.org/10.3389/fimmu.2016.00476

Objetivo 3

Estudiar la expresión de receptores, marcadores de activación y de diferenciación NK, tales como (CD11b, CD27, CD57, CD69, HLA-DR, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46 y NKp80) en subpoblaciones de células NK $CD56^{bright}$ y $CD56^{dim}$ y el porcentaje de CD107a e IFN-γ en células NK estimuladas con la línea celular K562, de pacientes con LMC y controles sanos, estratificados según la edad en donantes de mediana edad y ancianos. Abordamos este objetivo en el artículo: **"Effect of age on NK cell compartment in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors**". Frontiers in Immunology. Nov. 08; 2018, https://doi.org/10.3389/fimmu.2018.02587

4. Donantes, Materiales y Métodos, Resultados y Discusión

I. Expression of NKp30, NKp46 and DNAM-1 activating receptors on resting and IL-2 activated NK cells from healthy donors according to CMV-serostatus and age.

*Carmen Campos, **Nelson López**, Alejandra Pera, Juan J. Gordillo, Fakhri Hassouneh, Raquel Tarazona, Rafael Solana

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* La primera autora de este trabajo fue codirectora de tesis desde febrero de 2015 hasta septiembre de 2018 fecha en la que se vio obligada a presentar su renuncia a continuar con la dirección del trabajo al haber obtenido por oposición una plaza de Profesora Técnica de Formación Profesional fuera de Córdoba. La normativa de la UCO permite que el doctorando pueda “ocupar el segundo lugar de los autores siempre que el primer lugar sea ocupado por la persona que ostente la dirección de la tesis” como en este caso.

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RESEARCH ARTICLE

Expression of NKp30, NKp46 and DNAM-1 activating receptors on resting and IL-2 activated NK cells from healthy donors according to CMV-serostatus and age

Carmen Campos · Nelson López · Alejandra Pera · Juan J. Gordillo · Fakhri Hassouneh · Raquel Tarazona · Rafael Solana

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Abstract Human natural killer (NK) cells are innate lymphoid cells with capacity to kill tumor cells and virus-infected cells. According to the expression of CD56 and CD16 several NK cell subsets have been identified, a major CD56dimCD16+ subpopulation characterized by higher cytotoxic capacity, two CD56bright subsets (CD16−and CD16+) that represent different maturation stages and the fourth CD56−CD16+ subset that correspond to activated dysfunctional NK cells. Previous studies have shown quantitative changes in the frequency, phenotype and distribution of NK cell subsets depending on CMV-serostatus and age. We have analyzed the expression of NKp30, NKp46 and DNAM-1 NK activating receptors on resting and IL-2 activated NK cells from CMV-seronegative and seropositive healthy young donors and from CMV-seropositive elderly individuals. Our results showed that CMV-serostatus of healthy young donors is associated with phenotypic differences on both CD56bright and CD56dim NK cells with an increase of NKp46 and a decrease of NKp30 expression respectively. A reduced expression of DNAM-1 related to ageing and a lower NKp30 expression associated with CMV-seropositivity were observed. The expression of NKp46 and NKp30 was lower in CD57+ NK cells while the expression of DNAM-1 was increased. In vitro NK cell activation by IL-2 increased the expression of NKp46 and NKp30. In summary, both age and CMV-serostatus influence the expression of these cytotoxicity activating receptors that will have functional consequences. In elderly donors is difficult to isolate age from the effect of chronic CMV infection since in our study all elderly donors were CMV-seropositive. The possibility of modulating the expression of these activating receptors by cytokines such as IL-2 may open new opportunities for improving age-associated deterioration of NK cell function.

Keywords CMV · Ageing · NK cell subsets · CD57 · NKp30 · NKp46 · DNAM-1

Rafael Solana and Raquel Tarazona are co-senior authors with similar contribution to the manuscript

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C. Campos · N. López · A. Pera · F. Hassouneh · R. Solana (✉)
Department of Immunology, IMIBIC - Reina Sofia University Hospital, University of Cordoba, Av. Menéndez Pidal S/N, 14004 Córdoba, Spain
e-mail: rsolana@uco.es

J. J. Gordillo · R. Tarazona
Immunology Unit, University of Extremadura, Caceres, Spain

Introduction

Human natural killer (NK) cells are innate lymphoid cells representing 10–20 % of peripheral blood

lymphocytes with capacity to kill tumor cells and virus-infected cells without previous sensitization. NK cell functional capacity depends on a balance between activating and inhibitory signals triggered by activating and inhibitory receptors (Hamerman et al. 2005; Lanier 2008; Moretta et al. 2008). These cells do not rearrange T or B cell gene receptors and are characterized by the expression of CD56 and/or CD16. NK cells can be activated in vitro with interleukin-2 (IL-2) and other cytokines resulting in increased cytotoxic activity against NK susceptible and NK resistant target cells and higher capacity to produce cytokines (Zwirner and Domaica 2010). Based on the expression of CD56 and/or CD16, peripheral blood NK cells can be classified in more immature CD56bright CD16null/low that have an immune-modulatory role with high production of cytokines, and mature CD56dimCD16+, mainly cytotoxic and interferon-gamma producers (Cooper et al. 2001).In addition CD57 expression defines a subset of mature highly differentiated CD56dim NK cells (Bjorkstrom et al. 2010b; Lopez-Verges et al. 2010; Poli et al. 2009; Cichocki et al. 2013; Nielsen et al. 2013). A subset of NK cells that do not express CD56 but express other NK receptors such as CD16, CD94 and CD161 was originally defined in HIV-1 infected patients (Tarazona et al. 2002). CD56 negative NK cells were found to be expanded in hepatitis C virus infection (Gonzalez et al. 2009) and in old donors (Campos et al. 2014b; Solana et al. 2014) compared to young healthy subjects (Bjorkstrom et al. 2010a).The function of this subset and the relationship with other NK subsets are unclear. CD56−CD16+NK cells expanded in HIV-1 seropositive individuals are dysfunctional NK cells (Mavilio et al. 2005) and it has been recently shown that a subset of these cells are activated, mature NK cells with impaired effector function that have recently engaged target cells (Milush et al. 2013).

Previous studies have shown quantitative changes in the frequency, phenotype and distribution of NK cells in ageing and in circumstances of chronic activation of the immune system (Camous et al. 2012; Solana et al. 2012; Gayoso et al. 2011). Changes in NK cell subset distribution that have been associated with aging include a decrease of the CD56bright NK cell subset and the expansion of CD56−NK cells (Borrego et al. 1999; Chidrawar et al. 2006; Tarazona et al. 2009; Campos et al. 2014b; Solana et al. 2014). Neither the expression of CD16 nor its capacity to trigger antibody dependent NK cell cytotoxicity are affected by ageing (Mariani et al. 1998; Solana and Mariani 2000). There are evidences that alterations in the number and cytotoxicity of NK cells associate to a greater risk of infections and mortality in the elderly (Camous et al. 2012; Grubeck-Loebenstein et al., 2009; Larbi et al. 2008; DelaRosa et al. 2006).

Human cytomegalovirus (CMV) is a persistent ß-herpesvirus which infects all human populations with a variable prevalence. CMV-seropositivity increases with age and depends on geographic, ethnic and social factors (Cannon et al. 2010). CMV prevalence is very high in Spain and more than 80 % of individuals over the age of 40 years are CMV-seropositive (deOry et al. 2004). CMV infection of immunocompetent subjects is associated with an age-related deterioration of the immune system, with accumulations of late-differentiated CD8+ T cells and with the development of an “Immune Risk Phenotype” (IRP), predictive of early mortality in the elderly (Pawelec and Derhovanessian 2011; Derhovanessian et al. 2009; Wikby et al. 2005; Koch et al. 2007; 2006; Pawelec et al. 2005).

Cytomegalovirus infection of healthy individuals induces the expression of NKG2C on NK cells (Guma et al. 2004), that can also co-express CD57 (Wu et al. 2013; Lopez-Verges et al. 2011). This subset of NKG2C + CD57 + NK cells plays an important role on CMV control (Guma et al. 2004; Lopez-Verges et al. 2011; Della et al. 2012; Lopez-Verges et al. 2011; Wu et al. 2013; Muntasell et al. 2013b) and it is expanded in CMV-seropositive patients undergoing other viral infections (Petitdemange et al. 2011; Bjorkstrom et al. 2011; Beziat et al. 2012) suggesting (Muntasell et al. 2013b) that these cells might represent the equivalent of memory-like NK cells expressing Ly49H observed in CMV infected mice (Marcus and Raulet 2013; Min-Oo et al. 2013).

Considering that CMV infection contributes to age-associated changes in NK cells and that ageing also affects the frequency and cytotoxic capacity of NK cells, in this work we have investigated the effect of latent CMV infection and ageing in the expression of NK activating receptors in resting and IL-2 activated NK cells. Thus, we studied the expression of NKp46, NKp30 and DNAM-1 on NK cell subsets defined by the expression of CD56, CD16 and CD57 and on IL-2 activated NK cells from healthy individuals stratified by CMV status and age.

Materials and methods

Subjects and samples

Peripheral blood samples from healthy donors were obtained in heparinized tubes and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Histopaque-1077 density gradient centrifugation (Sigma Aldrich, St Louis, MO, USA). These cells were frozen in FBS (Sigma Aldrich, St Louis, MO, USA) 10 % DMSO (Panreac Química S.A.U., Barcelona, Spain) and preserved in liquid nitrogen for subsequent utilization.

A sample of plasma was extracted from all donors to analyze CMV-specific IgG and IgM. CMV-serology was determined by using automated CMV enzyme-linked immunosorbent assay (Genesis Diagnostics, Cambridge, UK), according to manufacturer's instructions. Concentrations below 3 IU/ml were considered negative for anti-CMV IgG. Young donors were stratified according to CMV-serology in CMV-seronegative and CMV-seropositive. All elderly donors were CMV-seropositive. The prevalence of CMV-seropositivity in Spain increases with age from 45 % in infants of 2–5 years to 93 % in the age group of 41–60 years old (deOry-Manchon et al. 2001; deOry et al. 2004). This prevalence is similar to the prevalence reported in other south European countries such as France (Gratacap-Cavallier et al. 1998). In our hands the prevalence of CMV-seropositivity in the 40–60 years old group is 90 % and in the > 61 years old group is 99 % in Andalusia (unpublished). All volunteer donors were selected and included in the study, according to the inclusion criteria shown in Table S1 (Electronic Supplementary Material). The study was approved by the Ethics Committee of the Reina Sofia University Hospital. All volunteers agreed and signed informed consent to participate.

Flow cytometric analysis and monoclonal antibodies

A sample of frozen cells from each donor was used for flow cytometric analysis. The thawing was carried out in RPMI 1640 (Sigma Aldrich, St Louis, MO, USA) 20 % FBS and then cells were left in an incubator at 37 °C and 5 % CO_2 for 1 h. Later, cells were stained with an appropriate combination of fluorochrome conjugated mAbs for the analysis of surface markers. Cells were resuspended in MACSQuant Running Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and multicolour flow data was acquired on a MACSQuant cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood lymphocytes were selected using forward and side scatter detectors and NK cell subpopulations were identified on CD3- cells by the differential expression of surface markers CD56 and CD16 (Figure S1, Electronic Supplementary Material). Data were analyzed using FlowJo software (Tree Star, OR, USA).

The expression of NKp30, NKp46 and DNAM-1, measured as normalized median fluorescent intensity (NMFI) was determined in the different NK cell subpopulations and analyzed by multiparametric flow cytometry. The expression of NKp30, NKp46 and DNAM-1 was also measured on CD57+ and CD57− NK cells. NMFI was calculated by dividing MFI of the stained sample by MFI of the negative control on the same channel.

The following monoclonal antibodies (mAbs) were used: anti-CD3 (clone SK7, BD Biosciences), anti-CD56 (clone B159, BD Pharmingen), anti-CD16 (clone 3G8, BD Pharmingen), anti-CD57 (clone TB03, Miltenyi Biotec), anti-NKp30 (clone p30-15, BD Pharmingen), anti-NKp46 (clone 195314, R&D System), anti-DNAM-1 (clone DX11, Miltenyi Biotec), labelled with peridinin chlorophyll protein, PE-Cy7, APC-Cy7, VioBlue, phycoerythrin, fluorescein isothiocyanate and allophycocyanin, respectively. Isotype controls labelled with the different fluorochromes were used in all experiments. All mAbs were IgG1 isotype, except anti-CD57 and anti-NKp46 that were IgM and IgG2b respectively.

IL-2 activation of NK cells and flow cytometry analysis of CD3-CD56+ activated cells

IL-2 activated cells were induced by culturing thawed PBMCs (2×10^6 PBMCs/mL) for 5 days in complete RPMI 1640 medium (Sigma Aldrich, St Louis, MO, USA) supplemented with 10 % Human Serum, 1 % Glutamax, 1 % pyruvate, 1 % penicilin/streptomicin and recombinant human IL-2 (at final concentration of 500 U/mL) at 37 °C in 5 % CO_2. The expression of NKp30, NKp46 and DNAM-1 (NMFI) was determined on CD3-CD56 + IL-2 activated NK cells and on CD57+ and CD57− subsets as indicated above. The fold change was calculated by dividing the NMFI

of NKp30, NKp46 and DNAM-1 on IL-2 activated NK cells by the NMFI of these receptors on resting NK cells.

Statistical analysis

Data were analyzed with SPSS for Windows version 17.0 (SPSS Inc., Chicago). The Shapiro–Wilk test was applied to check the normal distribution of the variables. For direct comparison of three independent samples, Kruskal–Wallis H test (non-parametric) was used. Mann–Whitney *U* test (non-parametric) was applied to analyze the specific sample pairs for significant differences. For comparison of two related samples, Wilconson test (non-parametric) was used.

For the graphic representation we used GraphPad Prism 5 software. All data were expressed as median with interquartile range and results were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Differences in the expression of NKp46, NKp30, and DNAM-1 on NK cells associated with age and CMV-seropositivity

The expression of NK cell receptors NKp30, NKp46 and DNAM-1 was determined in the different NK cell subpopulations from young and elderly healthy donors by multiparametric flow cytometry. Our results showed an increase in the expression of NKp46 on CD56bright NK cells (CD16+ and CD16−) from CMV-seropositive compared with CMV-seronegative young donors (Fig. 1A). We also observed a decrease in the expression of NKp46 on CD56bright NK cells (CD16+ and CD16−) of healthy elderly but only when it is compared with young CMV-seropositive individuals and not when compared with young CMV-seronegative donors. On the other hand, we found a decrease in the expression of NKp46 on CD56− CD16+ NK cells from healthy elderly (CMV-seropositive) when compared with young healthy individuals (CMV-seropositive and CMV-seronegative).

When we analysed the expression of NKp30 on the surface of NK cell subpopulations we found a decreased expression on total NK cells, CD56dimCD16+ NK cells and CD56− CD16+ NK cells from CMV-seropositive young individuals and elderly subjects when compared with young individuals CMV-seronegative (Fig. 1B).

Analysis of DNAM-1 on NK cell subpopulations also revealed a decreased expression on total and CD56dimCD16+ NK cells from elderly subjects when compared to young individuals CMV-seronegative and CMV-seropositive (Fig. 1C).

CD57+ NK cells differentially express NK activating receptors

The expression (NMFI) of NKp46, NKp30 and DNAM-1 was also measured on the different NK cell subsets according to the expression of CD57 (Figure S1, Electronic Supplementary Material). Since CD57 expression is very low or absent on the CD56bright cells these subsets were not considered.

The analysis of NKp46, NKp30 and DNAM-1 expression on CD57+ showed a decrease of NKp46 and NKp30 and an increase of DNAM-1 expression on total NK cells as well as on CD56dimCD16+ and CD56− CD16+ NK cell subsets compared with CD57− NK cells (Fig. 2A).

As shown in Fig. 2B the expression of NKp46 on CD57+ or CD57− is not significantly different among the groups of donors considered. On the contrary the expression of NKp30 on CD57+ NK cells is reduced in CMV-seropositive young and elderly groups compared with CMV-seronegative young donors. A decrease of NKp30 expression was also observed on CD57− NK cells in old donors when compared with CMV-seronegative young individuals. The expression of DNAM-1 is decreased on both CD57+ or CD57− NK cells in CMV-seropositive elderly groups compared with CMV-seropositive and seronegative young donors.

Expression levels of NKp46, NKp30 and DNAM-1 on IL-2 activated NK cells

The expression of NKp46, NKp30 and DNAM-1 was analysed on resting and IL-2 activated NK cells, according to CD57 expression. IL-2 activation induced an upregulation of CD56 and a downregulation of CD16 (not shown) that did not allow the analysis of NK cell subsets according to CD56 and/or CD16 expression, thus this part of our study focussed on resting and IL-2 activated NK cells defined as

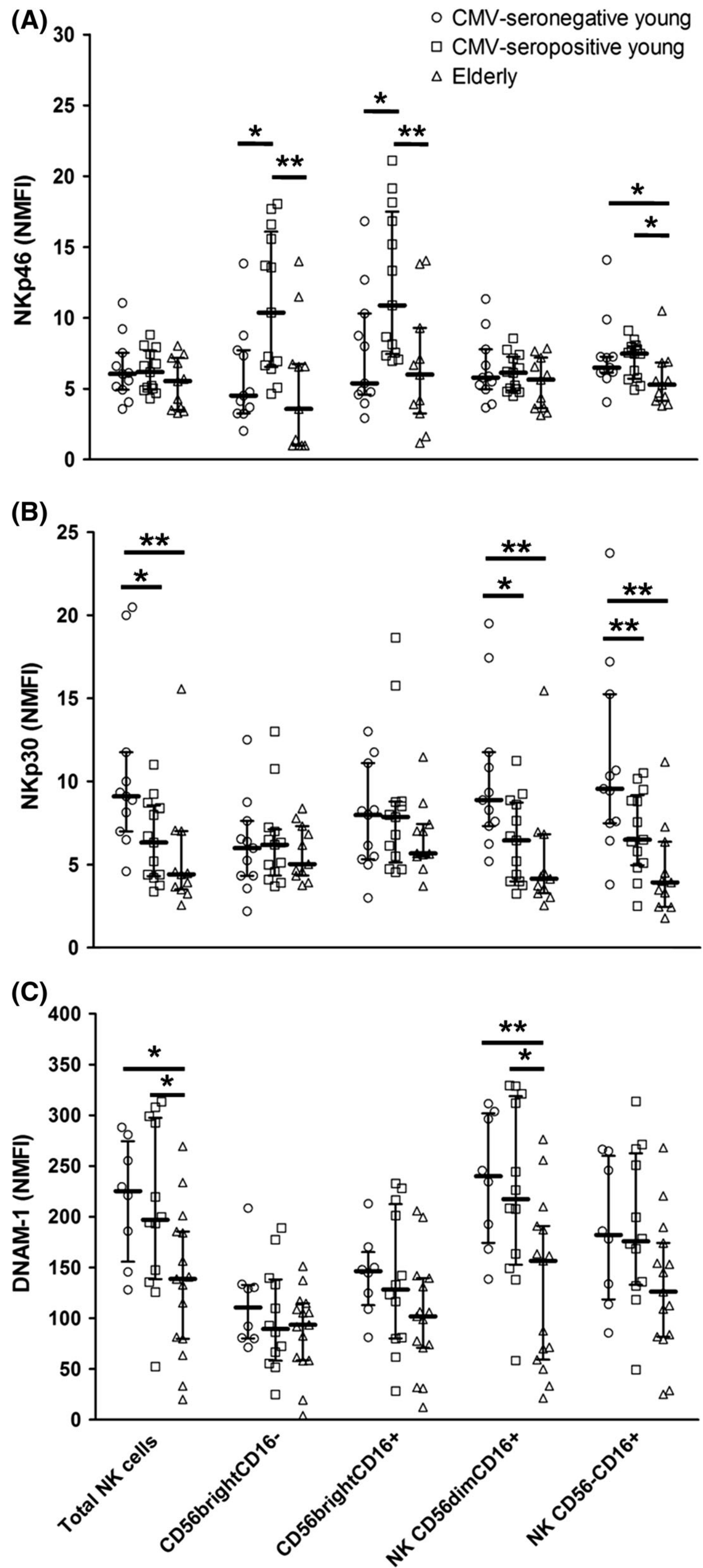
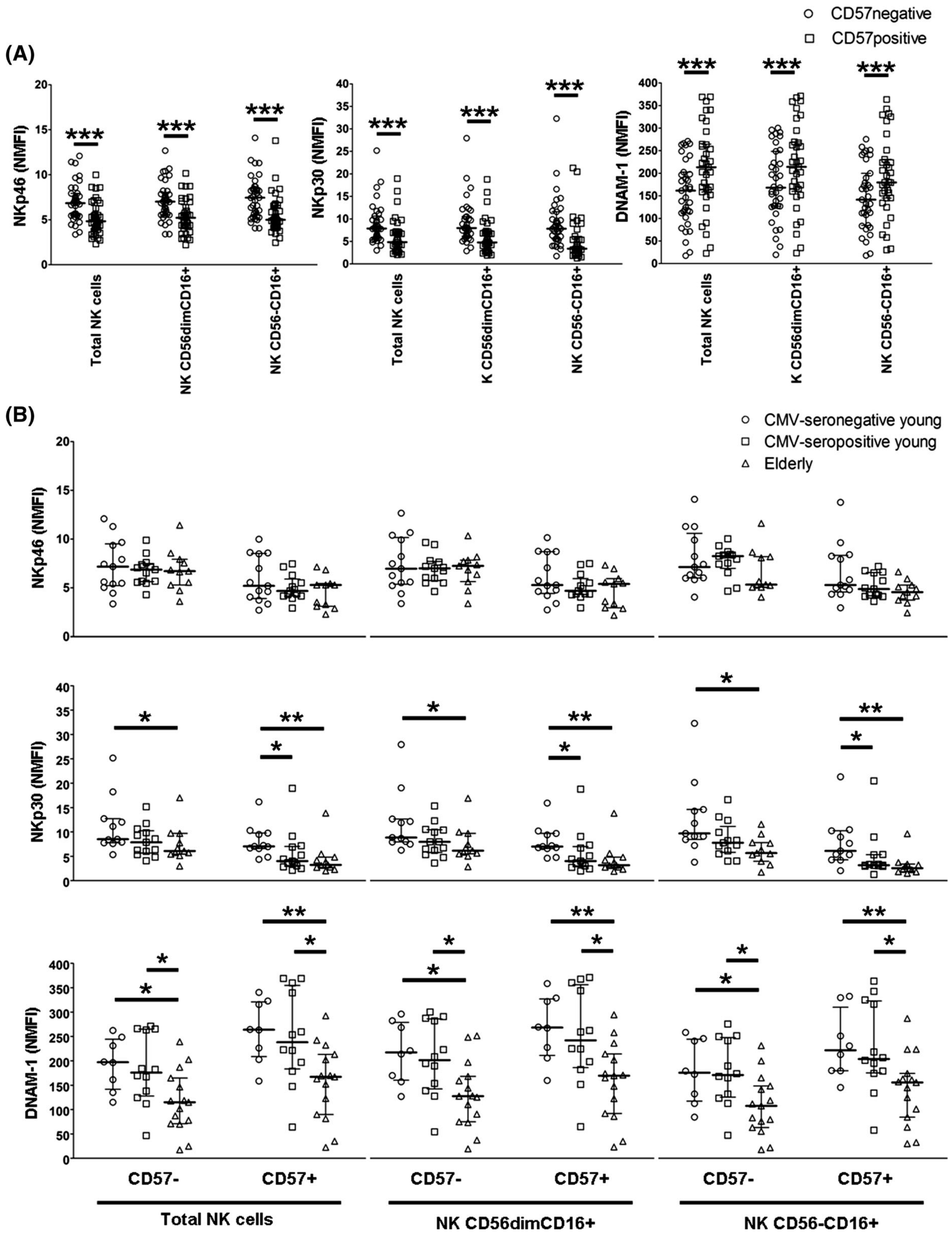


Fig. 1 Expression of NKp46, NKp30 and DNAM-1 on NK cells from young and elderly healthy donors. Expression of NKp46, NKp30 and DNAM-1, measured as normalized median fluorescent intensity (NMFI), was determined on the different NK cell subpopulations from three groups of donors: CMV-seronegative young (*circle*), CMV-seropositive young (*square*) and elderly donors (*triangle*). **a** Expression of NKp46 was determined in the different NK cell subpopulations from CMV-seronegative young (n = 13), CMV-seropositive young (n = 13) and elderly donors (n = 11; all CMV-seropositive). **b** Expression of NKp30 was determined in the different NK cell subpopulations from CMV-seronegative young (n = 11), CMV-seropositive young (n = 13) and elderly donors (n = 11). **c** Expression of DNAM-1 was determined in the different NK cell subpopulations from CMV-seronegative young (n = 8), CMV-seropositive young (n = 12) and elderly donors (n = 15). Kruskal–Wallis *H* test (non-parametric) was used to compare three independent samples, and Mann–Whitney *U* test was applied to analyze the specific sample pairs for significant differences. The data were expressed as median with interquartile range. Results were considered significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$



CD57negative
CD57positive
(A)
NKp46 (NMFI)
NKp30 (NMFI)
DNAM-1 (NMFI)
***
Total NK cells
NK CD56dimCD16+
NK CD56-CD16+
K CD56dimCD16+
(B)
CMV-seronegative young
CMV-seropositive young
Elderly
NKp46 (NMFI)
NKp30 (NMFI)
DNAM-1 (NMFI)
CD57-
CD57+
Total NK cells
NK CD56dimCD16+
NK CD56-CD16+

◀**Fig. 2** Analysis of NK activating receptors on total NK cells and on CD56dimCD16+ and CD56−CD16+ subsets in relation to CD57 expression. **a** Comparison of NKp46, NKp30 and DNAM-1 expression (NMFI) on CD57− versus CD57+ cells in all healthy individuals enrolled in the study (Wilconson test) **b** expression of NKp46, NKp30 and DNAM-1 on CD57+ and CD57− NK cell subsets was compared among CMV-seronegative young, CMV-seropositive young and elderly donors. (Kruskal–Wallis *H* test was used to compare independent samples, and Mann–Whitney *U* test was applied to analyze the specific sample pairs for significant differences). The data were expressed as median with interquartile range. Results were considered significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

CD3− CD56+ (Fig. 3A). Results in Fig. 3B showed an increased expression of NKp46 and NKp30 on IL-2 activated NK cells when compared with resting NK cells independently of the expression of CD57− in all donors independently of CMV-serostatus or age. No significant differences were observed in the expression of DNAM-1 in the CMV-seronegative and old donors, whereas a decreased expression of DNAM-1 was observed on CD57− NK cells from CMV-seropositive young donors (Fig. 3B). Analysis of the fold change (Fig. 3C) in the expression of NKp46, NKp30 and DNAM-1 on IL-2 activated NK cells versus resting NK cells revealed a significant increase in the expression of NKp30 in CMV-seropositive young individuals when compared with CMV-seronegative young or CMV-seropositive old individuals, whereas the fold change of NKp46 and DNAM-1 was not significantly different among the groups.

Discussion

Cytomegalovirus infection induces profound changes in the T cell compartment in human ageing (Koch et al. 2007; Pawelec et al2005; 2012; Pawelec and Derhovanessian 2011; Derhovanessian et al. 2009). Ageing is also associated with changes in NK cell phenotype and function (Solana et al. 2012; Solana et al. 2006) and CMV infection also has an impact on NK cells (Muntasell et al. 2013b; Monsivais-Urenda et al. 2010; Guma et al. 2006). CMV-seroprevalence shows substantial geographic variation, differing by as much as 30 % points between countries (Cannon et al. 2010), with a North–South gradient with people born in the southern regions having a higher seropositivity rate (Gratacap-Cavallier et al. 1998). As indicated in the materials and methods, the prevalence of CMV-seropositivity in Spain (Madrid region) increases with age from 45 % in infants of 2–5 years to 93 % in the age group of 41–60 years old (deOry-Manchon et al. 2001; deOry et al. 2004), percentages similar to those found in our geographic area (Andalusia) (unpublished observation).

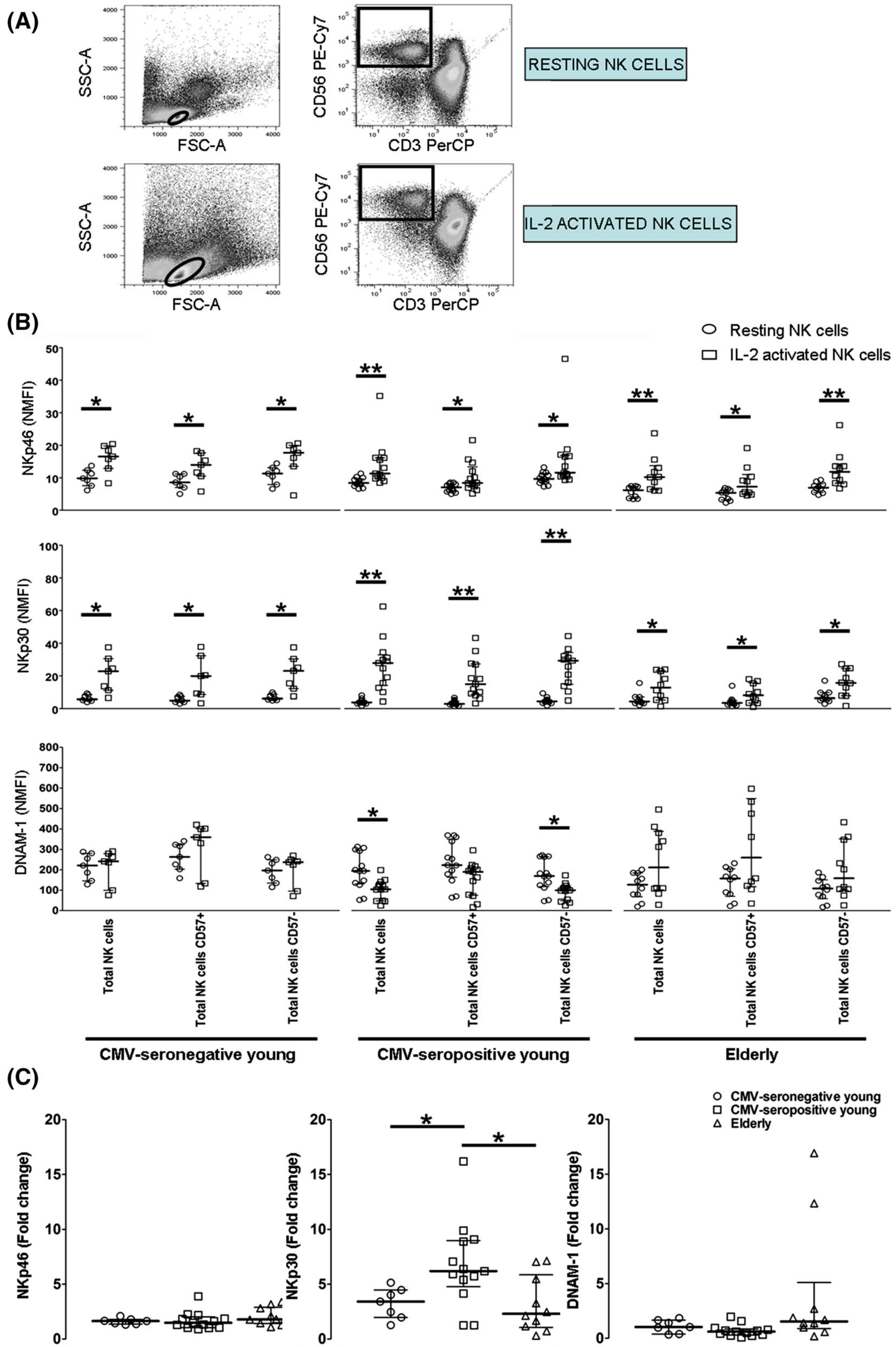
In this work we have analyzed the effect of CMV-seropositivity and ageing on the expression of NK activating receptors on NK cell subsets defined by the expression of CD56, CD16 and CD57.

Differences in CD56bright NK cells

Peripheral blood CD56brightCD16− NK cells are immature NK cells and CD56bright NK cells that co-express CD16 represent an intermediate differentiation stage to the more mature CD56dimCD16+ NK cells (Beziat et al. 2011). Our results show that CMV-seropositivity in young individuals is associated with a significant increase in the expression of NKp46 on CD56bright NK cells. The expression of this activating receptor on NK cells tends to increase in vitro when cocultured with CMV-infected myelomonocytic cells (Muntasell et al. 2013a) and it has been shown that it participates in the recognition of CMV-infected cells (Magri et al. 2011; Romo et al. 2011). However, in old donors (all of them CMV-seropositive) its expression is low, supporting that age has a negative impact on CD56bright NK cells additional to the decreased frequency of CD56bright NK cells previously shown in CMV-seropositive old individuals (Campos et al. 2014b; 2014a). This is likely the consequence of the decreased output of new NK cells (Zhang et al. 2007; Solana et al. 2014), possibility that is supported by the demonstration that old mice show a defective maturation of NK cells due to the decreased capacity of bone marrow stroma cells to support final stages of NK differentiation (Beli et al. 2014; Chiu et al. 2013).

Differences in CD56dimCD16+ NK cells

CD56dimCD16+ NK cells represent the major population of peripheral blood NK cells with cytotoxic capacity and producers of gamma interferon. CMV has developed mechanisms to evade recognition by NK cells that help CMV to persist in the human host and attain a state of chronic infection. One of these mechanisms is the antagonistic effect of the main


(A)
SSC-A
FSC-A
CD56 PE-Cy7
CD3 PerCP
RESTING NK CELLS
IL-2 ACTIVATED NK CELLS
(B)
Resting NK cells
IL-2 activated NK cells
NKp46 (NMFI)
NKp30 (NMFI)
DNAM-1 (NMFI)
Total NK cells
Total NK cells CD57+
Total NK cells CD57-
CMV-seronegative young
CMV-seropositive young
Elderly
(C)
CMV-seronegative young
CMV-seropositive young
Elderly
NKp46 (Fold change)
NKp30 (Fold change)
DNAM-1 (Fold change)

◀ **Fig. 3** Expression levels of NKp46, NKp30 and DNAM-1 on IL-2 activated NK cells. **a** Gating strategy to study resting and IL-2 activated NK cells (defined as CD3−CD56+) analysed by flow cytometry. **b** Expression of NKp46, NKp30 and DNAM-1 on resting and IL-2 activated NK cells according to CD57 expression in all healthy individuals enrolled in this study stratified by CMV-serostatus and age (n = 29, 7 CMV-seronegative young, 12 CMV-seropositive young and 10 elderly donors) (Wilconson test). **c** Fold change in the expression of NKp46, NKp30 and DNAM-1 between IL-2 activated and resting NK cells in the three groups of donors (Kruskal–Wallis *H* test was used to compare independent samples, and Mann–Whitney *U* test was applied to analyze the specific sample pairs for significant differences). The data were expressed as median with interquartile range. Results were considered significant at *$p < 0.05$

HCMV tegument protein, pp65, on NKp30 (Arnon et al. 2005; Rajagopalan and Long 2005). The analysis of the CD56dimCD16+ NK cell subsets shows a decreased expression of NKp30 in CMV-seropositive young and old individuals compared with CMV-seronegative young individuals.

Interestingly, NKp46 has been shown to play a significant role in the in vitro response of NK cells against CMV-infected monocyte-derived dendritic cells (moDCs) (Magri et al. 2011). NKp46 expression on CD56dim NK cell subset is similar in the three groups of donors analysed. The expression of NKp30 is decreased in the CD56dim NK cells in particular in the sub-populations that also express CD57, supporting that CMV promotes a maturation of these NK cells that is associated with the decrease of this activating receptor.

The percentage of CD56dimCD16+ NK cells expressing CD57 are increased in CMV-seropositive individuals and further increased by age (Campos et al. 2014b). These cells represent highly differentiated NK cells with low proliferative capacity and high cytotoxicity. Our results show that the expression of NKp30 and NKp46 are decreased in this subpopulation of NK cells as previously described (Lopez-Verges et al. 2010; 2011). Contrary to the observation for NKp30 and NKp46, the expression of the activating receptor DNAM-1 is increased on the CD57+ subpopulation of CD56dimCD16+ NK cells.

The analysis of DNAM-1 expression on CD56dimCD16+ NK cells show that it is reduced in elderly donors independently of CD57 expression. No statistically significant differences were observed according to CMV-serostatus in young donors suggesting that DNAM-1 expression is more affected by age than by CMV infection. DNAM-1 has been shown to be required for NK cell-mediated host defence against MCMV infection in mice since the blockade of DNAM-1 with mAbs inhibited the generation of MCMV-specific Ly49H+ memory-like NK cells and there was a defective expansion and differentiation to memory-like NK cells of DNAM-1-deficient Ly49H+ NK cells indicating that cooperative signalling through DNAM-1 and Ly49H are required for NK cell-mediated host defence against MCMV infection (Nabekura et al. 2014). Thus, it can be suggested that the expression of DNAM-1 in CD57+ NK cells can contribute to the anti-CMV response of human NK cells, although the role of DNAM-1 expressed on NK cells on the response to CMV requires to be confirmed in humans. In addition killing of moDCs infected by CMV is dependent on the interaction between DNAM-1 and DNAM-1 ligands stressing the importance of the downregulation of this activating receptor on CMV evasion of NK cells (Magri et al. 2011). This is further supported by evidences showing that CMV can downregulate the DNAM-1 ligands on CMV infected cells limiting DNAM-1-mediated activation of NK cells (Prod'homme et al. 2010; Tomasec et al. 2005).

A direct correlation exists between the surface density of NCR and the ability of NK cells to kill various target cells (Biassoni et al. 2001; Moretta et al. 2001). In a similar way the decreased expression of DNAM-1 on NK cells is associated with decreased NK cell cytotoxicity (Sanchez-Correa et al. 2012) supporting that the decreased expression of NKp30 and DNAM-1 on NK cells found in old individuals contributes to the decreased per-cell cytotoxicity observed in the elderly (Mariani et al. 1996; Solana and Mariani 2000).

Differences in CD56−CD16+NK cells

The differences on the expression of NKp30 and DNAM-1 observed in CD57+CD56−CD16+NK cells are similar to those observed in the CD56dim NK cell subset. This population represents dysfunctional NK cells increased in old donors (Campos et al. 2014b) and changes in this subset were associated with ageing (decreased DNAM-1 expression) and CMV (decreased NKp30 expression). In addition we observed that the expression of NKp46 on CD56−CD16+ is lower in elderly donors. Further analysis of this subset will be required in order to establish the relevance of these CMV and age related alterations.

Differences in the expression of activating receptors by IL-2 activation of NK cells

As indicated in results, IL-2 activation induces differences in the expression of CD56 and CD16 that make impossible to distinguish NK subsets according to the expression of these markers. Thus we compare the total population of CD3−CD56+ resting and IL-2 activated NK cells. IL-2 activation induces an increase in the expression of NKp46 and NKp30 on NK cells, both in the CD57+ and the CD57− subpopulations in all groups of donors. The expression of DNAM-1 in CMV-seropositive young donors is reduced in total and CD57− NK cells. The analysis of the fold change of NKp46 and DNAM-1 after IL-2 activation in relation with CMV-serostatus and age shows no differences among the donor groups studied. The NKp30 fold increases in CMV-seropositive young donors in comparison to CMV-seronegative young and CMV-seropositive old donors.

It has been shown that NK cells from healthy donors and cancer patients can acquire a potent cytolytic activity after in vitro stimulation with IL-2. This enhancement of NK cytotoxicity has been associated in several studies to the increased expression of activating NK receptors (deRham et al. 2007; Brehm et al. 2011), supporting that the expression of activating receptors can be enhanced in NK cells by IL-2 treatment. In a recent work, IL-2-cultured NK cells obtained from cancer patients pleural effusions display increased expression of certain activating NK receptors, including NKp30 and DNAM-1 but not NKp46 (Vacca et al. 2013), supporting that the expression of NK activating receptors can be enhanced by IL-2 treatment. The discrepancies with our data are likely due to the methodological differences including the individuals studied and the origin of NK cell populations.

In conclusion, in this study we have further characterized the previously described redistribution of NK cell subsets associated with ageing and CMV-serostatus. These results emphasize the significance of determining CMV-serostatus in those studies addressed to analyze the immune response in the elderly (Camous et al. 2012; Solana et al. 2012; Gayoso et al. 2011). CMV-serostatus of healthy young donors is associated with phenotypic differences on both CD56bright (increase of NKp46) and CD56dim NK cells (decrease of NKp30). Lower expression of DNAM-1 was, in general, associated with age whereas decreased NKp30 expression was related to CMV-seropositivity. A limitation of the analysis of the changes associated with CMV is the impossibility to include a group of CMV-seronegative elderly individuals in our geographic area since most elderly donors are CMV-seropositive. This fact does not permit us to totally exclude the possible role of long-term CMV infection in these NK cell alterations. Both age and CMV-associated differences observed in the expression of cytotoxicity activating receptors may have functional relevance not only against CMV infection but also against other age-associated diseases as cancer. The possibility of modulate activating receptor expression by cytokines may open new opportunities for improving age-associated deterioration of NK cell function.

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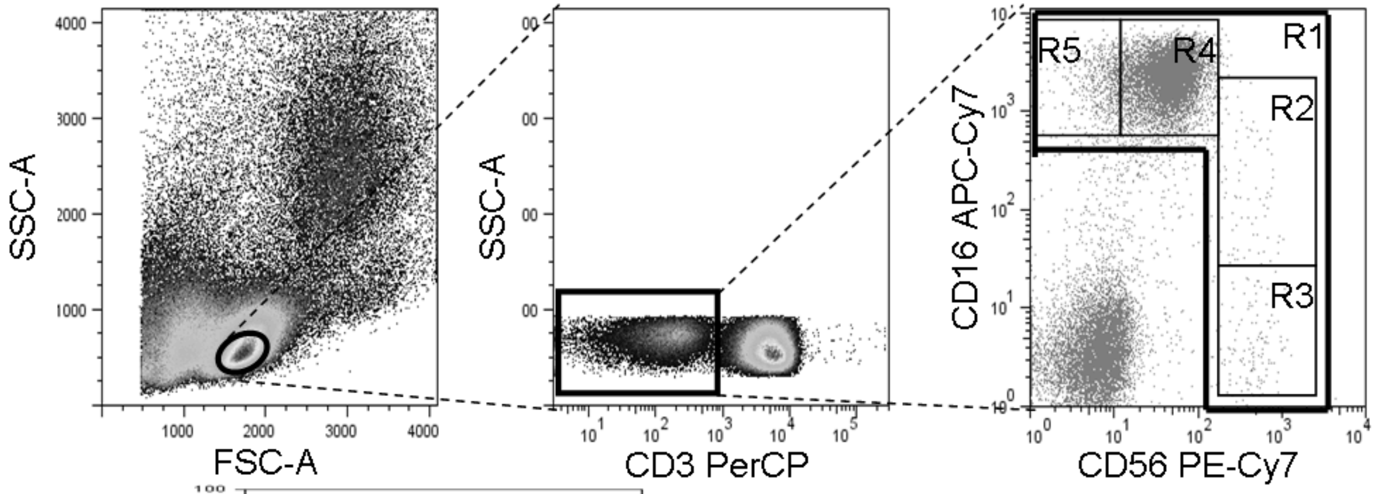
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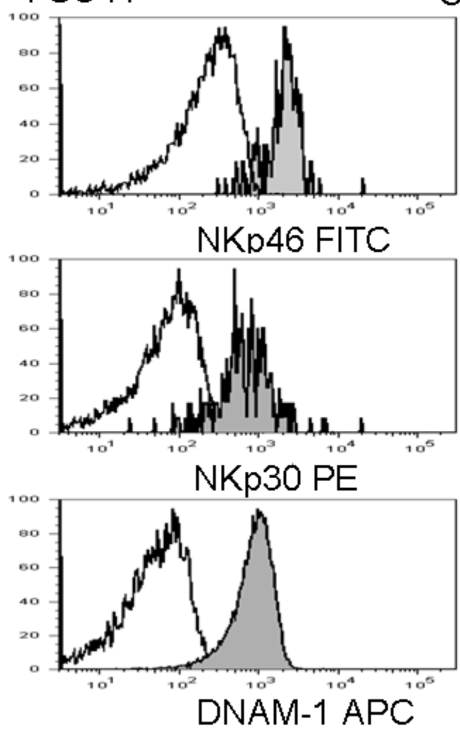
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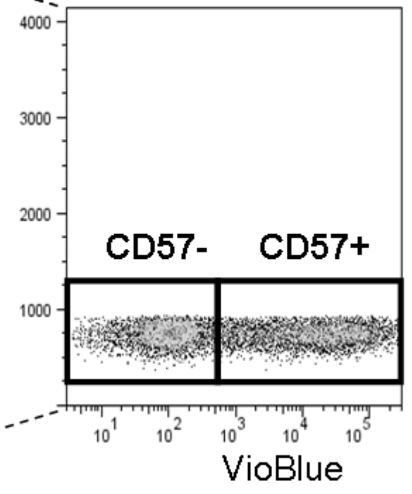
CD57-
CD57+
VioBlue

Supplementary Table.

Criteria for inclusion. Donors included in the study were selected based on the

- Aged 18-35 years (young) and >70 years (elderly)
- No infection at the time of extraction
- Not suffer or have suffered:
  - *Cancer*
  - *Rheumatoid arthritis*
  - *Ankylosing Spondylitis*
  - *Ulcerative colitis*
  - *Crohn's Disease*
  - *Celiac Disease*
  - *Systemic Lupus Erythematosus*
  - *Multiple Sclerosis*
  - *Psoriasis or any other autoimmune disease*
- Not be taking any immunosuppressive therapy, such as:
  - *Cyclosporin A*
  - *Prednisone*
  - *Cyclophosphamide*
  - *Azathioprine*
  - *Methotrexate*
  - *Mycophenolic acid (MPA)*
  - *Steroids*
  - *Tacrolimus*
  - *Sirolimus or rapamycin*
- Not be taking antihypertensive therapy whose active ingredient is calcium channels blocker, such as:
  - *Lercanidipine*
  - *Felodipine*
  - *Amlodipine*
  - *Bepridil*
  - *Diltiazem*
  - *Isradipine*
  - *Nicardipine*
  - *Nifedipine*
  - *Nimodipine*
  - *Verapamil*
  - *Clevidipine*
  - *Nisoldipine*

following criteria for inclusion / exclusion:

II. Effect of CMV and aging on the differential expression of CD300a, CD161, T-bet, and Eomes on NK cell subsets.

Nelson López-Sejas, Carmen Campos, Fakhri Hassouneh, Beatriz Sanchez-Correa, Raquel Tarazona, Alejandra Pera and Rafael Solana

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Effect of CMV and Aging on the Differential Expression of CD300a, CD161, T-bet, and Eomes on NK Cell Subsets

Nelson Lopez-Sejas[1†], Carmen Campos[1†], Fakhri Hassouneh[1], Beatriz Sanchez-Correa[2], Raquel Tarazona[2], Alejandra Pera[1‡] and Rafael Solana[1*]*

[1] Maimonides Biomedicine Institute of Cordoba (IMIBIC), Reina Sofia Hospital, University of Cordoba, Cordoba, Spain,
[2] Immunology Unit, Department of Physiology, University of Extremadura, Cáceres, Spain

Natural killer (NK) cells are innate lymphoid cells involved in the defense against virus-infected cells and tumor cells. NK cell phenotype and function is affected with age and cytomegalovirus (CMV) latent infection. Aging affects the frequency and phenotype of NK cells, and CMV infection also contributes to these alterations. Thus, a reduction of $CD56^{bright}$ NK cell subpopulation associated with age and an expansion of memory-like NK cells $CD56^{dim}CD57^{+}NKG2C^{+}$ probably related to CMV seropositivity have been described. NK cells express T-bet and Eomes transcription factors that are necessary for the development of NK cells. Here, we analyze the effect of age and CMV seropositivity on the expression of CD300a and CD161 inhibitory receptors, and T-bet and Eomes transcription factors in NK cell subsets defined by the expression of CD56 and CD57. CD300a is expressed by the majority of NK cells. $CD56^{bright}$ NK cells express higher levels of CD300a than $CD56^{dim}$ NK cells. An increase in the expression of CD300a was associated with age, whereas a decreased expression of CD161 in $CD56^{dim}$ NK cells was associated with CMV seropositivity. In $CD56^{dim}$ NK cells, an increased percentage of $CD57^{+}CD300a^{+}$ and a reduction in the percentage of $CD161^{+}CD300a^{+}$ cells were found to be associated with CMV seropositivity. Regarding T-bet and Eomes transcription factors, CMV seropositivity was associated with a decrease of $T\text{-}bet^{hi}$ in $CD56^{dim}CD57^{+}$ NK cells from young individuals, whereas Eomes expression was increased with CMV seropositivity in both $CD56^{bright}$ and $CD56^{dim}CD57^{+/-}$ (from middle age and young individuals, respectively) and was decreased with aging in all NK subsets from the three group of age. In conclusion, CMV infection and age induce significant changes in the expression of CD300a and CD161 in NK cell subsets defined by the expression of CD56 and CD57. T-bet and Eomes are differentially expressed on NK cell subsets, and their expression is affected by CMV latent infection and aging.

Keywords: aging, CMV, CD57, CD161, CD300a, Eomes, NK cell subsets, T-bet

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***Correspondence:**
Alejandra Pera alejandrapera@gmail.com;
Rafael Solana rsolana@uco.es

†Nelson Lopez-Sejas and Carmen Campos have contributed equally to the manuscript.

‡Present address:
Alejandra Pera, Brighton and Sussex Medical School, University of Sussex, Brighton, UK

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INTRODUCTION

Natural killer (NK) cells are lymphocytes of innate immune response responsible for killing virus-infected cells and tumor cells. Frequency, phenotype, and function of NK cell subsets change in aging, and these changes are considered part of a general process of age-associated immune dysfunctions defined as immunosenescence (1–4). Immunosenescence affects adaptive and innate immunity, and it is associated with increased incidence and severity of infections and decreased response to vaccination (5–11).

It has been shown that percentage of $CD56^{dim}$ NK cell subset (the main subset of NK cells and most cytotoxic) is increased (12, 13) or maintained (14) by age, whereas percentage of immature $CD56^{bright}$ NK cells is decreased (12–15). On the other hand, the expression of different receptors on NK cells is also altered in aging (16–18). It has been found a decrease in the expression of natural cytotoxicity receptors (such as NKp30 and NKp46) and activating receptor DNAM-1 on $CD56^{dim}$ NK subset (3, 19–21) an increased expression of CD57 (18, 20, 22), as well as reciprocal changes in NKG2A and killer immunoglobulin-like (KIR) inhibitory receptors, associated with age (18, 20, 22).

Human cytomegalovirus (CMV) chronic infection is related to a deterioration of the immune system that affect adaptive and innate immunity, and it has been postulated that CMV infection is a major driving force contributing to immunosenescence (10, 23, 24). CMV is a human herpesvirus type 5 (HHV5), which replicate in different cell types. Although CMV seropositivity is influenced by geographic, ethnic, and socio-economical factors, it has been shown that it increases with age in all populations studied (25). CMV prevalence is very high in Spain and more than 80% of individuals over the age of 40 years are CMV-seropositive (26).

Cytomegalovirus seropositivity can reshape the repertoire of NK cells (2, 27), particularly with an expansion of $NKG2C^{+}$ NK cells, which also coexpress CD57 marker (14, 28, 29). Recent studies stratifying donors according to CMV serology have shown that increase of CD57 expression on $CD56^{dim}$ NK cells is related to CMV seropositivity rather than aging (14) as well as the decreased expression of other surface receptors of NK cells, such as NKp30 (30) or CD161 (31). Thus, aging is associated with a loss of $CD56^{bright}$ NK cell subpopulation (probably due to a decrease in the production of new NK cells in the bone marrow) and an expansion of memory-like NK cells $CD56^{dim}CD57^{+}NKG2C^{+}$ that is mainly related to CMV seropositivity [reviewed in Ref. (2)].

Although it is well established that aging and CMV infection are associated with changes in NK cells, including alterations in the expression of activating (e.g., NCRs, NKG2C, and DNAM-1) and inhibitory receptors (e.g., KIR and NKG2A), little is known on the effect of aging and CMV on the expression of other inhibitory receptors such as CD300a and CD161 in NK cell subpopulations. CD300a is an inhibitory receptor expressed by NK cells that belongs to the CD300 family of molecules. These receptors are broadly expressed on immune cells and modulate their function *via* paired activating and inhibitory receptors that recognize lipids exposed on the plasma membrane of dead and activated cells including aminophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) (32). The analysis of its expression can be used in diagnosis and therapy in several pathological situations including infectious diseases, allergy, or cancer [for review, see Ref. (33)]. The human CD161 inhibitory receptor (also termed NKR-P1A, KLRB1, and CLEC5B) was originally described as a disulfide-linked homodimer of the C-type lectin superfamily expressed on subsets of NK cells and T lymphocytes (34) that binds the lectin-like transcript 1 (LLT1, also named CLEC2D, OCIL, and CLAX) (35, 36). The binding of CD161 on NK cells with its ligand on target cells results in inhibition of NK cell cytotoxicity by a mechanism involving the activation of acid sphingomyelinase (37). CD161 can also be expressed on subsets of other cells of the immune system, and different functional capacities have been shown after the interaction with its ligand, which can be upregulated during the immune response and during pathological circumstances. The current knowledge of NKRP1 receptors and their genetically linked CLEC2 ligand in human and other species has been recently reviewed (38, 39).

Natural killer cells are included in group 1 of the innate lymphoid cell (ILC), characterized by the release of interferon-gamma (IFN-γ) upon stimulation, and by the expression of T-bet and eomesodermin (Eomes) transcription factors (40–42). Both T-bet and Eomes are constitutively expressed by murine (43) and human (44, 45) NK cells and are necessary for the proper development of NK cells (46), sharing several functions. It has been observed that the frequency of $T\text{-}bet^{+}$ cells and the level of T-bet expression per cell is significantly greater in the $CD56^{dim}$ population compared to the $CD56^{bright}$ population from peripheral human immune cells, contrary to Eomes expression pattern, suggesting the existence of a relationship among the expression levels of both transcription factors and the functionality of these cells (45). Thus, T-bet is related to terminal stages of maturation, while Eomes is downregulated during peripheral maturation (47).

Considering that aging affects the frequency and phenotype of NK cells and that CMV infection contributes to age-associated changes in NK cells; in this work, we have analyzed the effect of age and CMV seropositivity on inhibitory receptors CD300a and CD161 in NK cell subpopulations. Additionally, we have investigated the effect of age and CMV infection on T-bet and Eomes transcription factors expression in the $CD56^{bright}CD57^{-}$, $CD56^{dim}CD57^{-}$, and $CD56^{dim}CD57^{+}$ NK cell subsets.

MATERIALS AND METHODS

Study Subjects

A total of 72 healthy adults voluntarily participated in the study, stratified according to age: 18–35 years (young), 40–65 years (middle age), and >70 years (old). Young and middle age donors were further divided according to CMV serology (CMV-seropositive and CMV-seronegative). However, all elderly donors included in the study were CMV-seropositive, given that the prevalence of CMV seropositivity in Spain in individuals over the age of 40 years is 80% (26) and, in our geographic area (Andalusia, Southern Spain), about 99% of individuals over

65 years are CMV-seropositive. Thus, the absence of a group of CMV-seronegative old donors represents a limitation of the study, making difficult to isolate age-related effect from the effect of chronic CMV infection in elderly individuals.

All donors were informed and signed informed consent to participate in the study and were included according to following inclusion criteria: no infection at the time of extraction, not suffer or have suffered cancer or autoimmune diseases, and not be under immunosuppressive drugs or calcium channel blockers. The study was approved by the Ethics Committee of Hospital Universitario Reina Sofia of Córdoba (Spain).

Sample Collection and Processing

Peripheral blood mononuclear cells (PBMCs) were obtained from blood samples (collected in lithium heparin tubes) and isolated by density gradient centrifugation using Ficoll Histopage-1077 (Sigma-Aldrich, St. Louis, MO, USA). Aliquots of cells were cryopreserved in FBS (Sigma-Aldrich, St Louis, MO, USA) with 10% DMSO (Panreac Chemistry SAU, Barcelona, Spain) in cryotubes at concentrations $5–6 \times 10^6$ cells/mL until further use.

A sample of plasma or serum was retrieved from all donors to analyze CMV-specific IgG and IgM. CMV serology was determined by using automated enzyme-linked immunosorbent assay (ELISA) (DRG International, Mountainside, NY, USA).

Analysis of NK Cell Receptors by Flow Cytometry

For surface marker analysis and computation of frequency of cells expressing CD57, CD161, or CD300a, cryopreserved PBMCs were used. Cell thawing was carried out in RPMI 1640 (Sigma-Aldrich) with 20% FBS (Gibco, Life Technologies California, USA). For flow cytometry staining, the following antibodies (mAbs) were used: anti-CD3 PerCP (clone: BW 264/56, Miltenyi Biotec), anti-CD56 PE-Cy7 (clone: B159, BD Pharmingen), anti-CD57 VioBlue (clone: TB03, Miltenyi Biotec), anti-CD300a PE (clone: E59.126, Beckman Coulter), and anti-CD161 APC (clone: DX12, BD Pharmingen). Cells were acquired on a MACSQuant cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Compensation for flow cytometry was performed using single cell staining.

Data were analyzed using FlowJo v10 (Tree Star, Inc.). $CD3^{-}CD56^{+}$ NK cells were gated from total peripheral blood lymphocytes (PBLs), after singlets gating (Figure S1 in Supplementary Material). Two NK cell subsets were defined ($CD56^{bright}$ and $CD56^{dim}$) according to the level of CD56 marker expression. Subsequently, cells were gated according to the coexpression of CD57, CD161, and CD300a markers. Fluorescence minus one controls (FMO control) were used to identify and gate cells. FMO controls contain all the fluorochromes of the panel, except the one that was being measured. Analysis of coexpression of the three receptors was performed using FlowJo's Boolean gating options.

Transcription Factors Expression Analysis

The expression of T-bet and Eomes transcription factors was analyzed using cryopreserved PBMCs, thawed as indicated above. Surface staining was performed using anti-CD7 APC (clone: M-T701, BD Pharmingen), anti-CD56 BV421 (clone: NCAM16.2, BD Horizon), anti-CD16 PE- Vio770 (clone: VEP13, Miltenyi Biotec), anti-CD57 Biotin-Anti-Biotin-Viogreen (Miltenyi Biotec), and anti-CD3/anti-CD14/anti-CD19 conjugated with APC-Vio770 (clones: BW264/56, TÜK4, LT19 Miltenyi Biotec). After cell fixation and permeabilization using the Kit FoxP3 Staining Buffer Set (Miltenyi Biotec), following the manufacturer's instructions, intracellular staining was realized with anti-T-bet PerCP Cy5.5 (clone: 04-46, BD Pharmingen) and anti-Eomes FITC (clone: WD1928, eBioscience) antibodies. Cells were then acquired on a 10 parameter MACSQuant cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Data were analyzed using FlowJo v10 (Tree Star, Inc.). Once selected the $CD7^{+}CD3^{-}CD19^{-}CD14^{-}$ cells from PBLs singlets, three populations of NK cells were described: $CD56^{bright}CD57^{-}$, $CD56^{dim}CD57^{-}$, and $CD56^{dim}CD57^{+}$. Then, the intracellular expression of T-bet and Eomes was measured in each of these three subpopulations (Figure S2 in Supplementary Material). FMO controls and flow cytometry compensation were performed as indicated above.

Processing of Data and Statistical Analysis

For statistical analysis, Shapiro–Wilk normality test was performed for different groups. Since the distribution of measured values was not normal, the groups were evaluated by the non-parametric Kruskal–Wallis test (for comparison multiple) and Mann–Whitney test (for comparison sample pairs). Non-parametric Friedman test (for comparison multiple) and Wilcoxon test were used to test for differences between groups when the samples are related. The results are shown in graphs with interquartile medium range using GraphPad Prism (version 5.0), and analysis of Boolean gating data was performed by SPICE 5.35 software (Mario Roederer, ImmunoTechnology Section, Vaccine Research Centre, NIH, Bethesda, MD, USA; http://www.niaid.nih.gov) (48). To compare the pie charts, we used SPICE's permutation analysis, which asks how often the samples that comprise two different pies charts, would be different simply by chance (10,000 permutations). All statistical analysis was performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). p-Values <0.05 were considered significant.

RESULTS

Increase of CD300a and Decrease of CD161 Expression from CMV-Seropositive Old Individuals

We analyzed by flow cytometry the expression of CD300a and CD161 inhibitory receptors on NK cells ($CD56^{bright}$ and $CD56^{dim}$) from healthy individuals stratified by age and CMV latent infection. Our data showed that both receptors exhibit a differential expression pattern. Particularly, the majority of $CD56^{dim}$ and $CD56^{bright}$ NK cells expressed CD300a, whereas CD161 was expressed on a subpopulation of NK cells regardless of the NK cell subset analyzed (Figure S1 in Supplementary Material). Moreover, when we gated the NK cell subsets according to CD56

and CD300a (referred to CD3⁻ cells), we observed that the majority of CD56^{bright} NK cells were CD300a^{hi}, whereas a high percentage of CD56^{dim} NK cells were CD300a^{lo} (**Figure 1A**). Analysis of the effect of age and CMV seropositivity on the expression of these receptors by NK cell subsets (gating strategy in Figure S1 in Supplementary Material) showed an increase of CD300a on CD56^{bright} and CD56^{dim} subsets from CMV-seropositive old individuals compared with middle-aged and young donors (independently of CMV seropositivity) (**Figure 1B**).

The analysis of CD161 expression on CD56^{dim} NK cells showed that young CMV-seronegative donors expressed higher levels of CD161 than young CMV-seropositive or middle age CMV-seronegative individuals, supporting that CMV and age independently associated with decreased expression of CD161 in

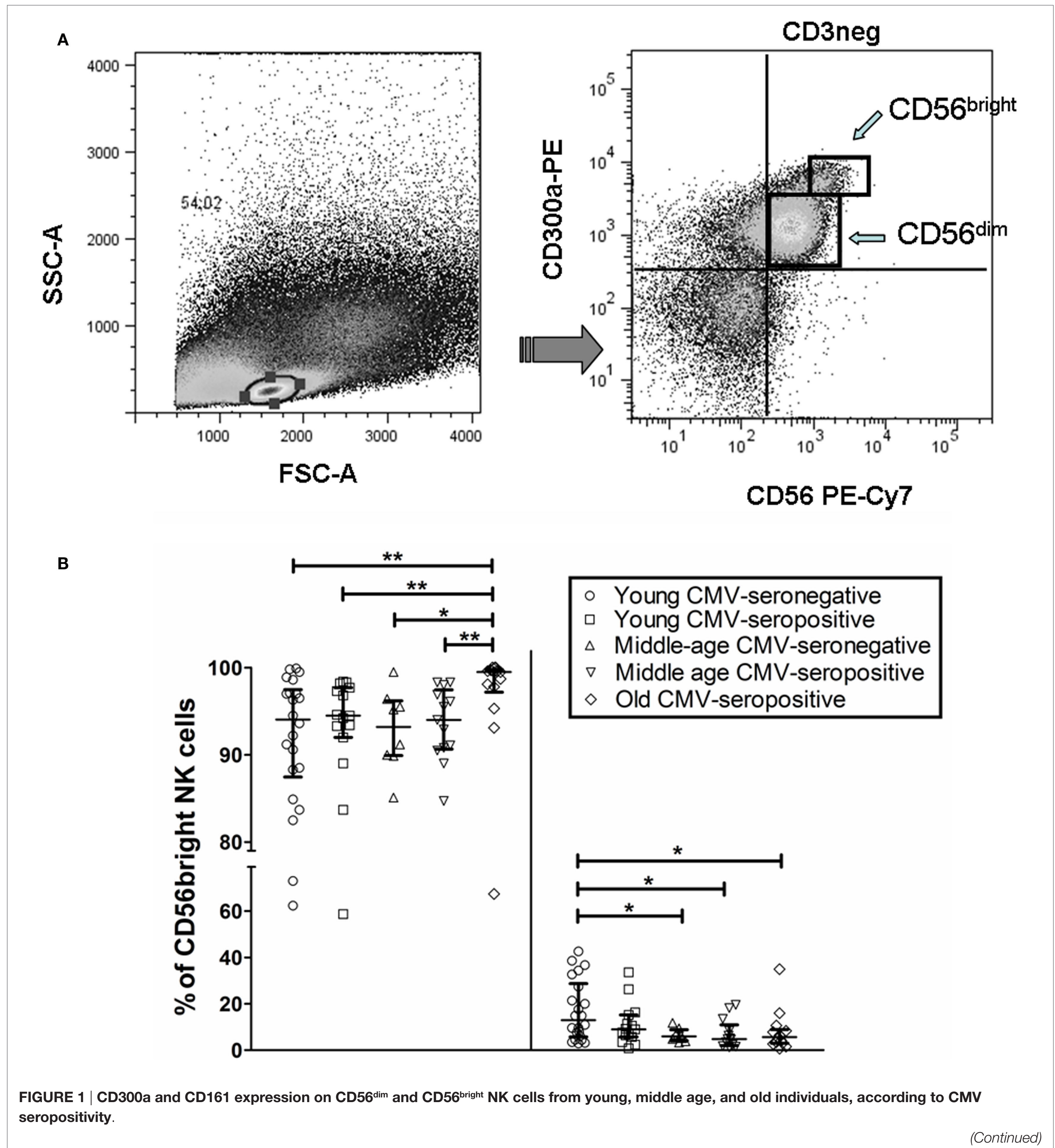

FIGURE 1 | **CD300a and CD161 expression on CD56^{dim} and CD56^{bright} NK cells from young, middle age, and old individuals, according to CMV seropositivity**.

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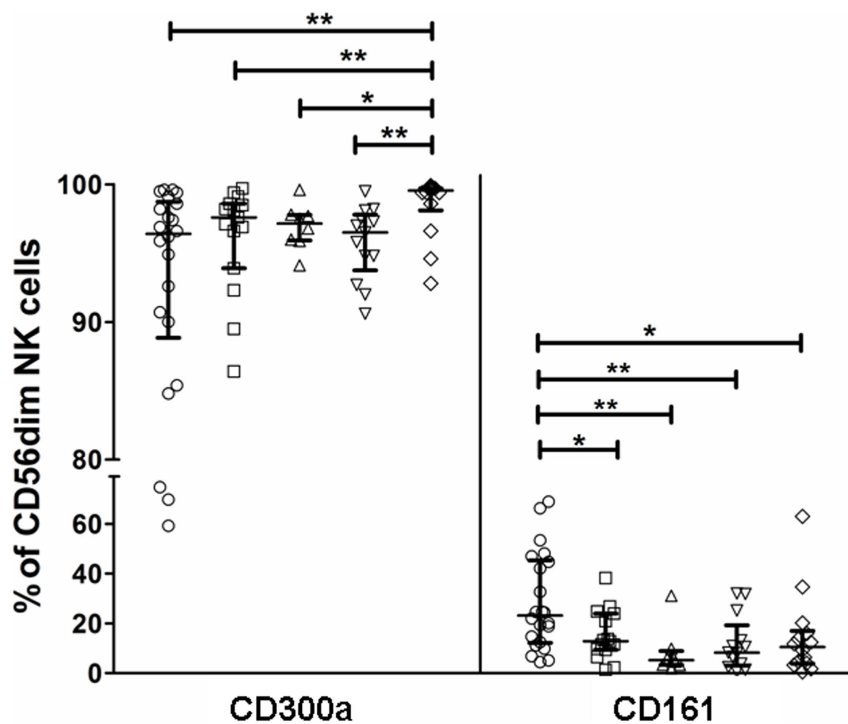

FIGURE 1 | **Continued**
(A) Differential expression of CD300a on NK cell subsets. NK cells were defined as CD3⁻CD56⁺ and characterized by CD300a expression as CD56^{bright}CD300a^{hi} and CD56^{dim}CD300a^{lo}. **(B)** Effect of age and CMV seropositivity on the expression of CD300a and CD161. Expression (percentage) of CD300a and CD161 markers was determined on NK cell subsets from young CMV-seronegative (n = 22), young CMV-seropositive (n = 15), middle age CMV-seronegative (n = 8), middle age CMV-seropositive (n = 13), and old CMV-seropositive donors (n = 14). CD56^{+}CD3^{-} NK cells were gated from singlets peripheral blood lymphocytes (PBLs). Then, two NK cell subsets were defined (CD56^{bright} and CD56^{dim}) according to the level of CD56 marker expression, and cells were gated according to the expression of CD57, CD161, and CD300a markers. Non-parametric Kruskal–Wallis test (for multiple comparisons) and Mann–Whitney test (for paired comparisons) was used. Graphs showed the median with interquartile range, and results were considered significant at *p < 0.05, **p < 0.01, and ***p < 0.001.

this NK cell subset. On the contrary, the expression of CD161 on CD56^{bright} NK cells was not affected by CMV seropositivity in young donors, whereas it was decreased in middle age CMV-seronegative individuals. The expression of CD161 was lower on both NK cell subsets in middle age and old CMV-seropositive donors compared with young CMV-seronegative individuals (**Figure 1B**).

These results indicate an expansion of CD300a^{+} on NK cell subsets (CD56^{bright} and CD56^{dim}) from healthy old individuals (all CMV-seropositive), likely associated with the combined effect of CMV infection and age, and a decrease of CD161^{+} NK cells related to CMV seropositivity (CD56^{dim} NK cells) and age (CD56^{bright} and CD56^{dim} NK cells).

In this analysis, we have also observed an increased percentage of CD56^{dim}CD57^{+} NK cells associated with CMV seropositivity. Also, CD56^{bright} NK cells do not express or express very low levels of CD57 on their surface (data not shown).

Coexpression of CD300a, CD161, and CD57 on NK Cell Subsets

The coexpression of CD300a, CD161, and CD57 was measured on CD56^{dim} and CD56^{bright} NK cells using FlowJo's Boolean gating options. The majority of CD56^{bright} and CD56^{dim} NK cells expressed CD300a on their surface, either alone or in combination with CD161 or CD57. A minor subset of CD300a^{+} NK cells coexpressed CD161 and CD57 (**Figure 2A**).

The results on the effect of age and CMV seropositivity on the coexpression of CD300a, CD161, and CD57, revealed that CMV seropositivity, but not age, was associated with an increase of CD56^{dim} NK cells coexpressing CD300a and CD57. We also observed a decrease of CD56^{dim}CD300a^{+}CD161^{+} NK cells related to both CMV seropositivity and age. No significant differences were found in the percentage of CD56^{dim}CD300a^{+}CD57^{-}CD161^{-} NK cells among the different groups studied (data not shown). On the other hand, we have also observed an increase of CD56^{bright}CD300a^{+}CD57^{-}CD161^{-} NK cells and a decrease of CD56^{bright} coexpressing CD300a and CD161 in old individuals (**Figures 2A,B**).

Different Expression Patterns of T-bet and Eomes Transcription Factors in NK Cell Subsets

We analyzed the expression of T-bet and Eomes transcription factors in three subpopulations of NK cells, according to the

expression of CD56, CD16, and CD57 markers: CD56^bright^CD16^+/−^CD57^−^, CD56^dim^CD16^+^CD57^−^, and CD56^dim^CD16^+^CD57^+^ NK cells (Figure S2 in Supplementary Material). Although Eomes was expressed in most NK cells in all groups studied, the analysis of its expression pattern showed higher levels in the most immature NK cells ($CD56^{bright}CD16^{+/-}CD57^{-}$) with a decline in its expression in the $CD56^{dim}CD16^{+}CD57^{-}$ and in the most differentiated NK cells ($CD56^{dim}CD16^{+}CD57^{+}$) (**Table 1**).

The expression of T-bet in NK cell subsets showed a gradient of expression and two subsets can be distinguished: T-bet^{hi} and

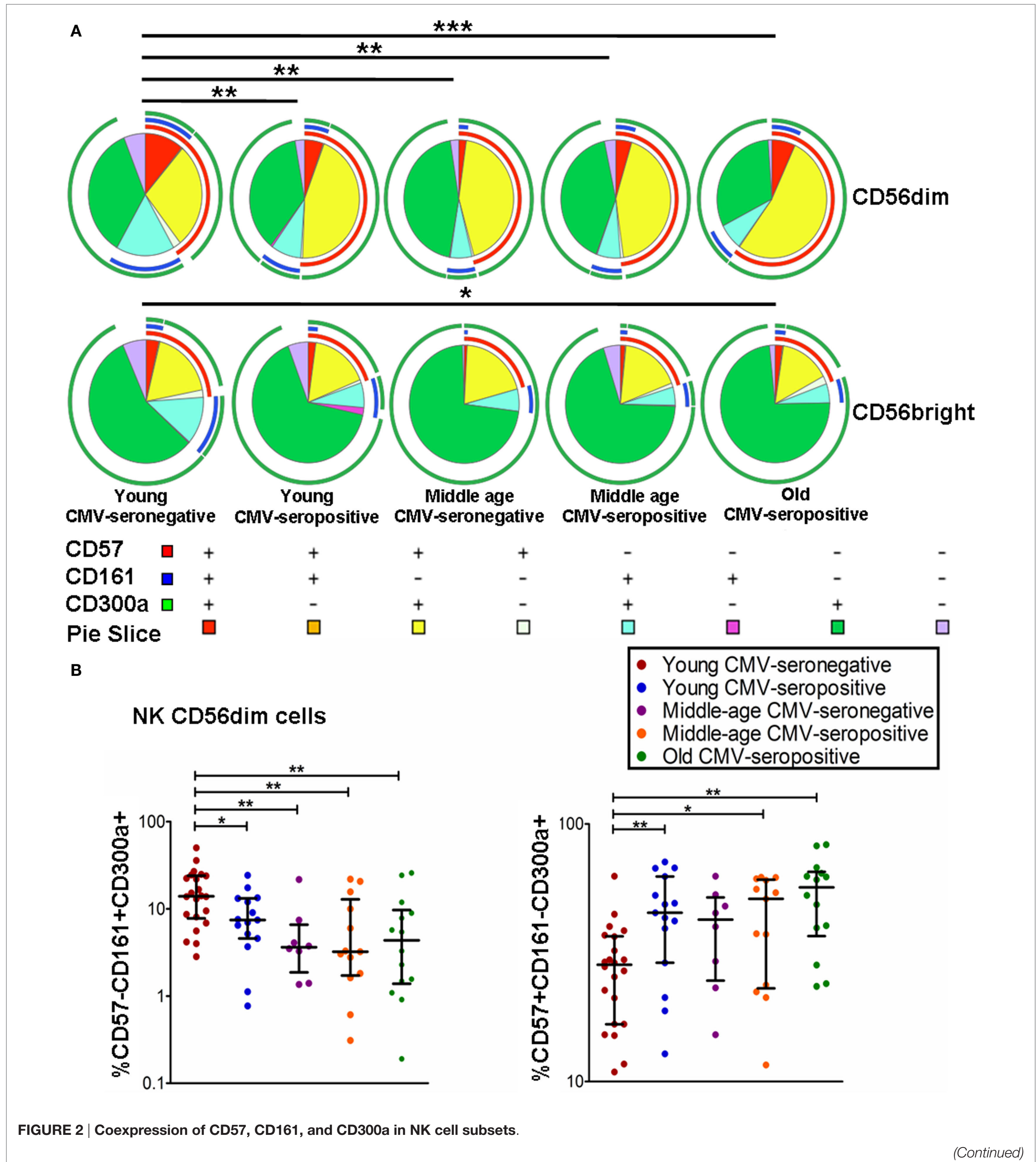

FIGURE 2 | **Coexpression of CD57, CD161, and CD300a in NK cell subsets.**

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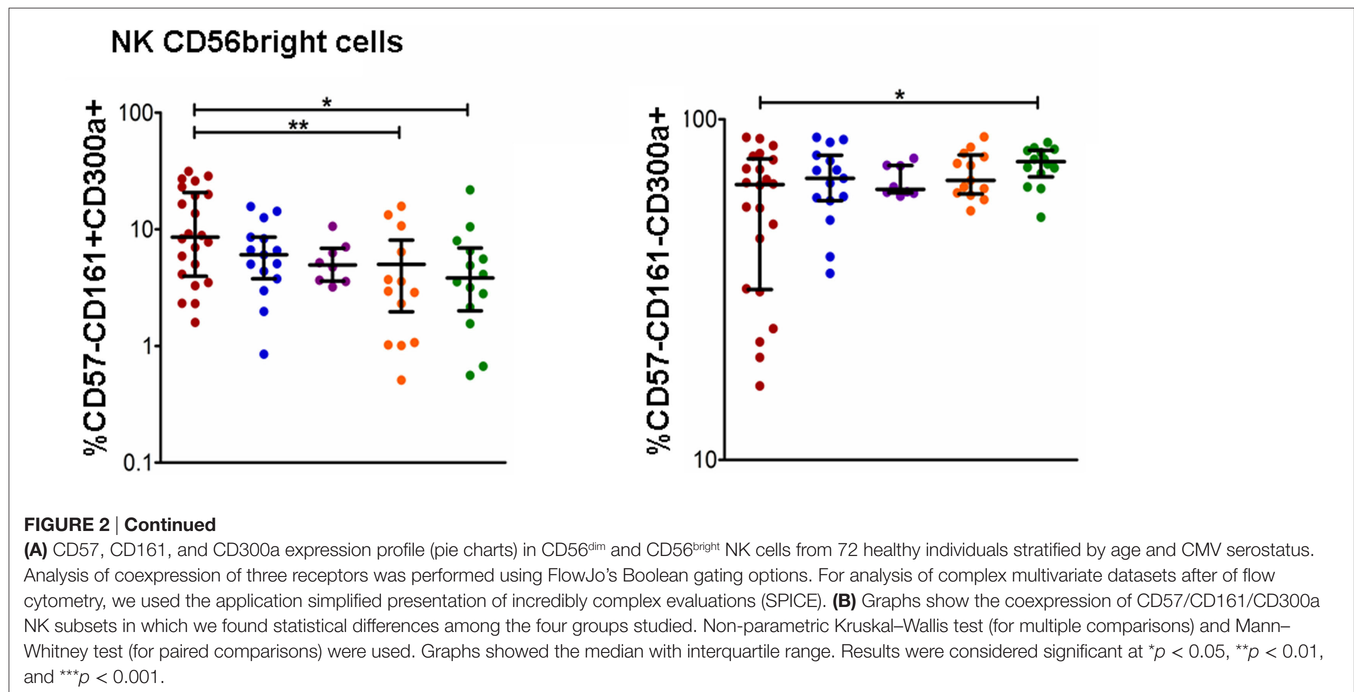

FIGURE 2 | **Continued**
(A) CD57, CD161, and CD300a expression profile (pie charts) in CD56^{dim} and CD56^{bright} NK cells from 72 healthy individuals stratified by age and CMV serostatus. Analysis of coexpression of three receptors was performed using FlowJo's Boolean gating options. For analysis of complex multivariate datasets after of flow cytometry, we used the application simplified presentation of incredibly complex evaluations (SPICE). **(B)** Graphs show the coexpression of CD57/CD161/CD300a NK subsets in which we found statistical differences among the four groups studied. Non-parametric Kruskal–Wallis test (for multiple comparisons) and Mann–Whitney test (for paired comparisons) were used. Graphs showed the median with interquartile range. Results were considered significant at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

TABLE 1 | **Eomes expression (MFI) in NK subsets from donors stratified according to age and CMV seropositivity.**

	CD56^{bright}CD16$^-$CD57$^-$ (1)	CD56^{dim}CD16$^+$CD57$^-$ (2)	CD56^{dim}CD16$^+$CD57$^+$ (3)	ρ	ρ (1–2)	ρ (2–3)	ρ (1–3)
Young CMV$^-$	3.47 (4.46–2.58)[a]	2.12 (2.23–1.35)	1.59 (1.78–0.84)	0.000	0.005	0.005	0.005
Young CMV$^+$	3.75 (4.91–2.35)	2.28 (2.67–1.9)	2.18 (2.34–1.58)	0.007	0.007	0.169	0.007
Middle age CMV$^-$	3.48 (4.74–2.33)	1.87 (2.41–1.65)	1.45 (2.4–1.28)	0.001	0.018	0.018	0.018
Middle age CMV$^+$	3.32 (4.54–2.96)	2.15 (2.52–1.67)	1.6 (2.1–1.41)	0.000	0.008	0.008	0.008
Old CMV$^+$	3.02 (3.79–2.46)	2.01 (2.71–1.59)	1.8 (2.05–1.2)	0.000	0.005	0.005	0.005

[a]*Values expressed as median (interquartile range, 75–25).*
ρ value obtained by the Friedman rank sum test.
ρ values comparing (1–2), (2–3), and (1–3) groups obtained by the Wilcoxon test.

T-bet^{int} NK cells. The percentage of NK cells expressing T-bet^{hi} was higher than the percentage of NK cells expressing T-bet^{int} in the three subsets studied (Figure S2 in Supplementary Material). The analysis of T-bet MFI in the different NK cell subsets showed that T-bet expression was lower in CD56^{bright} CD16$^{+/-}$CD57$^-$ NK cells from each group studied than in CD56^{dim}CD16$^+$CD57$^-$ and CD56^{dim}CD16$^+$ CD57$^+$ NK cells into the same group (**Table 2**).

The analysis of Eomes expression in NK cell subsets from CMV-seronegative and CMV-seropositive individuals showed that the percentage of positive cells was significantly increased in CD56^{bright}CD16$^{+/-}$CD57$^-$ NK cells from CMV-seropositive middle age donors and in CD56^{dim}CD16$^+$CD57$^+$ and CD56^{dim}CD16$^+$CD57$^-$ from CMV-seropositive young donors compared with the CMV-seronegative counterparts (**Figures 3A,B**). The analysis of the effect of age on Eomes expression showed a decreased expression in CD56^{bright}CD 16$^{+/-}$CD57$^-$ NK cells from CMV-seronegative middle age donors compared with CMV-seronegative young individuals (**Figure 3A**). Eomes expression in CMV-seropositive donors was significantly higher in the young compared with the middle age and old groups in the three NK cell subsets considered (**Figures 3A,B**).

The effect of age and CMV seropositivity on T-bet expression was also analyzed. The results only showed a significant decrease in the percentage of T-bet^{hi} within the CD56^{dim}CD16$^+$CD57$^+$ NK cell subset from CMV-seropositive compared with CMV-seronegative young donors (**Figures 3C,D**).

DISCUSSION

Cumulative evidences in the last decade support that aging and CMV latent infection combine to influence the immune phenotype and function of immune cells, including NK cells, in different ways that have been often overlooked in studies aiming to analyze the effect of aging on NK cells without considering the CMV serostatus of the individuals studied.

In this work, we have analyzed the effect of CMV seropositivity and aging on the expression of CD300a and CD161 receptors and transcription factors T-bet and Eomes on peripheral blood NK cell subsets with different levels of maturation.

TABLE 2 | T-bet expression (MFI) in NK subsets from donors stratified according to age and CMV seropositivity.

	$CD56^{bright}CD16^{-}CD57^{-}$ (1)	$CD56^{dim}CD16^{+}CD57^{-}$ (2)	$CD56^{dim}CD16^{+}CD57^{+}$ (3)	ρ	ρ (1–2)	ρ (2–3)	ρ (1–3)
Young CMV^{-}	6.31 (10.2–2.91)[a]	8.84 (12.6–5.89)	9.31 (12.5–6.24)	0.000	0.008	0.022	0.005
Young CMV^{+}	7.17 (10.3–3.47)	10.84 (12.2–7.26)	9.64 (11.4–7.49)	0.000	0.005	0.011	0.008
Middle age CMV^{-}	10.10 (13–1.89)	12.8 (13.8–5.77)	13.2 (13.8–5.84)	0.004	0.043	0.042	0.018
Middle age CMV^{+}	7.09 (10.8–3.85)	10.3 (13.5–6.97)	10.7 (13.9–6.79)	0.003	0.008	0.285	0.011
Old CMV^{+}	4.85 (11.3–2.79)	7.57 (11.6–6.42)	7.22 (10.6–5.95)	0.007	0.017	0.013	0.114

[a]*Values expressed as median (interquartile range, 75–25).*
ρ value obtained by the Friedman rank sum test.
ρ values comparing (1–2), (2–3), and (1–3) groups obtained by the Wilcoxon test.

Our results show that $CD57^{+}CD56^{dim}$ NK cells are expanded in CMV-seropositive individuals and that these cells are not further expanded by aging. In addition, the majority of these cells also coexpress CD300a, but not CD161. The expansion of $CD57^{+}CD56^{dim}$ NK cells support previous data showing that acute and latent CMV infection leads to the expansion of $CD57^{+}CD56^{dim}$ NK cells that also express $NKG2C^{+}$ (28, 49–54). CMV seropositivity is also associated with a decreased expression of other NK receptors, in some cases, as a consequence of the shift of NK cells to the more differentiated $NKG2C^{+}CD57^{+}$ phenotype (14, 27, 30, 54). These cells have some characteristics of adaptive immunity and are considered "memory" or "adaptive" NK cells (55–58). The magnitude of the expansion of $NKG2C^{high}$ NK cells is determined by the magnitude of the proinflammatory cytokine secretion upon NK cell activation (59), and it has been proposed that these cells contribute to the proinflammatory environment based on the relation between the percentage of $NKG2C^{+}$ cells, elevated levels of PCR, and cardiovascular risk determinants of CMV-seropositive individuals (60, 61).

Whereas it has been show that both aging and CMV infection are associated with a decreased expression of several NK activating receptors (with the exception of NKG2C that is increased in CMV-seropositive individuals), their effect on the expression of inhibitory receptors is still controversial. Thus, whereas different studies have shown an age-associated increased expression of KIR (20, 62) and a decreased expression of NKG2A (20), others have not found significant differences in the expression of KIR or NKG2A in the elderly (14, 17, 18, 30) or even a decreased expression of KIR (CD158a) in middle age donors if they are CMV-seropositive (63). The expression of another inhibitory receptor KLRG-1 is significantly reduced with aging (21) and with CMV seropositivity in the young individuals (63).

The inhibitory receptor CD300a is expressed on the majority of NK cells, and its expression increases with aging in CMV-seropositive individuals both in $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. As stated in Section "Materials and Methods," in our geographic area (Andalusia, Southern Spain), about 99% of individuals over 65 years are CMV-seropositive. Thus, the high prevalence of CMV in our geographic area supposes a limitation of the study as we lack of a group of CMV-seronegative elderly donors. This limitation makes difficult to assess the differential effect of CMV and aging when we observe changes only in the group of old CMV-seropositive individuals compared with the other groups. This is the case of the higher expression of CD300a found in old CMV-seropositive individuals compared with the other groups studied. Since we do not observe an effect of CMV seropositivity between in the young and middle age groups, it could be thought that the higher expression of CD300a found in old CMV-seropositive individuals is likely due to aging. However, due to this limitation, we can only conclude that aging has an effect on CD300a expression on CMV-seropositive donors. CD300a is an inhibitory receptor that can be expressed by NK cells and that deliver inhibitory signals upon binding to PS expressed by tumor cells (64). The binding of CD300a on human or porcine NK cells to the surface of the pseudorabies virus porcine infected cell is increased by the US3 protein kinase of this alpha-herpesvirus in an aminophospholipids and p21-activated kinases dependent way, providing protection of infected cells against NK cell cytotoxicity (65). These results support the possible relevance of CD300a as a possible NK cell evasion strategy by CD300a-modulating viruses and cancer cells.

Although CD300a is highly homologous to CD300c and both receptors are considered as paired receptors with inhibitory and activating roles, respectively, very little is known on the expression and function of CD300c on NK cells. It has been recently shown that NK cells do not express (or express very low levels of) CD300c and that its expression is induced uniquely on $CD56^{bright}$ NK cells after their treatment with IL-2 or IL-15 (66). The analysis of the interaction of CD300a and CD300c with their ligands shows differences on their binding affinity to the lipid ligands. Whereas both CD300a and CD300c show similar binding to PS, it has been shown that CD300a exhibits a stronger binding to dead cells and to PE than CD300c and PE induces a negative response of IL-2 preactivated $CD56^{bright}$ NK cells, supporting that the inhibitory signals triggered by CD300a after binding to its lipid ligands overrides the signals triggered by its activating counterpart CD300c (66). These findings are consistent with these shown for other paired receptors such as KIR2DL1 and CD94/NKG2A that have higher binding affinity to HLA-CLys80 and HLA-E, respectively, than their activating counter parts KIR2DS1 and CD94/NKG2C. The increased expression of CD300a with aging both in $CD56^{bright}$ and $CD56^{dim}$ subsets can contribute together with other age-associated alterations of NK cells to the decreased functional capacity of these cells in the elderly.

The human CD161 receptor, expressed in a subset of NK cells, is an inhibitory receptor that, after interacting with its ligand LLT1, inhibits NK cell cytotoxicity by a mechanism involving the activation of acid sphingomyelinase (37). Our results show that the expression of CD161 on $CD56^{dim}$ NK cells is decreased

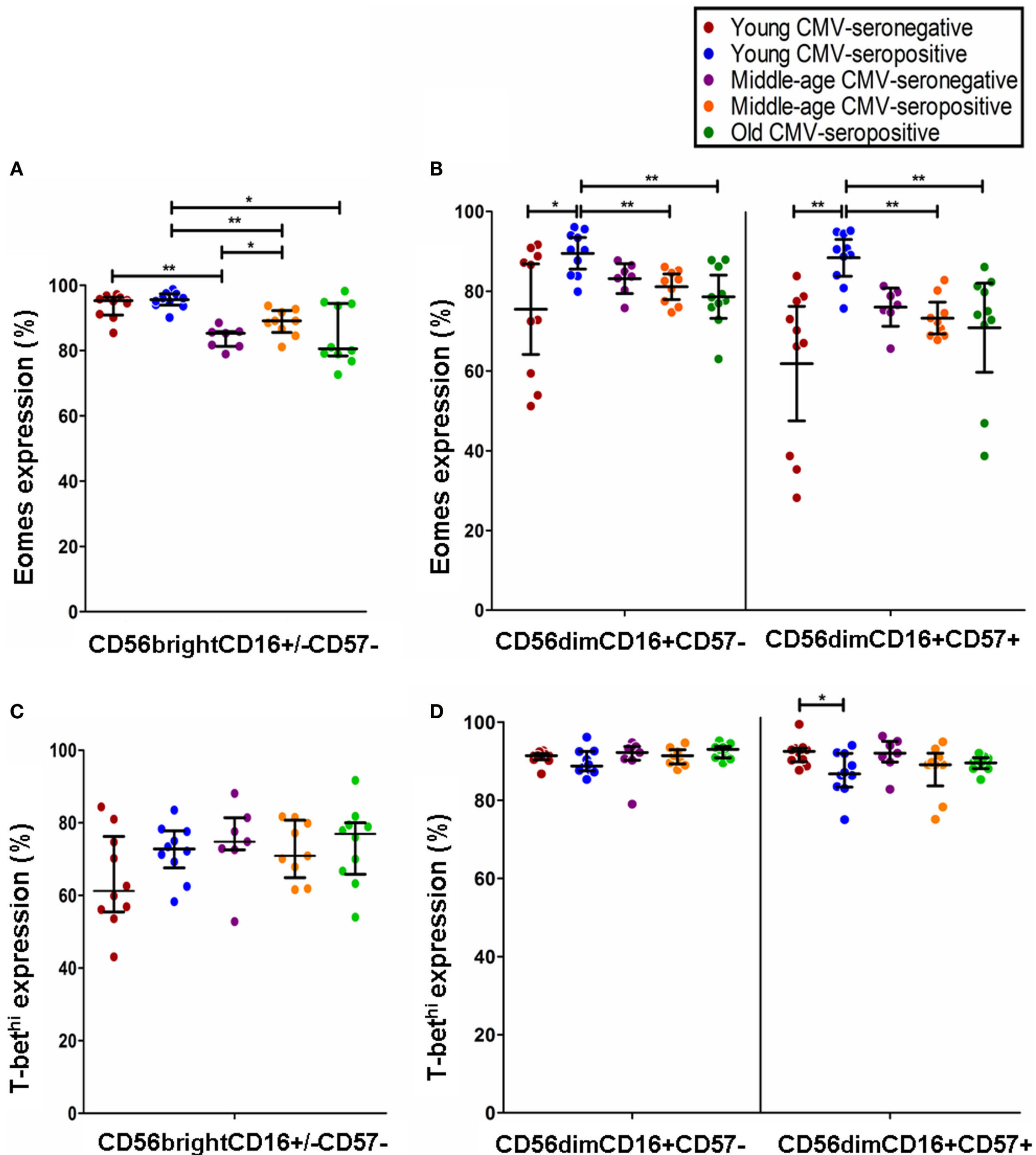

FIGURE 3 | Effect of age and CMV seropositivity on Eomes and T-bet expression. The expression of T-bet and Eomes was measured in CD56^{bright}CD16$^{+/-}$CD57^{-}, CD56^{dim}CD16^{+}CD57^{-}, and CD56^{dim}CD16^{+}CD57^{+} NK cells from 46 healthy individuals (10 young CMV-seronegative, 10 young CMV-seropositive, 7 middle age CMV-seronegative, 9 middle age CMV-seropositive, and 10 old CMV-seropositive donors). **(A)** Analysis of Eomes expression in CD56^{bright}CD16$^{+/-}$CD57^{-} NK cells. **(B)** Analysis of Eomes expression in CD56^{dim}CD16^{+}CD57^{-} and CD56^{dim}CD16^{+}CD57^{+} NK cells **(C)** Analysis of T-bet expression in CD56^{bright}CD16$^{+/-}$CD57^{-} NK cells. **(D)** Analysis of T-bet expression in CD56^{dim}CD16^{+}CD57^{-} and CD56^{dim}CD16^{+}CD57^{+} NK cells. Non-parametric Kruskal–Wallis test (for multiple comparisons) and Mann–Whitney test (for paired comparisons) were used. Graphs showed the median with interquartile range. Results were considered significant at *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

in CMV-seropositive donors compared with young CMV-seronegative donors. Little is known regarding the effect of CMV and aging on the expression of CD161 on NK cell subsets. The analysis of telomere length in NK cells has shown that the average telomere length in human NK cells decrease with age. In addition, the telomere length was significantly shorter in CD56^{dim}CD16^{+} NK cells compared to CD56^{bright}CD16^{-} NK cells from the same donor indicating that this subset represents more immature NK cells (67). In this study, it was shown that although CD161 can be expressed on CD56^{bright} and CD56^{dim} subsets, its expression

is independent of the level of differentiation estimated by the telomere length (67).

The expression of CD161 in T lymphocytes and NK cells in PBLs from healthy children appears unrelated to CMV serostatus (68). On the contrary, it has been reported that NKG2C^{+}CD56^{dim} NK cells, expanded in CMV-seropositive chronic hepatitis patients, have a significant decrease in the expression of CD161 and a significant increase in the expression of CD57 (69). In agreement with these data, our results show that the majority of NK cells do not coexpress CD57 and CD161, and that CMV seropositivity is associated not only with an increase in the expression of CD57 in CD56^{dim} NK cells but also with a decrease in the expression of CD161 in this subset. The decreased expression of CD161 in NK cells from CMV-seropositive NK cells parallels the finding that CD161 is decreased on CD56^{+} NKT-like cells in CMV^{+} subjects compared with CMV-seronegative donors (70). Interestingly, it has also been shown that the expression of CD161 in CMV-specific cytotoxic T lymphocytes is very low (71). Considering the inhibitory capacity of CD161 on NK and T lymphocytes cytokine production, these results support that the CMV-induced downregulation of CD161 receptor together with the expansion of polyfunctional response of CD57^{+} NK cells and T- and NKT-like lymphocytes (14, 30, 69, 72, 73), contribute to the proinflammatory environment observed in CMV-seropositive healthy individuals.

Recent reports have strengthened the significance of T-bet and Eomes in NK cell biology (47). In mice, T-bet and Eomes are necessary for maintenance of peripheral NK cells, their deletion in mature NK cells results in reversion to an immature phenotype (46). These transcription factors also modulate many NK cell effector functions, including cytotoxicity and cytokine production (74, 75). In human NK cells, T-bet and Eomes are differentially expressed on NK cell subsets (45), supporting that they can regulate different functions in different NK cell subpopulations. Our results confirm and extend these results showing that the levels of T-bet and Eomes are modulated in peripheral blood NK cell subsets representing different maturation stages, independently of aging and CMV serostatus. The expression of T-bet is lower in the CD56^{bright} NK cells than in the CD56^{dim} subset, whereas the expression of Eomes is higher in the CD56^{bright} NK cells. The CD56^{dim}CD57^{+} subset shows higher levels of T-bet and lower levels of Eomes than the CD56^{bright} and CD56^{dim}CD57^{-} NK cells. These results agree with the finding that not only the expression of T-bet and Eomes but also the expression of CD57 marker, modulate in parallel with the increase of KIRs on NK cells, but they do not differ in licensed or unlicensed NK cells (76), supporting that NK cell maturation but not NK cell licensing is dependent on T-bet and Eomes modulation. The significance of T-bet and Eomes in the maturation of other NK cell subpopulations is also supported by the demonstration that other subsets of NK cells display distinct patterns of expression of these transcription factors. Thus, a subpopulation of tissue-resident hepatic CD56^{bright} NK-cells, adapted to the tolerogenic liver microenvironment, with reduced proinflammatory potential, and characterized by the expression of CXCR6, express high levels of Eomes and low levels of T-bet, a phenotype virtually absent from peripheral blood (77, 78).

Very little is known on the expression patterns of T-bet and Eomes within human NK cell subpopulations in clinical situations. In this work, we have analyzed the expression of these transcription factors in two circumstances, such as CMV chronic infection and aging, which have a profound impact in NK cell differentiation.

Our results show that CMV seropositivity in young individuals associates with a significant increase in the percentage of CD56^{dim} NK cells expressing Eomes and a decreased percentage of T-bet^{hi} NK cells within the CD56^{dim}CD57^{+} subset, suggesting that changes in the expression of these transcription factors are involved in CMV-induced remodeling of NK cells, characterized by the expansion of CD56^{dim}CD57^{+} NK cells coexpressing NKG2C activating receptors (2, 14, 30, 60, 79). It has been reported that the level of expression of Eomes and T-bet is strongly reduced in NK cells from allogeneic hematopoietic stem cell transplantation recipients compared with healthy control subjects and that acute graft-versus-host disease and CMV reactivation are associated with further downregulation of T-bet (80) supporting the importance of these transcription factors in the generation and differentiation of NK cells and in the response against CMV after hematopoietic stem cell transplantation.

The analysis of the effect of age on Eomes expression showed a decreased expression in CD56^{bright} NK cells from CMV-seronegative middle age donors compared with CMV-seronegative young individuals, whereas in CMV-seropositive donors, its expression was significantly higher in the three NK cell subsets, in young donors compared with the middle age and old groups. The percentage of T-bet^{hi} NK cells was not significantly affected by age, independently of CMV serostatus. The effect of aging on the expression of transcription factors in NK cells is not known. It has been recently shown that impaired NK cell maturation in old mice is associated with a decreased expression of T-bet and Eomes in aged bone marrow NK cells. The use of bone marrow chimeras has revealed that the non-hematopoietic environment is responsible for the impaired maturation and function of NK cells, including the defective expression of T-bet and Eomes expression on NK cells (81). Considering that the defect in NK cell generation and in T-bet and Eomes expression in old mice is due to age alterations in non-hematopoietic environment, a better understanding of these non-hematopoietic factors involved in NK cell differentiation is required for the definition of new strategies aiming to improve NK cell function in the elderly.

It has been shown that the expression of T-bet and Eomes is increased in non-naïve CD8^{+} T cells from aged subjects and that this increase correlated closely with the levels of CD57 and KLRG1. In addition, it was shown that aging is associated with a decreased functionality of influenza virus-specific CD8^{+} T cells and increased expression of CD57, KLRG1, and T-bet (82), supporting that the increased expression of these transcription factors is related to the expansion of highly differentiated, senescent or exhausted, CD8^{+} T cells found in elderly individuals.

In conclusion, CMV latent infection has a profound impact on NK cells inducing significant changes in the expression of NK receptors, including the inhibitory receptors CD300a and

CD161. T-bet and Eomes are differentially expressed on NK cell subsets defined by the expression of CD56 and CD57, and its expression is affected by CMV latent infection and aging, which can be involved in the age-associated changes observed in the differentiation and function of NK cells.

AUTHOR CONTRIBUTIONS

RS, CC, and AP designed the study. NL-S collected the data and performed the laboratory experiments. NL-S and FH performed the laboratory analysis. CC and NL-S performed the statistical analysis and wrote the draft. RT, BS-C, CC, and AP made significant conceptual contributions to the manuscript. RS, RT, and CC reviewed the final version of the paper. All the authors provided intellectual content and approved the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00476/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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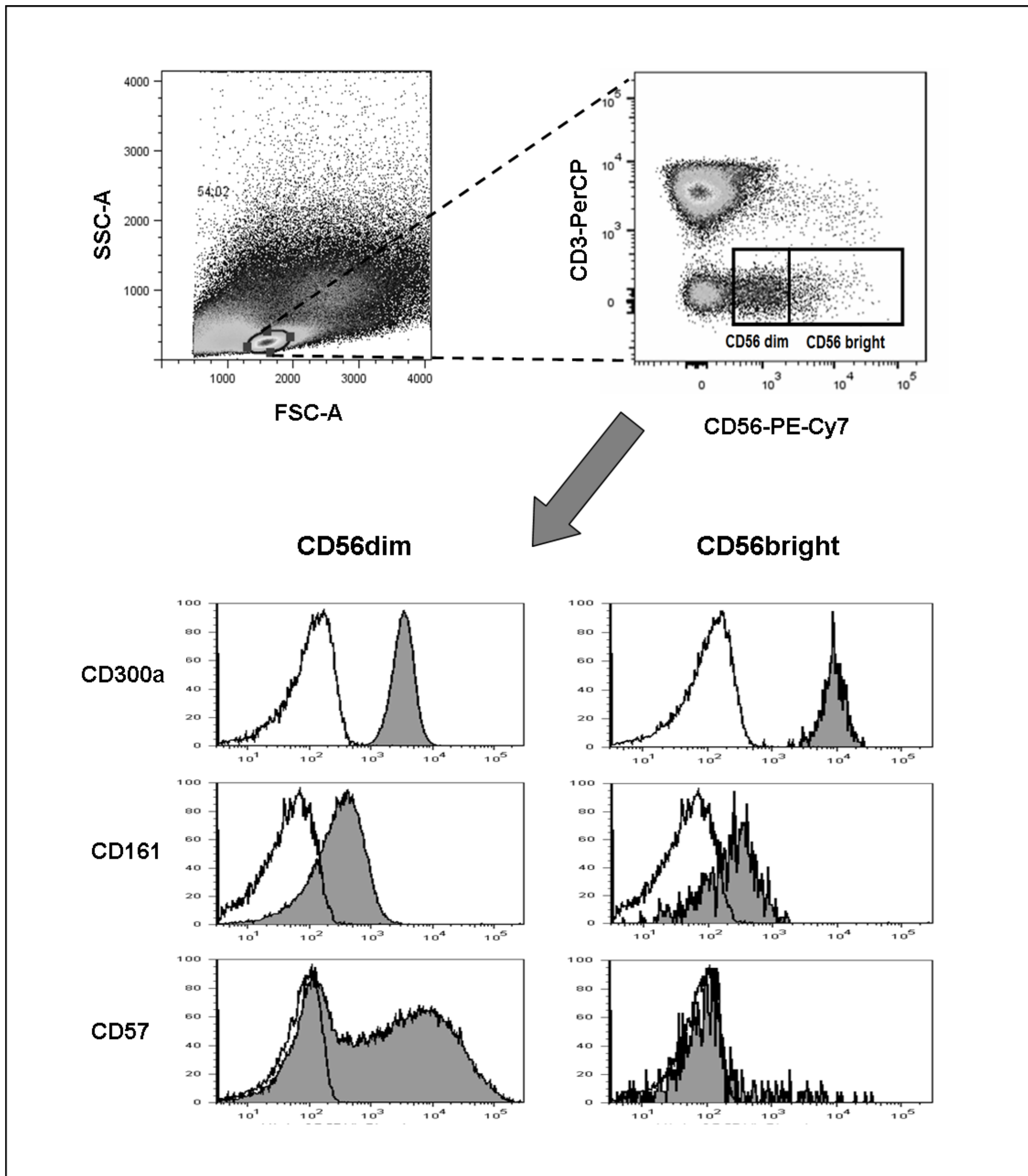

Figure S1. Gating strategy used for the analysis of CD57, CD300a and CD161 on NK cells by Flow Cytometry. Peripheral blood lymphocytes (PBLs) were selected using forward (FSC) and side scatter (SSC) detectors and $CD3^{-}CD56^{+}$ NK cells were gated from PBLs after single cells gating. CD57, CD300a and CD161 expression (measured as percentage) was determined on $CD56^{dim}$ and $CD56^{bright}$ NK cells.

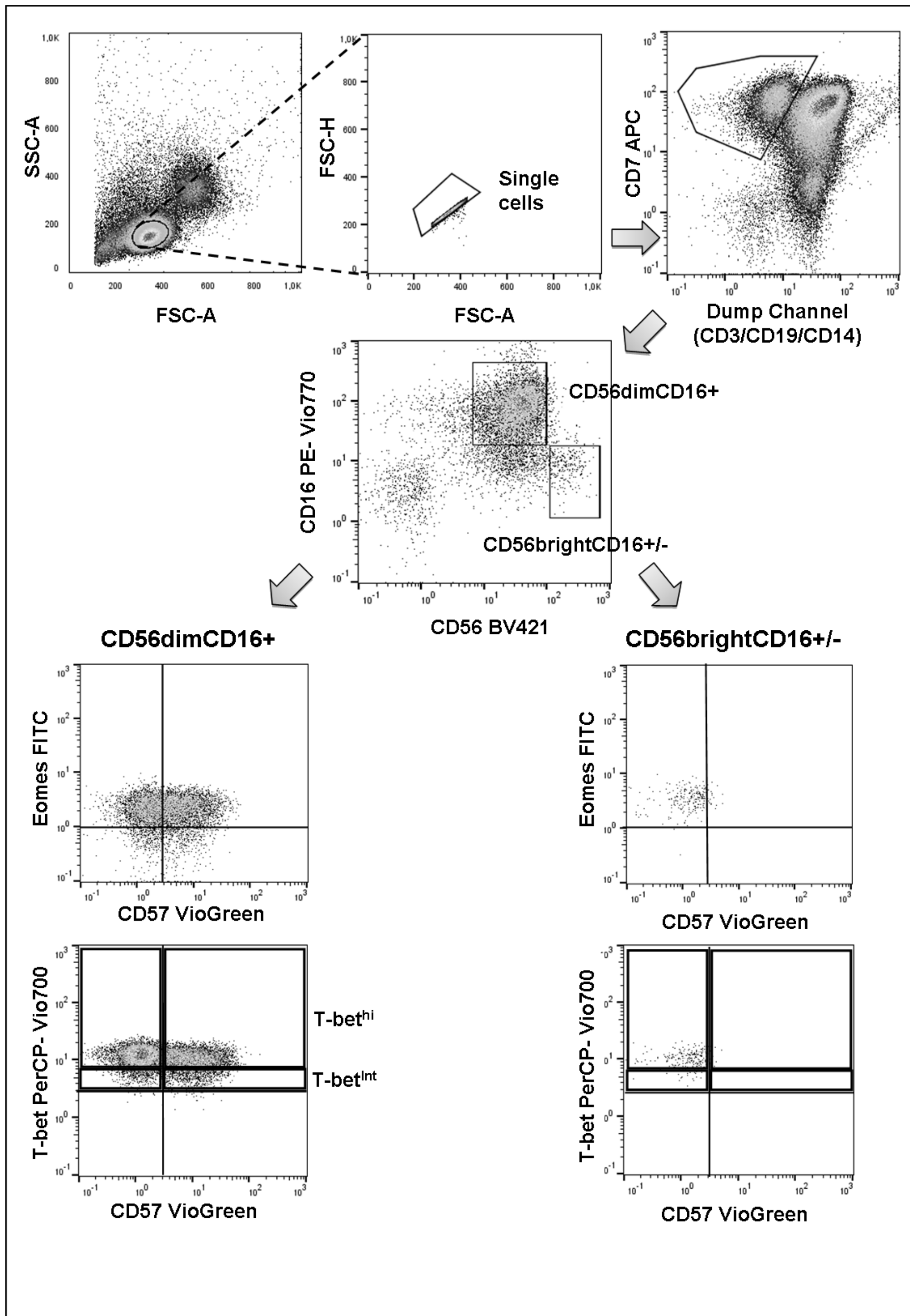

Figure S2. Gating strategy used for the analysis of Eomes and T-bet expression on NK cell subsets. $CD7^{+}CD3^{-}CD19^{-}CD14^{-}$ cells were gated of total PBLs, after isolating the single cells. NK cells subsets were selected according to expression density of CD56 and CD16 markers, followed by CD57 expression. Three subpopulations of NK cells were described: $CD56^{bright}CD57^{-}$, $CD56^{dim}CD57^{-}$ and $CD56^{dim}CD57^{+}$. The expression of T-bet and Eomes was measured in these NK subsets by Flow Cytometry. Two subsets of cells were defined according to the level of T-bet expression: T-bet^{hi} and T-bet^{int}.

III. Effect of age on NK cell compartment in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors.

Paulo Rodrigues-Santos*, **Nelson López-Sejas***, Jani Sofia Almeida, Lenka Ruzicková, Patricia Couceiro, Vera Alves, Carmen Campos, Corona Alonso, Raquel Tarazona, Paulo Freitas-Tavares, Rafael Solana* and Manuel Santos-Rosa*

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Effect of Age on NK Cell Compartment in Chronic Myeloid Leukemia Patients Treated With Tyrosine Kinase Inhibitors

Paulo Rodrigues-Santos[1,2,3†‡], Nelson López-Sejas[4†‡], Jani Sofia Almeida[2,3], Lenka Ruzičková[5], Patricia Couceiro[2,3], Vera Alves[1,3], Carmen Campos[4*], Corona Alonso[4], Raquel Tarazona[6], Paulo Freitas-Tavares[5], Rafael Solana[4*†§] and Manuel Santos-Rosa[1,3†§]

[1] Faculty of Medicine, Institute of Immunology, University of Coimbra, Coimbra, Portugal, [2] Laboratory of Immunology and Oncology, Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal, [3] Faculty of Medicine, Center of Investigation in Environment, Genetics and Oncobiology - CIMAGO, University of Coimbra, Coimbra, Portugal, [4] Department of Immunology, Instituto Maimónides de Investigación Biomédica de Córdoba - Reina Sofia University Hospital - University of Córdoba, Córdoba, Spain, [5] Hematology Service, Coimbra Hospital and Universitary Centre, Coimbra, Portugal, [6] Immunology Unit, University of Extremadura, Cáceres, Spain

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Edited by:
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***Correspondence:**
Carmen Campos
mccampos1977@gmail.com
Rafael Solana
rsolana@uco.es

† These authors have contributed equally to this work

‡ These authors share co-first authorship

§ These authors share co-senior authorship

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Natural killer (NK) cells are a very important component of the innate immune response involved in the lysis of virus infected and tumor cells. Aging has a profound impact in the frequency, phenotype and function of NK cells. Chronic Myeloid Leukemia (CML) is caused by the BCR-ABL gene formation encoding aberrant oncoprotein tyrosine kinase. Treatment with tyrosine kinase inhibitors (TKIs) induces durable deep molecular response. The response to treatment and life expectancy is lower in older patients with chronic phase of CML than in younger patients. In this work we analyse NK cells from TKI-treated CML patients and healthy controls stratified according to age. We have analyzed the expression of NK receptors, activation markers, NK cell differentiation in CD56^{bright} and CD56^{dim} NK cell subsets and the expression of CD107a and IFN-γ in NK cells stimulated with K562. Whereas significant differences on the phenotype and function of NK cells were found between middle-aged (35–65 years old) and elderly (older than 65) healthy individuals, NK cells from TKI-treated CML patients do not show significant differences related with age in most parameters studied, indicating that age is not a limitation of the NK cell recovery after treatment with TKI. Our results also revealed differences in the expression of NK receptors, activation markers and functional assays in NK cells from TKI-treated CML patients compared with age-matched healthy controls. These results highlight the relevance of NK cells in TKI-treated patients and the need of an extensive analysis of the effect of aging on NK cell phenotype and function in these patients in order to define new NK-cell based strategies directed to control CML progression and achieve long-term disease remission after TKI cessation.

Keywords: aging, CML, NK receptors, activation markers, differentiation markers, cytokines, NK cell subsets, tyrosine kinase inhibitors

INTRODUCTION

Natural Killer cells (NK) are innate lymphoid cells (ILCs) that represent ∼15% of peripheral blood lymphocytes (PBLs). NK cells share many features with ILC1 although they are developmentally distinct (1). NK cells can be classified in two major subpopulations according to CD56 expression. CD56^{bright} NK cells are less differentiated subpopulation that represents <10% of peripheral blood NK cells and have an immune-modulatory role with high production of cytokines and chemokines. CD56^{dim} NK cells, a more differentiated subpopulation that represents about 90% of NK cells, are mainly cytotoxic and interferon-gamma (IFN-γ) producers after direct contact with target cells (2, 3). A model of differentiation from immature CD56^{bright} that leads to more mature CD56^{dim} NK cells in the periphery has been proposed (4). Another subpopulation of NK cells, that do not express CD56 but express other NK receptors, expanded in healthy old and HIV-1 or hepatitis C infected individuals (5, 6), has been defined. NK cell function depends on a balance between activating and inhibitory signals triggered by activating and inhibitory receptors (7).

Chronic Myeloid Leukemia (CML) is an aging-associated disease (approximately half of cases are diagnosed in people older than 65) caused by reciprocal translocation between chromosomes 9 and 22 that give rise to the Philadelphia chromosome (Ph) and the BCR-ABL gene formation that encodes an oncoprotein tyrosine kinase with an aberrant activity in the hematopoietic stem cells (8, 9). Age has been included as a poor prognostic factor for survival in CML (10, 11). About half of the patients diagnosed with CML are between 60 and 65 years old (12–14) and the response to disease and life expectancy is lower in older patients with chronic phase of CML than in younger patients (15, 16). However, elderly CML patients are underrepresented in clinical studies having a reduced access to investigational therapies and median age of CML patients in cancer registries and patients included in clinical trials differs by 10–20 years (13, 14), supporting the interest to study immune parameters in elderly CML patients.

Immunosenescence is defined as age-associated dysregulation and dysfunction of the immune system characterized by impaired protective immunity and decreased efficacy of vaccines (17–20). These changes mainly affect the adaptive immune response (21) although consistent findings reveal that innate immune response is also affected (22, 23). In addition to age, situations of chronic activation of the human immune system, such as viral infections, autoimmune diseases and cancer, are involved in the development of immunosenescence (24–27).

Age-related alterations in frequency, distribution, phenotype, and function of NK cell subsets have been described, including an increased expression of CD57 (considered a marker of 'memory-like' NK) and a decreased expression of Natural Cytotoxicity Receptors (NCRs) and other NK activating receptors (22, 23, 28–31), CD69 (32), and CD94/NKG2A, and an increase of killer Ig-like receptors (KIR) (33–35) in older individuals.

Several studies have also found a decrease in the frequency and function of NK cells in CML patients at the time of diagnosis, with a progressive functional deterioration during all phases of the disease (36–39). It has been described a decreased expression of NKG2A, NKp30, and NKp46 at the time of diagnosis and changes in the NKG2C and KIR receptors (40, 41). Patients with CML also show a decrease in NKG2D expression, that mediates NK anti-CML response through its ligands MICA/B, when compared with healthy controls (42). NK cells from acute myeloid leukemia patients also have a downregulated expression of activating receptors NKp30 and NKp46 (27, 43–45) and DNAM-1 (27, 46), likely as a consequence of the interaction with their ligands in leukemic blasts.

The standard treatment for CML patients is based in the use of tyrosine kinase inhibitors (TKIs) such as imatinib, and more potent second-generation nilotinib and dasatinib, that have improved CML poor prognosis (47, 48). TKIs have a direct effect inhibiting the BCR-ABL1 kinase activity to induce a durable deep molecular response, a prelude to successful treatment-free remission that occurs in ∼50% of all CML patients who cease TKI therapy (48–51). In addition to their direct anti-kinase activity, TKIs contribute to the restoration of immune cell function leading to the efficient immunological control of CML (41). Recent studies show the impact of TKIs on NK cells, finding that the expression of NK activating receptors is restored to normal levels compared to their low level at the time of diagnosis (40, 41).

Considering that both aging and CML induce changes in phenotype and function of NK cells, in this work we have studied the expression of several markers (CD11b, CD27, CD57, CD69, HLA-DR, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, and NKp80) in NK cell subsets, and CD107a and IFN-γ in K562 stimulated NK cells, from CML patients and healthy controls, stratified according to age in middle-aged and elderly donors.

MATERIALS AND METHODS

Study Subjects

A total of 80 individuals were included in the study, 38 CML patients treated with first-line TKIs Imatinib, and 42 healthy controls, stratified in two groups according to age: middle-aged (35–65 years) and old-age (over 65 years) (**Table 1**). All the participants in the study were CMV-seropositive (Non-reactive IgM and reactive IgG, data not shown). Controls were excluded of the study if they had infection at the time of sample collection, suffered or had suffered cancer or autoimmune diseases, were under immunosuppressive drugs or calcium channel blockers. The Ethical Committees of the Faculty of Medicine of the University of Coimbra and the Coimbra Hospital and University Centre (Portugal) and the Ethics Committee of the Reina Sofia University Hospital of Cordoba (Spain) approved this study and all volunteers agreed and signed informed consent to participate.

Procedures of Sample Collection and Processing

Peripheral blood samples from all individuals were obtained in heparinized tubes. Flow cytometry studies were performed on freshly-obtained cells. After antibody staining, BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) was used for lysis of red blood cells. Subsequently, cells were washed and resuspended in Dulbecco's Phosphate Buffered Saline (PBS)

TABLE 1 | Demographics characteristics of individuals ($n = 80$).

Group name	No.	Sex (male: female)	Mean (SD)[a]	p[b]
Middle age (Control)	19	8:11	51 (7.85)	0.924
Middle age (CML*)	19	12:7	51 (8.97)	
OLD (Control)	23	10:13	74 (6.17)	0.814
OLD (CML*)	19	12:7	75 (5.71)	

[a]*Average age (Standard Deviation) of the group.*
[b]*p-value for the comparison of means between controls and CML, within the same age group (t-Student test).* [*]*TKI-treated CML patients.*

pH 7.4 (Ambion, Austin, TX, USA) for later acquisition on the cytometer. Cell suspensions were acquired in a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA). These procedures were performed according to the manufacturer protocols.

Flow Cytometric Analysis and Monoclonal Antibodies

Fresh blood was used for the analysis of the surface receptors by flow cytometry in different tubes. The following mouse anti-human conjugated monoclonal antibodies (mAbs) were used: anti-CD3 V500 (clone UCHT1, BD Horizon), anti-CD14 V500 (clone M5E2, BD Horizon), anti-CD19 V500 (clone HIB19, BD Horizon), anti-CD3 APC-H7 (clone SK7, BD Biosciences), anti-CD56 PerCP-Cy5.5 (clone HCD56, BD Pharmingen), anti-CD11b V450 (clone ICRF44, BD Horizon), anti-CD27 FITC (clone 0323, Biolegend), anti-CD57 Pacific Blue (clone HNK-1, Biolegend), anti-CD69 FITC (clone FN50, Biolegend), anti-HLA-DR V500 (clone G46-6, BD Pharmingen), anti-NKp30 Alexa Fluor 647 (clone P30-15, Biolegend), anti-NKp44 Alexa Fluor 647 (clone P44-8, Biolegend), anti-NKp46 PE (clone 9E2, Biolegend), anti-NKp80 PE (clone SD12, Biolegend), anti-NKG2A PE (clone 131411, R&D Systems), anti-NKG2C APC (clone 134591 R&D Systems), anti-NKG2D APC (clone 1D11, Biolegend). The expression of HLA-DR, NKp30, NKp44, NKp46, NKp80, NKG2D, (measured as Median Fluorescence Intensity, MFI) and the expression of CD11b, CD27, CD57, CD69, NKG2A, and NKG2C (measured as relative frequency) were determined in the different NK cell subpopulations and analyzed by multiparametric flow cytometry. The data were analyzed using FlowJo v10 (Tree Star, Inc.) from PBLs, selecting singlets. NK cells ($CD56^+$ and $CD3^-/CD14^-/CD19^-$) were selected and two subpopulations of NK cells were described, in relation to their expression of CD56, as $CD56^{bright}$ and $CD56^{dim}$ (**Figure S1A**). Isotype matched antibodies, labeled with the appropriate fluorochromes, were used as negative controls. Representative histograms for the NK cell markers, and the isotype and fluorochrome of the antibodies used in the studies, are shown in **Figure S1B**.

NK Cell Stimulation With K562, Degranulation Assay, and IFN-γ Staining

Effector peripheral blood mononuclear cells were isolated from heparinized blood samples by a standard density gradient procedure (Ficoll-Paque PLUS, Merck KGaA, Darmstadt, Germany) and counted to adjust the concentration to 50×10^6 cells/mL. Target cells (K562 cell line) were prepared; viability determined and counted to adjust the concentration to 1×10^5 cells/mL. Then, effector and target cells were mixed in a 25:1 effector-to-target ratio into 12×75 mm tubes with anti-CD107a PE (clone H4A3, BD Pharmingen®) antibody and brefeldin A (Merck, 10 μg/mL). Tubes were incubated in a humidified CO_2 incubator in the water reservoir at the bottom for 4 h. At the end of incubation cells were washed and resuspended in 100 μL of 1xPBS (phosphate-buffered saline) and the extracellular antibodies were added, anti-CD56 APC (clone B159, BD Pharmingen®) and anti-CD3V500 (clone UCTH1, BD Horizon®). After 15 min of incubation at RT in the dark, suspensions were treated with Fix and Perm A solution (Invitrogen®) for 15 min, in the dark at room temperature. Cells were centrifuged at 453 g for 5 min and the supernatant discarded. Next, cells were incubated with Fix and Perm B solution (Invitrogen®) and the intracellular antibody anti-IFN-γ PE-Cy7 (clone B27, BD Horizon®) for 20 min, in the dark at room temperature. After centrifugation at 453 g for 5 min and supernatant discarded cells were resuspended in 1x PBS and acquired in the flow cytometer (BD FACS Canto II).

Statistical Analysis

Shapiro-Wilk test was used for checking the normal distribution of the data. We used Kruskall-Wallis (non-parametric) test for multiple comparison and Mann-Whitney *U*-test (non-parametric) was used to compare specific groups. All tests were performed using statistical software package SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Values $p < 0.05$ were considered significant. The results were shown as median with interquartile range and the graphics were performed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Expression of Activating and Inhibitory Receptors on NK Cells From TKI-Treated CML Patients

We studied the expression of activating and inhibitory receptors on $CD56^{dim}$ and $CD56^{bright}$ NK cells in healthy donors and CML patients, stratified in middle age and old age. The expression of Natural Killer Group 2 (NKG2) receptors was measured as the percentage of positive cells or as MFI measured in the total of cells **(Figure 1A)**. Our results showed a significant decrease in the expression of the inhibitory receptor NKG2A on $CD56^{dim}$ NK cells in middle-aged CML patients compared with middle-aged healthy donors and a decrease in the percentage of $NKG2A^+CD56^{bright}$ NK cells in old CML patients compared with old healthy donors. Age-associated changes in the expression of NKG2A were only observed in healthy donors showing an increase with age in the percentage of $NKG2A^+CD56^{bright}$ NK cells. In contrast, NKG2C receptor expression was not influenced by CML or age. Regarding the activating receptor NKG2D, we found a significant decrease in

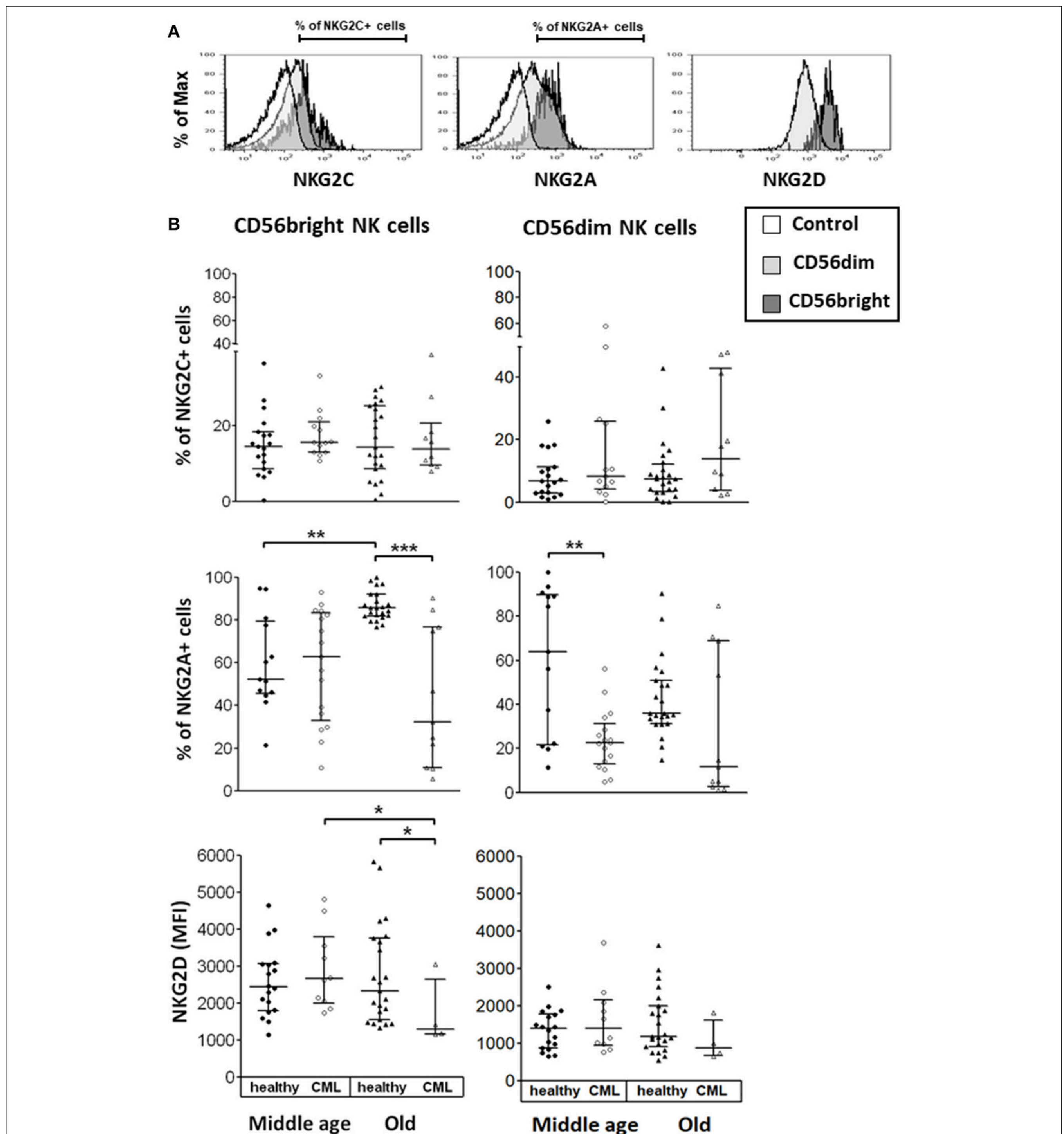


FIGURE 1 | Expression of Natural Killer Group 2 (NKG2) receptors on NK cell subpopulations. **(A)** Representative histograms for each marker are shown (the non-shaded area represents the control, the shaded area of light gray, the CD56^{dim} cells, the shaded one of gray, the CD56^{bright} cells). The percentage of cells expressing NKG2C, NKG2A, and NKG2D as (MFI) measured in the total of cells, was determined on the surface of each subset by multiparametric flow cytometry. **(B)** Expression of activating receptors (NKG2C and NKG2D) and of inhibitory receptor (NKG2A) on CD56^{bright} and CD56^{dim} NK subsets from healthy individuals and TKI-treated CML patients, stratified according to age (middle-aged 35–65 years and old >65 years). Number of donors: NKG2C middle-aged healthy $n = 19$, middle-aged CML $n = 13$, old healthy $n = 23$, and old CML $n = 10$; NKG2D middle-aged healthy $n = 18$, middle-aged CML $n = 10$, old healthy $n = 23$, and old CML $n = 4$; NKG2A middle-aged healthy $n = 13$, middle-aged CML $n = 17$, old healthy $n = 23$, and old CML $n = 11$. The results, expressed as median with interquartile range, were considered significant at $p < 0.05$. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. *p <0.05; **p <0.01; ***p <0.001.

the MFI of NKG2D on CD56^{bright} NK cells in old CML patients compared with old healthy donors, and a decrease with age in CML patients **(Figure 1B)**.

We have also studied the expression of NCRs on NK cell subpopulations. Results were expressed as MFI measured in the total number of cells **(Figure 2A)**. We have found that the expression of NKp30 and NKp80 (not a NCR) on CD56^{bright} NK cells and NKp80 on CD56^{dim} NK cells was lower in old CML patients compared with old healthy donors. NKp80 expression on CD56^{bright} cells and NKp46 expression on CD56^{dim} NK cells were significantly decreased in middle-aged CML patients compared with middle-aged healthy donors. The analysis of age-associated changes in the expression of NCRs in CML patients showed a decrease in NKp30 expression on CD56^{bright} NK cells. No significant differences associated with age (middle-aged vs. old age) were observed in healthy donors **(Figure 2B)**.

Expression of Activation Markers of NK Cells From TKI-Treated CML Patients

In this study, we have analyzed the expression of several activation markers (HLA-DR, CD69, and NKp44) on the surface of NK cell subsets in resting conditions. CD56^{bright} NK cells in elderly CML patients showed a decreased expression of HLA-DR (measured as MFI) and a reduction in the percentage of CD69 and NKp44 positive cells compared with elderly healthy donors. An age-associated increase in the expression of these three markers was observed on CD56^{bright} NK cells in elderly healthy donors compared with middle-aged healthy donors. In contrast, in CML patients a decrease of CD69 expression on CD56^{bright} NK cells was associated with age **(Figure 3A)**.

Regarding the expression of these activation markers on the CD56^{dim} NK cell subset, our results showed a decreased expression of these markers in elderly CML patients, as well as a decreased expression of CD69 and HLA-DR in middle-aged CML patients compared with age-matched healthy donors. Nevertheless, it is interesting to highlight the increase observed in the percentage of NKp44^{+} cells in middle-aged CML patients compared with middle-aged healthy donors. We also found an age-associated increase in the expression of HLA-DR and NKp44 in healthy individuals and a decrease of CD69 and NKp44 expression in CD56^{dim} NK cells from elderly CML patients compared with CD56^{dim} NK cells from middle-aged CML patients **(Figure 3B)**.

Analysis of NK Cell Differentiation Markers on NK Cells From TKI-Treated CML Patients

The analysis of NK cell subsets in healthy donors showed a decreased percentage of CD56^{bright} that correlated with an increased percentage of CD56^{dim} NK cells in elderly donors compared with middle-aged healthy donors. In contrast, no statistical significant differences in NK cell subset distribution was observed in CML patients according to age. Percentage of total NK cells was not influenced by CML or age (**Figure 4A**). The expression of CD57 was increased on CD56^{dim} NK cells from CML patients compared with age-matched healthy donors. Moreover, in healthy individuals, CD57 expression on both CD56^{bright} and CD56^{dim} NK cells increased with age (**Figure 4B**). The co-expression of CD11b and CD27 markers was also analyzed in each NK cell subset **(Figure 4C)**. Whereas, a high percentage of CD56^{bright} NK cells were CD11b^{+}CD27^{+}, the majority of CD56^{dim} NK cells from peripheral blood were CD11b^{+}CD27^{-}. Our results revealed a decrease of CD11b^{+}CD27^{+}CD56^{dim} NK cells associated with CML in middle-aged individuals and a decrease of this cell subset related to age in healthy donors (**Figure 4C**). Not significant differences were found in CD56^{bright} NK cells.

CD107a Expression and IFN-γ Production in NK Cells From TKI-Treated CML Patients Activated With K562

We analyzed the percentage of NK cells expressing CD107a or IFN-γ after stimulation with the K562 cell line. Representative analysis of middle-aged and old controls and TKI-treated CML patients are shown **Figures 5A,C**. The results did not show significant differences on CD107a expression and IFN-γ production in K562 stimulated NK cells between middle-aged healthy donors and middle-aged CML patients. NK cells from healthy elderly donors have higher expression of CD107a or IFN-γ compared with middle-aged healthy donors. In a similar way the percentage of NK cells expressing CD107a or IFN-γ was higher in healthy elderly donors than in TKI-treated CML old patients. On the contrary no significant age-associated differences on CD107a expression and IFN-γ production were observed in K562 stimulated NK cells from CML patients (**Figures 5B,D**).

As shown in **Figure S2**, the comparison of cytokine production in healthy controls vs. TKI-treated CML patients shows that the expression of IFN-γ by unstimulated NK cells is higher in middle-aged TKI-treated CML patients and lower in elderly patients compared with their age-matched controls, whereas the expression of IL-10 is higher in TKI-treated CML patients from both age groups compared with their respective controls.

DISCUSSION

A decrease in the frequency and function of NK cells in CML patients at the time of diagnosis has been demonstrated, with a progressive functional deterioration during disease progression to advanced and blast crisis phase (36–39, 52). In addition, NK cells from CML patients at diagnosis show a reduced expression of activating and inhibitory NK receptors compared to healthy donors (40, 53). NK cells play an important role in the control of CML not only during TKI treatment (40, 41) but also after TKI cessation (54, 55).

It has been shown that life expectancy is lower in older than in younger patients with chronic phase of CML and that aging is a poor prognostic factor for survival and response to treatment in CML (10, 11, 15, 16). Cumulative evidences support that aging affects NK cell subsets, phenotype and

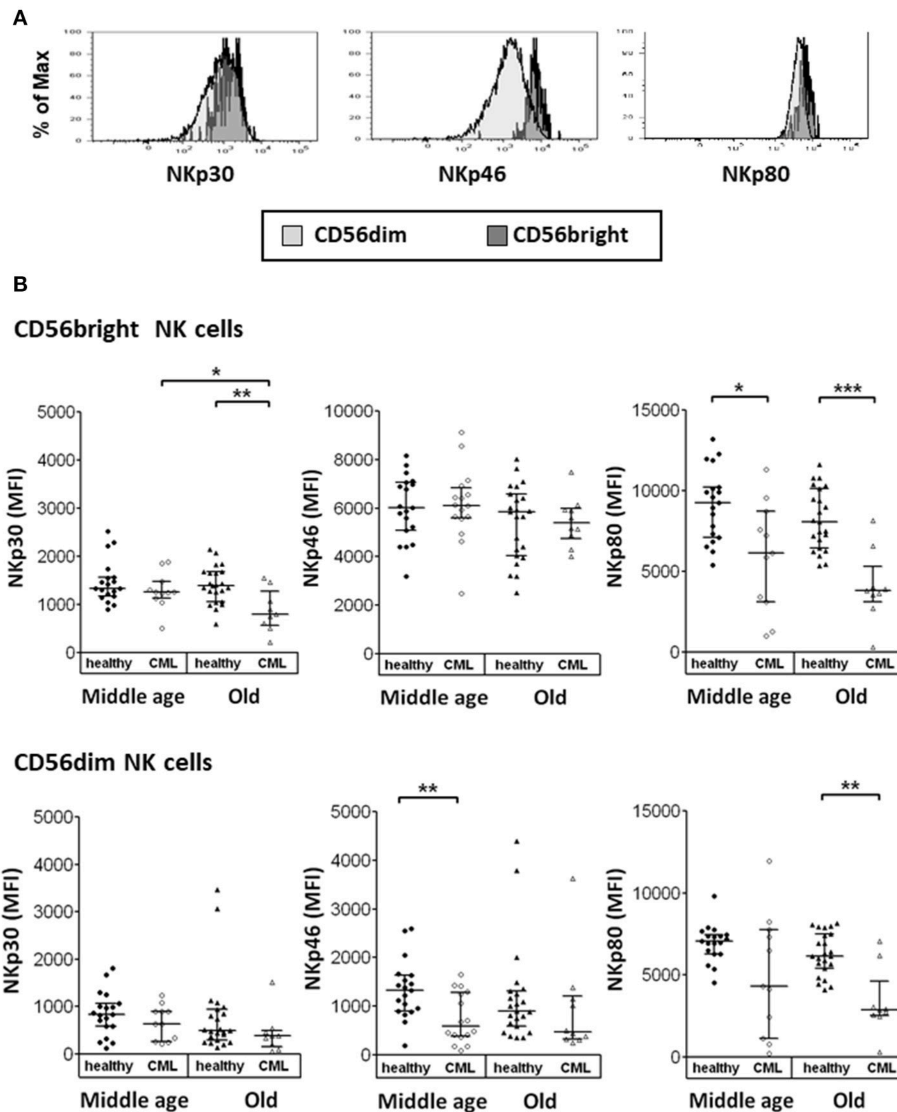


FIGURE 2 | Expression of Natural Cytotoxicity Receptors (NCRs) on $CD56^{bright}$ and $CD56^{dim}$ NK cells. **(A)** Representative histograms for NKp30, NKp46, and NKp80 are shown (the shaded area of light gray, the $CD56^{dim}$ cells, the shaded one of gray, the $CD56^{bright}$ cells). Results were expressed as Median Fluorescence Intensity (MFI), measured in the total of cells. **(B)** Expression of activating receptors NKp30, NKp46, and NKp80 on $CD56^{bright}$ and $CD56^{dim}$ NK subsets from healthy individuals and TKI-treated CML patients, stratified according to age (middle-aged 35–65 years and old >65 years). Number of donors: NKp30 and NKp80 middle-aged healthy $n = 19$, middle-aged CML $n = 11$, old healthy $n = 23$, and old CML $n = 9$; NKp46 middle-aged healthy $n = 19$, middle-aged CML $n = 16$, old healthy $n = 23$ and old CML $n = 10$. The results were expressed as median with interquartile range. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients and were considered significant at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

function (22, 23, 56), including the expression of activating and inhibitory NK cell receptors (28–30, 57). The analysis of NK cells from middle-aged and elderly healthy donors, summarized in **Figure 6A**, is in line with previous data on the differences between NK cells from young and old healthy donors. Thus, there are significant differences in the frequency of NK cells subsets with different maturation stages. The percentages of more immature $CD56^{bright}$ NK cells (2, 3), and $CD11b^{+}/CD27^{+}$ NK cells, that represent a minor subset of immunoregulatory NK cells described as an intermediate differentiation stage (58), are lower in elderly than in middle-aged healthy donors, whereas the percentage of mature highly cytotoxic $CD56^{dim}CD57^{+}$ NK cells is higher in the elderly (**Figure 6A**), confirming the age-associated shaping of NK cell subsets (22, 30). The expression of NK cell activation markers HLA-DR, CD69, and NKp44 is also higher in NK cells from elderly healthy donors, likely as a consequence of low grade age-associated inflammation (inflamm-aging) (59). Inflamm-aging is also consistent with the observation that NK cells from old healthy donors show higher expression of CD107a or IFN-γ in response to K562

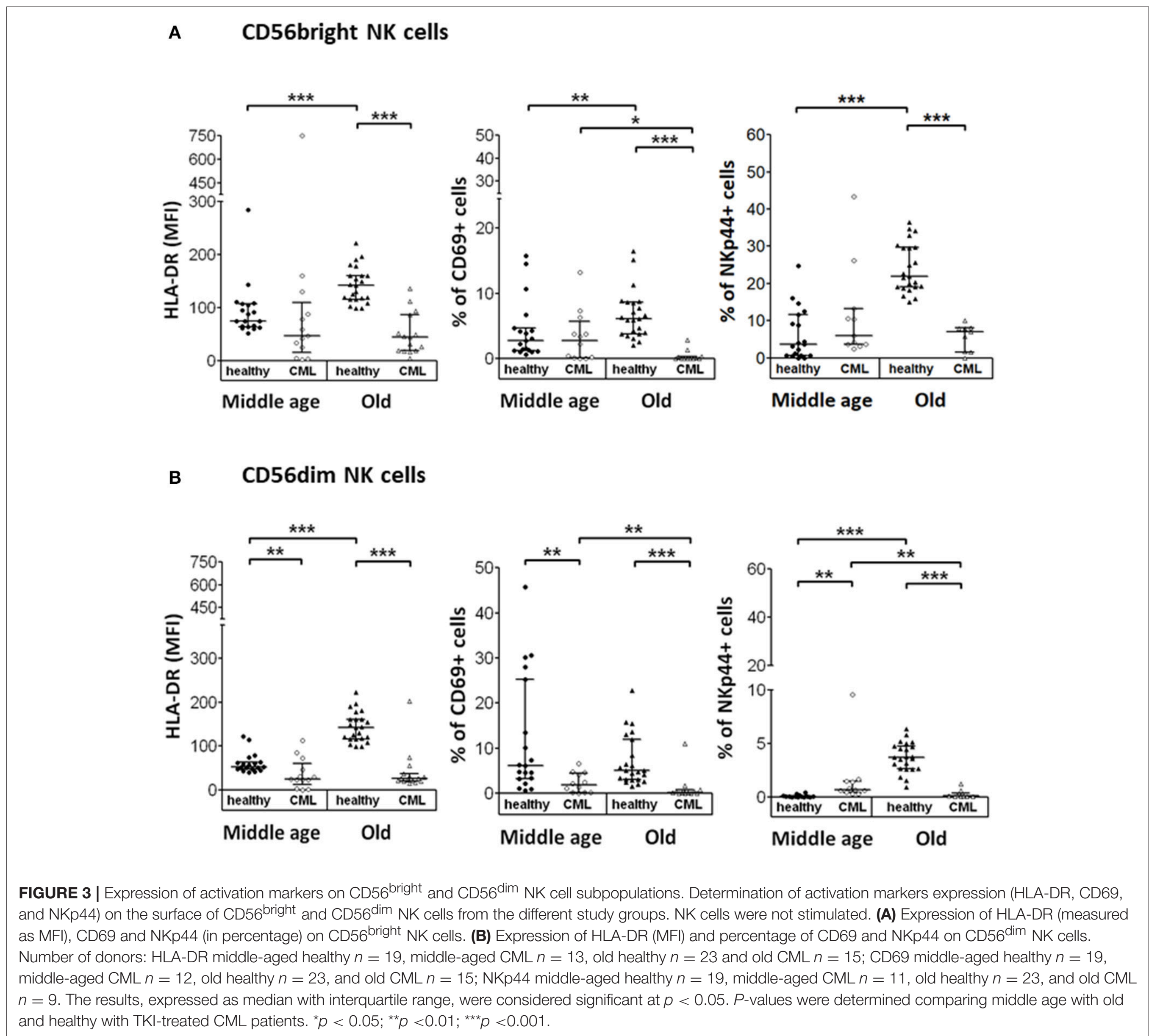


FIGURE 3 | Expression of activation markers on CD56^{bright} and CD56^{dim} NK cell subpopulations. Determination of activation markers expression (HLA-DR, CD69, and NKp44) on the surface of CD56^{bright} and CD56^{dim} NK cells from the different study groups. NK cells were not stimulated. **(A)** Expression of HLA-DR (measured as MFI), CD69 and NKp44 (in percentage) on CD56^{bright} NK cells. **(B)** Expression of HLA-DR (MFI) and percentage of CD69 and NKp44 on CD56^{dim} NK cells. Number of donors: HLA-DR middle-aged healthy $n = 19$, middle-aged CML $n = 13$, old healthy $n = 23$ and old CML $n = 15$; CD69 middle-aged healthy $n = 19$, middle-aged CML $n = 12$, old healthy $n = 23$, and old CML $n = 15$; NKp44 middle-aged healthy $n = 19$, middle-aged CML $n = 11$, old healthy $n = 23$, and old CML $n = 9$. The results, expressed as median with interquartile range, were considered significant at $p < 0.05$. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

stimulation than NK cells from middle-aged donors. The effect of aging on NK cell cytotoxicity and IFN-γ has been extensively analyzed in discrepant results have been found among different groups probably due to different selection age ranges, technical procedures, and health status of the individuals studied although it is generally accepted that the total number and cytotoxic function of NK cell are preserved or increased in healthy aging compared with young and middle-aged individuals (22, 23, 56).

The study of NK cells in middle-aged TKI-treated CML patients shows that the percentage of CD56^{bright} NK cells is similar to the percentage found in heathy middle-aged individuals, whereas the minor population of CD56^{dim} NK cells co-expressing CD11b and CD27 is dramatically decreased **(Figure 6B)**. In addition, CD56^{dim} NK cells from TKI-treated CML patients have higher expression of CD57, a marker of highly differentiated NK cells (22, 30), and NKp44 indicating that NK cells from TKI-treated CML patients are highly differentiated activated NK cells, as it has been recently suggested (40, 54). However, there is a lower expression of NKp46, NKG2A, CD69, and HLA-DR on CD56^{dim} NK cells and of NKp80 on CD56^{bright} NK cells **(Figure 6B)**. The comparison of NK cells from old TKI-treated CML patients with those from old healthy individuals shows a lower expression of NK receptors NKp44, NKp80, CD69, and HLA-DR in both CD56^{bright} and CD56^{dim} NK cells and also a reduced expression of NKp30, NKG2D, and NKG2A in CD56^{bright} NK cells **(Figure 6C)**. Thus, despite the well-established observations that CML patients

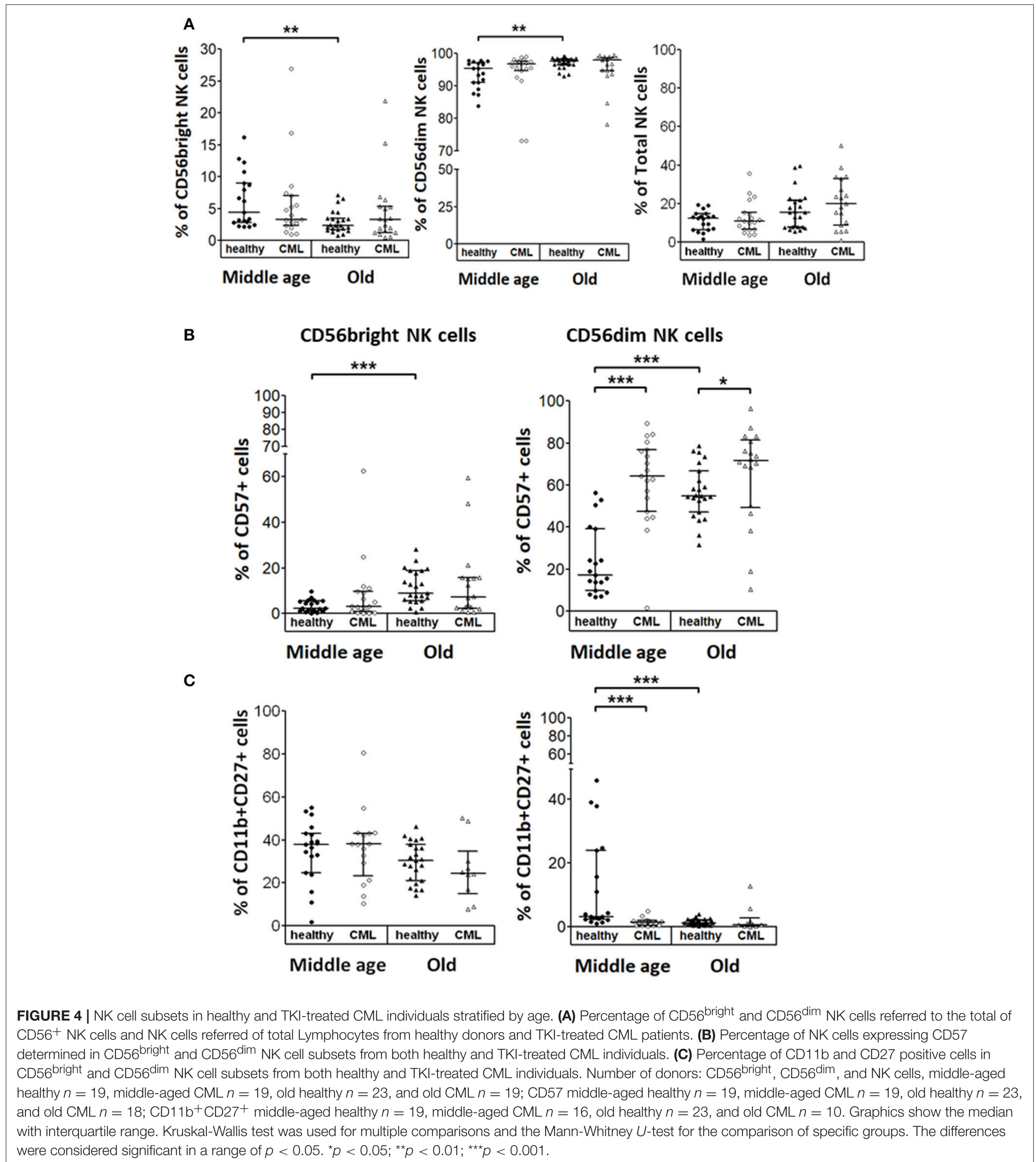


FIGURE 4 | NK cell subsets in healthy and TKI-treated CML individuals stratified by age. **(A)** Percentage of CD56^{bright} and CD56^{dim} NK cells referred to the total of CD56^{+} NK cells and NK cells referred of total Lymphocytes from healthy donors and TKI-treated CML patients. **(B)** Percentage of NK cells expressing CD57 determined in CD56^{bright} and CD56^{dim} NK cell subsets from both healthy and TKI-treated CML individuals. **(C)** Percentage of CD11b and CD27 positive cells in CD56^{bright} and CD56^{dim} NK cell subsets from both healthy and TKI-treated CML individuals. Number of donors: CD56^{bright}, CD56^{dim}, and NK cells, middle-aged healthy $n = 19$, middle-aged CML $n = 19$, old healthy $n = 23$, and old CML $n = 19$; CD57 middle-aged healthy $n = 19$, middle-aged CML $n = 19$, old healthy $n = 23$, and old CML $n = 18$; CD11b^{+}CD27^{+} middle-aged healthy $n = 19$, middle-aged CML $n = 16$, old healthy $n = 23$, and old CML $n = 10$. Graphics show the median with interquartile range. Kruskal-Wallis test was used for multiple comparisons and the Mann-Whitney *U*-test for the comparison of specific groups. The differences were considered significant in a range of $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

have a decreased expression of NK receptors at diagnosis (36–39), our results confirm that NK cells from TKI-treatment CML patients can express NK activating receptors (40, 41) such as NKp30, NKp46, and NKp80, although this expression is heterogeneous and in some cases their levels are lower than those found in age-matched healthy controls. NKp30 and NKp46 are NCRs involved in NK cells cytotoxicity after interaction with their ligands on target cells (60). NKp80, is

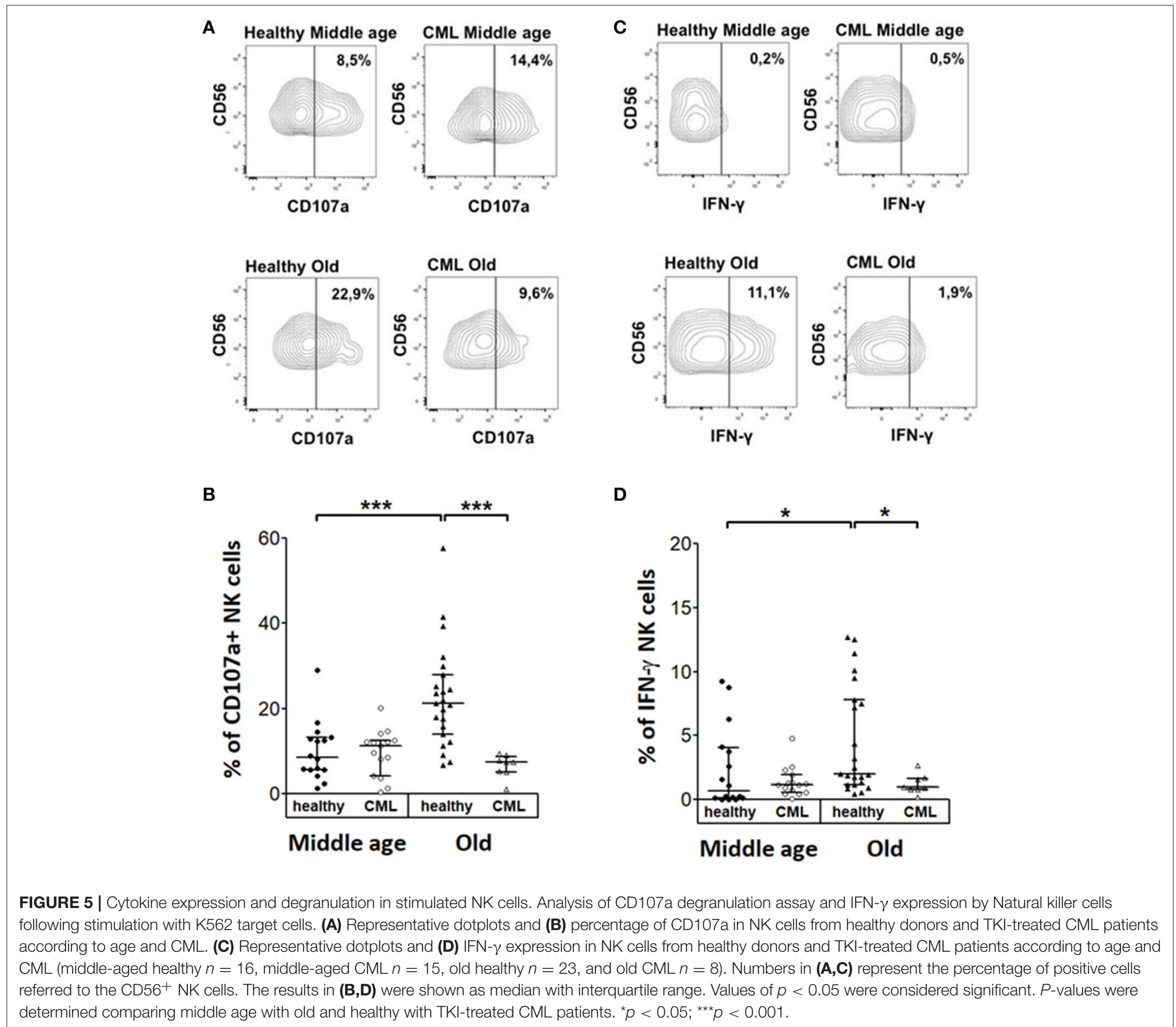
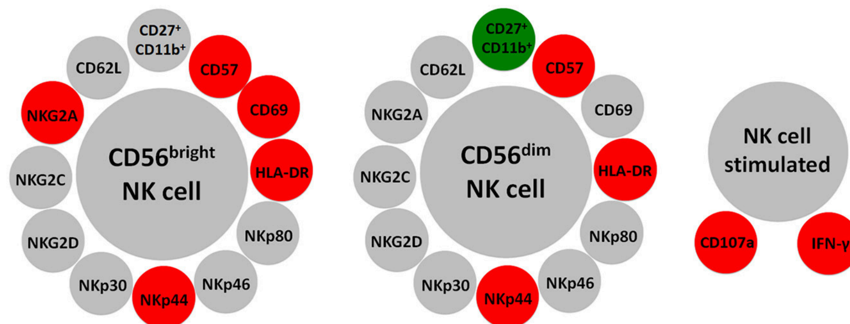


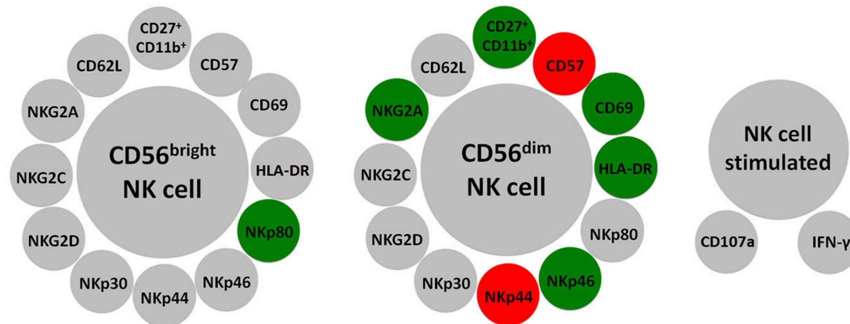
FIGURE 5 | Cytokine expression and degranulation in stimulated NK cells. Analysis of CD107a degranulation assay and IFN-γ expression by Natural killer cells following stimulation with K562 target cells. **(A)** Representative dotplots and **(B)** percentage of CD107a in NK cells from healthy donors and TKI-treated CML patients according to age and CML. **(C)** Representative dotplots and **(D)** IFN-γ expression in NK cells from healthy donors and TKI-treated CML patients according to age and CML (middle-aged healthy $n = 16$, middle-aged CML $n = 15$, old healthy $n = 23$, and old CML $n = 8$). Numbers in **(A,C)** represent the percentage of positive cells referred to the $CD56^{+}$ NK cells. The results in **(B,D)** were shown as median with interquartile range. Values of $p < 0.05$ were considered significant. *P*-values were determined comparing middle age with old and healthy with TKI-treated CML patients. $^{*}p < 0.05$; $^{***}p < 0.001$.

an activating C-type lectin-like receptor expressed on NK cells that interact with its ligand activation-induced C-type lectin (AICL) expressed on myeloid cells, including myeloid leukemia cells (61). It has been suggested that after TKI treatment NK cells are involved in the control of CML blasts (40, 41), thus the lower expression of these receptors compared with healthy controls can be the consequence of the interaction of NK cells with their ligands expressed on leukemic blasts, as suggested for NK cells from AML patients (44–46). Downregulation of NKG2D after its interactions with MICA/B ligand is a well-defined phenomenon in different tumors (62, 63). However, the expression of NKG2D is well preserved in all NK cell subsets from TKI treated CML patients, with the exception of $CD56^{bright}$ from elderly patients, confirming recent studies showing that the downregulated expression of NKG2D at the time of CML diagnosis, is restored to normal levels after TKI treatment (40, 41). The comparison of NK cell response to K562 in healthy controls vs. TKI-treated CML patients did not show significant differences on CD107a expression in K562 stimulated NK cells when middle-aged healthy donors were compared with middle-aged or old CML patients **(Figures 6B,C)** supporting previous findings that TKI treatment is associated with immune system re-activation and restoration of NK cell immune surveillance in CML patients (40, 41). On the contrary the observation that the percentage of NK cells expressing CD107a in TKI-treated CML old patients was lower than in healthy elderly donors, together with the lower expression of NK receptors in old TKI-treated CML patients compared with healthy elderly healthy donors **(Figure 6C)**, support that the alterations on NK cells observed in healthy elderly donors likely associated

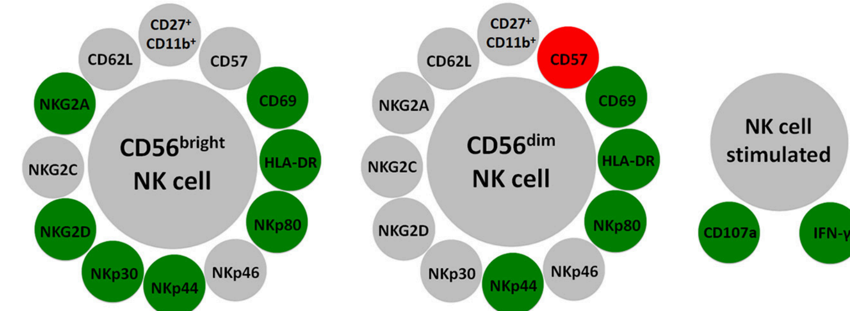
A **Age effect on NK cell compartment from healthy individuals: Middle-aged vs Old**



B **NK cell compartment in middle-age: CML patients vs healthy individuals**



C **NK cell compartment in old age: CML patients vs healthy individuals**



D **Age effect on NK cell compartment from CML patients : Middle-aged vs Old**

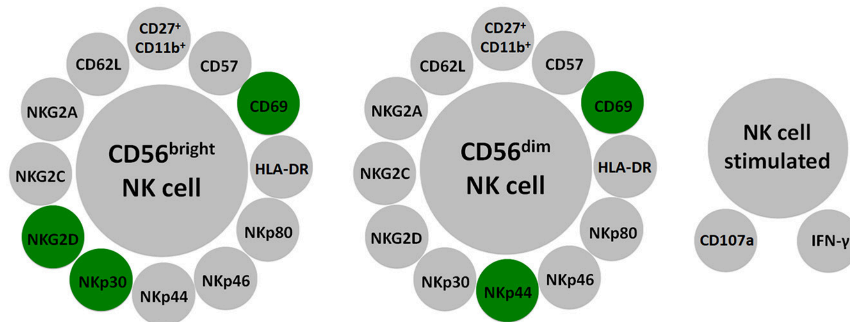


FIGURE 6 | Schematic representation of major differences in resting and K562 stimulated NK cells according to age and CML. Circles represent NK cell subpopulations expressing the indicated membrane markers in CD56^{bright} and CD56^{dim} resting NK cells and the expression of CD107a and IFN-γ in K562 stimulated NK cells. Red color was used for increased expression and green color for decreased expression of the membrane markers in NK cells from healthy old compared with healthy middle-aged individuals **(A)**, from CML patients compared with age-matched healthy donors **(B,C)**, or in old CML patients compared with middle-aged CML patients **(D)**.

with chronic virus infection, such as CMV, and inflamm-aging are not observed in old TKI-treated CML patients. The high variability observed in IFN-γ production by NK cells in response to K562 in healthy donors and the low response observed in most CML patients, represent a limitation of the study that precludes to obtain a conclusion on the significance of cytokine production in the disease control. The NK cell functional capacity and cytokine production during TKI-treatment and after TKI cessation in CML patients requires further analysis to discriminate a possible role in the long-term elimination of CML blasts (54, 55).

The analysis of the possible effect of age on NK cells from TKI-treated CML patients shows a lower expression of activation markers NKp44 and CD69 in elderly compared with middle-aged TKI-treated CML patients whereas no significant differences related with age are found in the other parameters studied, including CD107a expression and IFN-γ production in K562 stimulated NK cells from TKI-treated CML patients **(Figure 6D)**, indicating that age is not a limitation of the NK cell recovery after treatment with TKI.

In conclusion, despite the deleterious effect of aging in CML prognosis, our results showing that activating NK cell receptors can be expressed both in middle-aged and elderly TKI-treated CML patients highlight the interest to extensively analyse the effect of aging on NK cell phenotype and function in these patients. The possibility of enhancing NK cell activity by using cytokines and immunomodulating agents open new perspectives for the design of novel clinical trials aiming effective long-term treatment-free remission after TKI cessation in CML patients.

AUTHOR CONTRIBUTIONS

PR-S, NL-S, CC, RS, RT, and MS-R: research study design; PR-S, NL-S, JSA, PC, and VA: experiments conduction and data acquisition; LR and PF-T: clinical data and patient management; PR-S, NL-S, RT, JSA, CC, and CA: data analysis; PR-S, CA, and MS-R: reagents providing; PR-S, NL-S, CC, RT, and RS: manuscript writing; All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02587/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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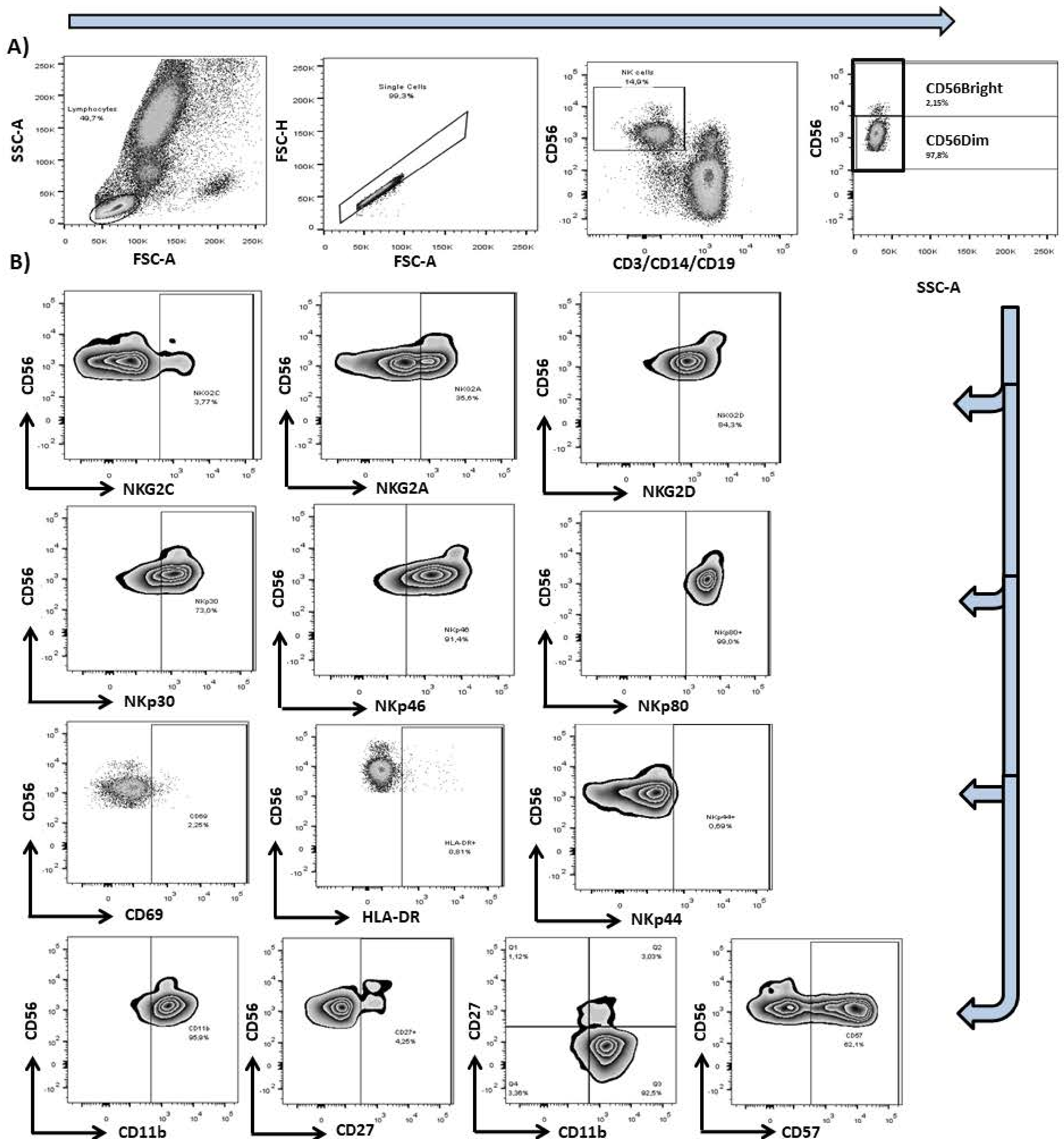
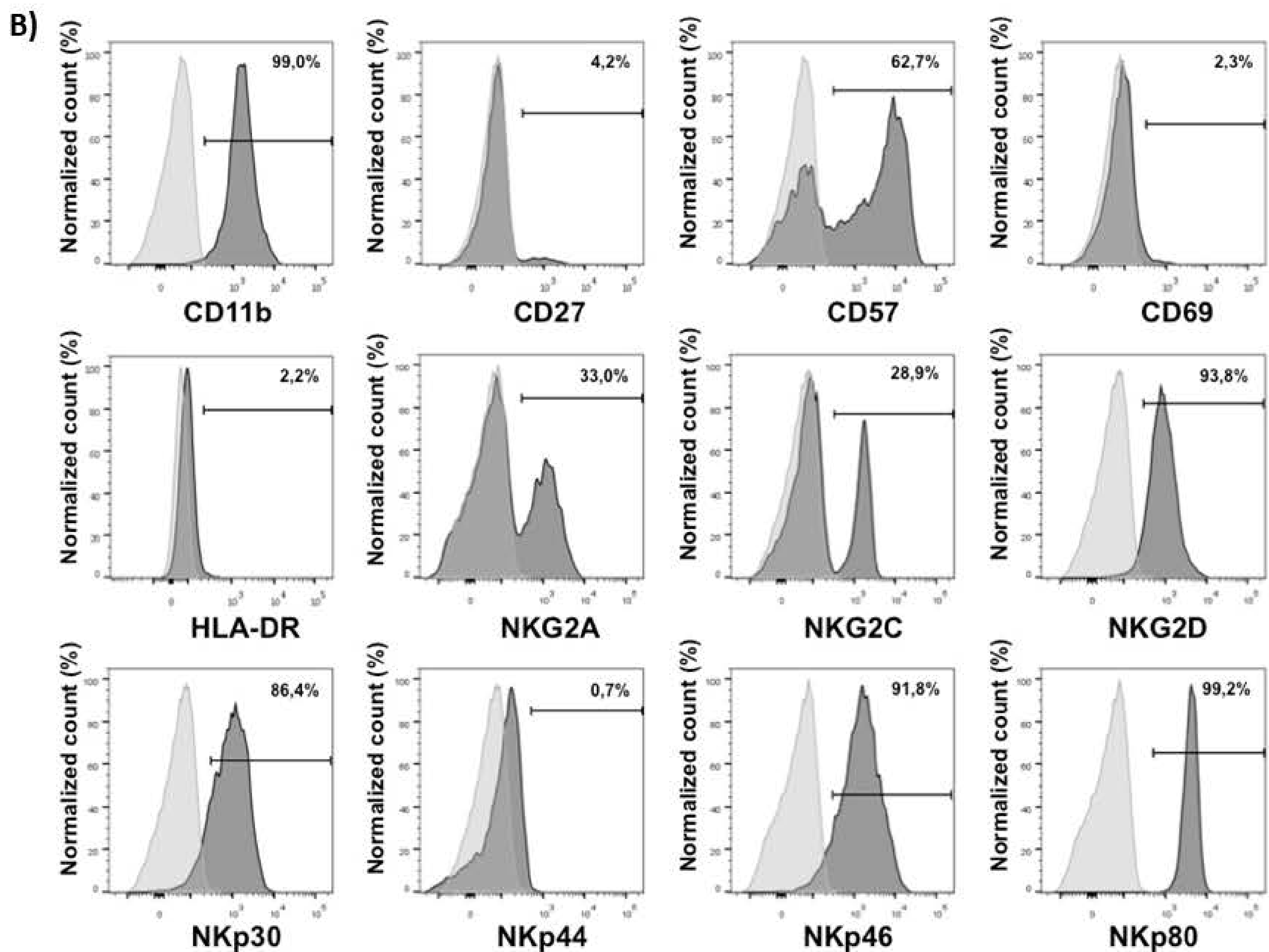


Figure S1A. Gating strategy used for the analysis NK cell markers. Lymphocytes were selected from total cells, doublets were removed using FSC-A and FSC-H, the dump channel (CD3/CD14/CD19) was excluded and finally NK cells were selected according to the CD56 expression. **B)** Receptors studied in the subpopulations CD56bright and CD56dim NK cells.



Specificity	Fluorochrome	Isotype
CD11b	V450	IgG1 κ
CD27	FITC	IgG1 κ
CD57	PacificBlue	IgM κ
CD69	FITC	IgG1 κ
HLA-DR	V500	IgG2a κ
NKG2A	PE	IgG2a κ
NKG2C	APC	IgG1 κ
NKG2D	APC	IgG1 κ
NKp30	AF647	IgG1 κ
NKp44	AF647	IgG1 κ
NKp46	PE	IgG1 κ
NKp80	PE	IgG1 κ

Figure S1B. Representative histograms used for analysis of NK cell markers. Representative histograms for each marker are shown in the upper part of the figure. The light gray area represents the isotype controls and the dark gray area represents the positive region. The antibodies used for the analysis of NK cell markers, indicating the fluorochrome and the isotype, are listed in the lower part of the figure. Isotype matched antibodies labelled with the appropriate fluorochrome were used as negative control.

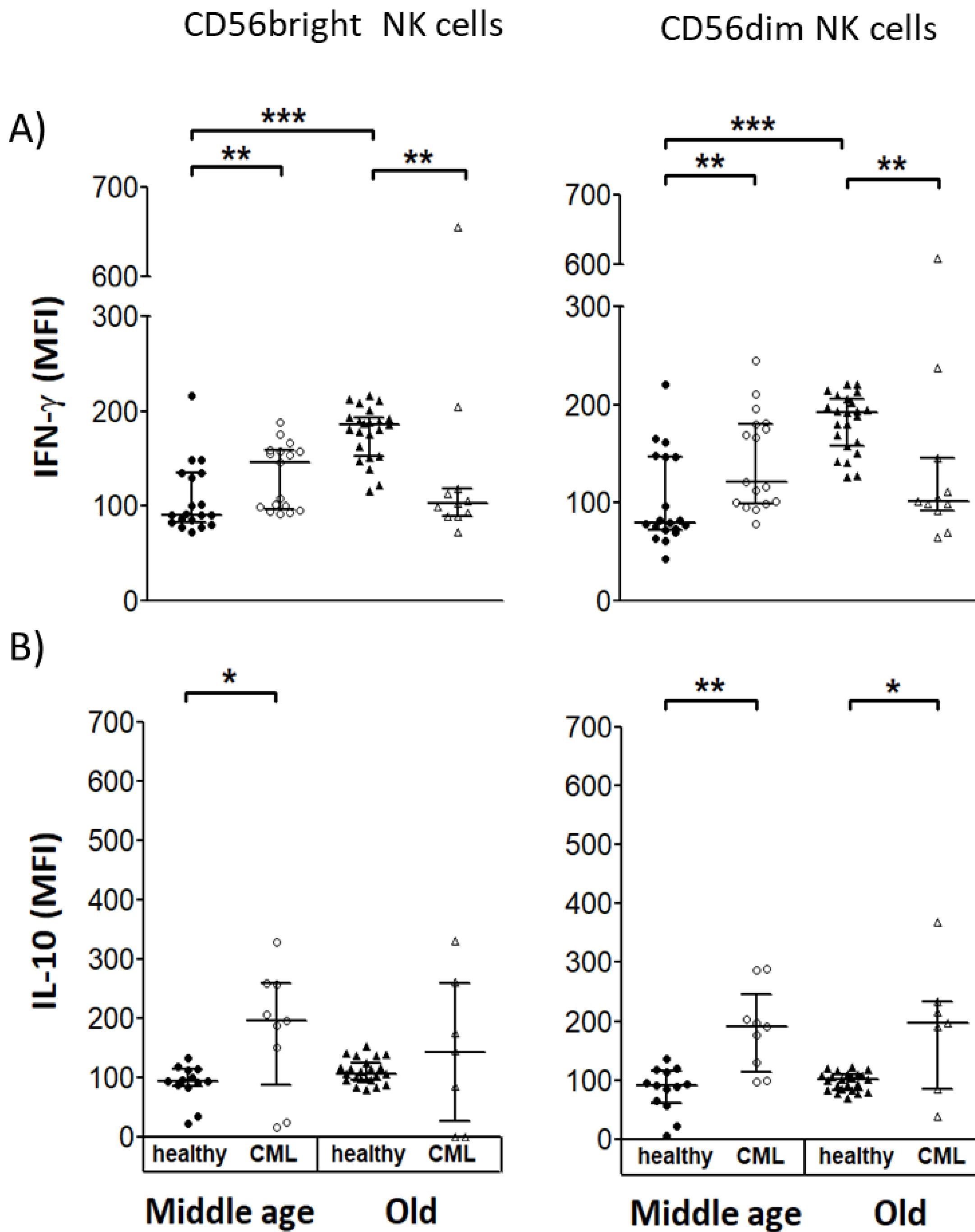


Figure S2. Cytokine expression in unstimulated NK cells.

A) Analysis of effect of age and CML in the expression of IFN-γ, and

B) IL-10 in CD56^{bright} and CD56^{dim} NK cells. Phenotypic characterization of the NK cell subpopulations was performed by surface labeling, and the determination of the expression of the cytokines into of each subset, was realized by intracellular labeling and flow cytometry. IFN-γ middle-aged healthy n=19, middle-aged CML n=17, old healthy n=23 and old CML n=11. IL-10 middle-aged healthy n=13, middle-aged CML n=9, old healthy n=23 and old CML n=7. The results were shown as median with interquartile range and were expressed in median fluorescent intensity (MFI). Values of *p* <0.05 were considered significant. (* *p*<0.05; ** *p*<0.01; *** *p*<0.001). *P* values were determined comparing middle age with old and healthy with TKI-treated CML patients.

5. Discusión General

Evidencias acumuladas con el tiempo apuntan que la infección crónica por virus, en particular por el CMV, y diferentes tipos de cáncer, tanto tumores sólidos como leucemias y linfomas, son responsables de cambios profundos en las células del sistema inmune tanto innato como adaptativo que son similares a las alteraciones del sistema inmunológico asociadas al envejecimiento y que definen el proceso de inmunosenescencia. Así se habla de inmunosenescencia prematura o inmunosenescencia temprana cuando se encuentran modificaciones del fenotipo y función de las células del sistema inmunológico similares a las que aparecen en el envejecimiento en individuos más jóvenes asociados a diferentes patologías como infecciones víricas crónicas o cáncer [56-59, 80, 110]. Entre las infecciones víricas crónicas que participan en el proceso de la inmunosenescencia destaca la infección por CMV y se ha postulado que la infección por CMV desempeña un papel muy importante en este proceso.

Alteraciones en la expresión de diferentes receptores activadores en subpoblaciones de células NK

En este trabajo hemos estudiado los cambios de las células NK asociados a la edad y a la infección por CMV. En primer lugar hemos analizado las alteraciones en la expresión de diferentes receptores NK en subpoblaciones de células NK definidas por la expresión de CD56, CD16 y CD57, en individuos sanos en relación con su grupo de edad y la seropositividad por CMV. Nuestros resultados confirman la redistribución de las células NK descrita en la literatura con el envejecimiento[119], y revelan diferencias en la expresión de los receptores activadores NK asociadas a la seropositividad CMV y a la edad. Las células NK $CD56^{bright}$ representan una subpoblación de células NK en un estadio inmaduro de diferenciación, que maduran en la periferia hacia células $CD56^{dim}$. Además, esta subpoblación presenta un descenso en la expresión del receptor activador NKp46. Este receptor activador se expresa constitutivamente en todas células NK, tiene una elevada expresión en las células $CD56^{bright}$, reconoce la hemaglutinina viral y desempeña un importante papel en la inmunidad antitumoral y antiviral participando en la lisis de células tumorales e infectadas por virus [120]. Nuestros resultados que muestran el descenso del porcentaje en esta subpoblación NK unido a una expresión disminuida de NKp46 en

ancianos CMV seropositivos, apoyan el impacto de la edad en la diferenciación de células NK posiblemente a consecuencia de una menor producción de células NK [77, 83]. Esta hipótesis está respaldada por la demostración de un defecto en la maduración de las células NK en ratones asociado a la edad como consecuencia de la disminución de la capacidad de las células del estroma de la médula ósea para mantener las etapas finales de la diferenciación de NK [121, 122].

El estudio de la población de células NK maduras definidas por la coexpresión de CD56 y CD16 confirma la expansión de la población que también expresa CD57 no solo en ancianos sino también en jóvenes CMV seropositivos[84]. Esta población de células $CD56^{dim}CD16^{+}CD57^{+}$representa la población de células NK maduras, altamente diferenciadas, con capacidad citotóxica y productoras de interferon gamma y se ha postulado que constituyan células NK de larga vida o células NK "tipo memoria" *(memory-like NK cells)*. Nuestros datos mostraron una expansión de células NK, $CD56^{dim}CD57^{+}$ relacionadas con el CMV y no con la edad, lo que apoya otros estudios que relacionan la expansión de células similar a memoria NK $CD56^{dim}CD57^{+}$que expresan NKG2C, con la infección por el CMV [83, 84]. El estudio de los receptores activadores en estas en estas células NK muestra la disminución de NKp30 relacionada con la seropositividad CMV y de DNAM-1 relacionada con la edad. El estudio de estos receptores en células NK $CD57^{+}$ han mostrado patrones de expresión similares, lo que confirma el papel del CMV en la disminución del receptor NKp30 [84]. DNAM-1 es un correceptor de citotoxicidad en las células $NKCD56^{dim}CD16^{+}$ y se ha observado que es esencial para la diferenciación de células NK de memoria durante la infección por citomegalovirus de ratón[123] y se ha sugerido que su expresión en células NK en humanos puede contribuir a la respuesta anti-CMV por las células NK. Su expresión esta disminuida en ancianos, lo que podría contribuir a un descenso en la capacidad de las células NK de individuos ancianos de controlar la replicación de este virus.

Con el objetivo de analizar si las alteraciones en la expresión de esto receptores activadores asociados a la edad y la infección por CMV, incubamos células NK procedentes de donantes de los diferentes grupos estudiados con IL-2 ya que varios trabajos han demostrado que la estimulación con IL-2 de células NK de donantes sanos y pacientes con cáncer potencian la actividad citolítica y aumentan su expresión de receptores activadores [124, 125]. Los

resultados demostraron que el tratamiento de las células NK con IL-2 incrementó la expresión de NKp46 y NKp30 en células NK en todos los grupos de edad estudiados, incremento que se observó tanto en las subpoblaciones $CD57^+$ como $CD57^-$, si bien no modificó de forma significativa la expresión de DNAM-1. Sin embargo, la imposibilidad de incluir un grupo CMV seronegativo de edad avanzada (la prevalencia de CMV en España para individuos mayores de 40 años es más del 80%)[(126)] representó un limitación en el estudio.

La posibilidad de modular la expresión de receptores activadores por el tratamiento con citoquinas abre nuevas oportunidades para mejorar el deterioro de la función de las células NK asociado con la edad y como consecuencia mejorar su respuesta antitumoral.

Tabla 2. Resumen receptores activadores NK

	CMV[1]			EDAD[2]			IL-2[3]
Fenotipo	$CD56^{bright}$	$CD56^{dim}CD16^+CD57^-$	$CD56^{dim}CD16^+CD57^+$	$CD56^{bright}$	$CD56^{dim}CD16^+CD57^-$	$CD56^{dim}CD16^+CD57^+$	NK Total
NKp30	–	–	↓	–	–	–	↑
NKp46	↑	–	–	↓	–	–	↑
DNAM-1	–	–	–	–	↓	↓	–

CMV[1] Referido a jóvenes CMV– vs jóvenes CMV+
EDAD[2] Ancianos CMV+ vs jóvenes CMV+
IL-2[3] Células NK (CD57+ y CD57–) de jóvenes CMV–, jóvenes CMV+ y ancianos CMV+

Expresión de los receptores inhibidores CD300a y CD161 en subpoblaciones NK

En segundo lugar, dentro del estudio de los cambios de las células NK asociados a la edad y a la infección por CMV, estudiamos la expresión de los receptores inhibidores CD300a y CD161 en subpoblaciones NK. Al contrario de otros receptores inhibidores como los KIR o NKG2A/CD94, cuyas alteraciones han sido estudiadas en ancianos o en pacientes con infecciones víricas, no existen datos disponibles sobre la expresión de los receptores CD300a y CD161 en el envejecimiento o la infección por CMV. CD300a es un receptor inhibidor, con motivos ITIMs intracelulares, cuyos ligandos, fosfatidilserina (PS) y fosfatidiletanolamina (PE), se encuentran en células apoptóticas, células infectadas por virus y células tumorales [(43-46)]. La mayoría de las células NK en todos los grupos de edad expresan CD300a. No obstante, su expresión se encuentra elevada en células NK de sujetos ancianos CMV seropositivos. Este aumento de expresión se debe posiblemente al efecto de la edad más que al de la infección por CMV, ya que no se encuentran diferencias en los

demás grupos de estudio estratificados por seropositividad al CMV, si bien la ausencia de sus controles ancianos CMV seronegativos no permiten descartar el papel de la infección por CMV durante la vida del individuo en este aumento de CD300a. La elevada expresión de este receptor inhibidor en las células NK de individuos ancianos puede contribuir a la evasión de células tumorales o infectadas por virus que expresan ligandos de CD300a como PS y PE a la vigilancia antitumoral por células NK.

El receptor inhibidor CD161 (NKR-P1A) se expresa en una subpoblación de células NK y reconoce a su ligando LLT1, que se encuentra en leucocitos activados. Existen pocos datos disponibles sobre la relación de CD161 con el envejecimiento y la infección por CMV [(47-49)]. Nuestros resultados muestran que la mayoría de las células NK $CD56^{dim}$ no coexpresan CD57 y CD161, y que la seropositividad a CMV está asociada no solo a un aumento en la expresión de CD57 en células NK $CD56^{dim}$, sino también a una disminución en la expresión de CD161 en comparación con su expresión en células NK de individuos CMV seronegativos. En un estudio reciente se ha observado que las células NK $CD56^{dim}NKG2C^{+}$ de pacientes con hepatitis crónica CMV seropositivos, presentan no solo un aumento de CD57 sino también una disminución de CD161 relacionados con la infección por CMV [(127)], lo que apoya nuestros resultados que muestran que la infección por CMV se asocia a un descenso de CD161. Teniendo en cuenta la capacidad inhibitoria de CD161 en la producción de citoquinas por los linfocitos NK y T, estos resultados respaldan que el CMV puede inducir un descenso en la expresión de receptor CD161 junto con la expansión de la respuesta polifuncional de las células NK y los linfocitos de T y NKT $CD57^{+}$ [(84, 127-130)], contribuyendo al entorno proinflamatorio observado en individuos sanos seropositivos al CMV.

El estudio de la coexpresión de CD300a, CD161 y CD57 mostró el efecto del CMV y de la edad en las subpoblaciones definidas por estos marcadores dentro de las células NK CD56dim (Figura 7). En particular la seropositividad por CMV, pero no la edad, se asocia al aumento de células NK $CD56^{dim}CD300a^{+}CD57^{+}$, mientras que el descenso de la subpoblación que coexpresa $CD56^{dim}CD300a^{+}CD161^{+}$ se asocia tanto a la seropositividad CMV como a la edad.

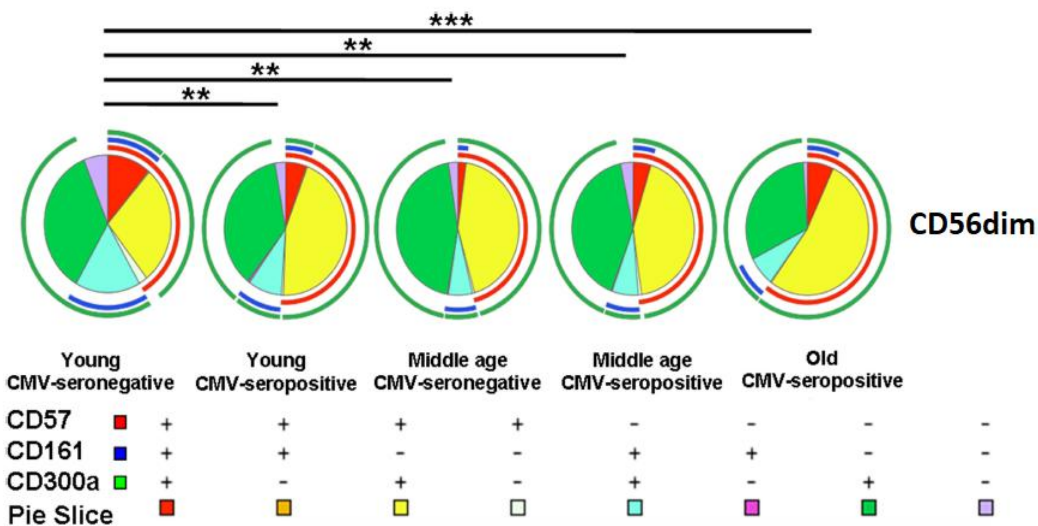


Figura 7. Estudio de la coexpresión (gráficos circulares) de CD300a, CD161 y CD57 en células NK $CD56^{dim}$, las diferencias entre grupos se consideraron significativas a un valor de p <o.o5

Efecto de la edad y la infección por CMV en la expresión de los factores de transcripción de T-bet y Eomesodermin (Eomes)

Asimismo, hemos investigado el efecto de la edad y la infección por CMV en la expresión de los factores de transcripción de T-bet y eomesodermin (Eomes) en las diferentes subpoblaciones onjuntos de células NK y en relación con la expresión de CD57 ($CD56^{bright}CD57^{-}$, $CD56^{dim}CD57^{-}$ y $CD56^{dim}CD57^{+}$). El análisis de T-bet y Eomes en células NK de jóvenes donantes CMV seronegativos muestra que la mayoría de las células NK de sangre periférica tienen una alta expresión de T-bet y Eomes. La expresión de T-bet en células NK permite diferenciar dos subpoblaciones: células NK $T\text{-}bet^{hi}$ y células NK $T\text{-}bet^{int}$ según la intensidad media de fluorescencia (MFI). El análisis del MFI de T-bet mostró un gradiente de incremento de la expresión de T-bet que es menor en las células NK $CD56^{bright}$ aumenta en las $CD56^{dim}CD57^{-}$ y alcanza los niveles más altos en las células $CD56^{dim}CD57^{+}$, dentro de cada grupo de individuos estudiado. Por el contrario, la expresión de Eomes es mayor en las células NK $CD56^{bright}$ y desciende a niveles más bajos en las células NK $CD56^{dim}CD57^{-}$ y en las células NK $CD56^{dim}CD57^{+}$. Estos resultados confirman y amplían los datos previos que analizan la expresión de T-bet y Eomes en subconjuntos de células NK [131, 132] e indican que la diferenciación a células terminales efectoras se asocia a un aumento en la expresión de T-bet y un descenso de Eomes, independientemente de la edad y el serostatus CMV.

La influencia del CMV en la expresión de receptores activadores e inhibidores en las subpoblaciones NK, se observa también en la expresión de los factores de transcripción Eomes y T-bet. Los individuos jóvenes CMV seropositivos mostraron un alto porcentaje de células $CD56^{dim}$ que expresan Eomes y un menor porcentaje de células $CD56^{dim}CD57^{+}T\text{-}bet^{hi}$ que los jóvenes CMV seronegativos (Figura 8a), lo que indica que estos factores de transcripción se encentran implicados en la remodelación de las células NK inducida por el CMV caracterizado por la expansión de la subpoblación $CD56^{dim}CD57^{+}$. En este sentido estudios previos han mostrado la reducción de T-bet en la enfermedad aguda de injerto contra huésped asociado y en la reactivación de CMV en pacientes sometidos a trasplante de progenitores hematopoyéticos, lo que pone de manifiesto la importancia de T-bet en la respuesta contra el CMV en las células NK caracterizada por la expansión de las células NK $CD56^{dim}CD57^{+}$[(90)].

No existen estudios sobre el efecto del envejecimiento en la expresión de factores de transcripción en células NK. En relación a la edad nuestros resultados muestran una disminución en la expresión de Eomes en las células $CD56^{bright}$ de sujetos de mediana edad CMV seronegativos comparado con jóvenes CMV seronegativos y un descenso en la expresión de Eomes en las células $CD56^{dim}$ tanto $CD57^{-}$como $CD57^{+}$en individuos de mediana edad y ancianos CMV seropositivos en comparación con jóvenes CMV seropositivos, en comparación con todos los grupos de edad estudiados. Por el contrario, T-bet^{hi} no mostró diferencias según la edad independientemente del seroestatus CMV (Figura 8b).

En modelos experimentales se ha demostrado que la maduración de las células NK en ratones viejos se asocia con una disminución de la expresión de T-bet y Eomes en células NK de médula ósea envejecidas. El uso de quimeras de la médula ósea ha revelado que el ambiente no hematopoyético es responsable del defecto en la maduración de las células NK, incluidas las alteraciones en la expresión de T-bet y Eomes en células NK [(133)]. Teniendo en cuenta que el defecto en la generación de células NK y en la expresión de T-bet y Eomes en ratones viejos se debe a alteraciones de la edad en el entorno no hematopoyético, se requiere una mejor comprensión de estos factores no hematopoyéticos involucrados en la diferenciación de células NK para la definición de nuevas estrategias dirigidas a mejorar la función de las células NK en ancianos.

La ausencia o alteración de la expresión de estos factores de transcripción conduce a un retroceso en la diferenciación y la pérdida del linaje NK. Eomes y T-bet desempeñan un papel crucial durante el desarrollo y maduración de las células NK, confiriéndolas de características fenotípicas y funcionales completas. Este equilibrio puede romperse a causa del envejecimiento y en enfermedades como el cáncer donde se produce un agotamiento de células NK por una disminución en la expresión de Eomes y T-bet.[88, 90]

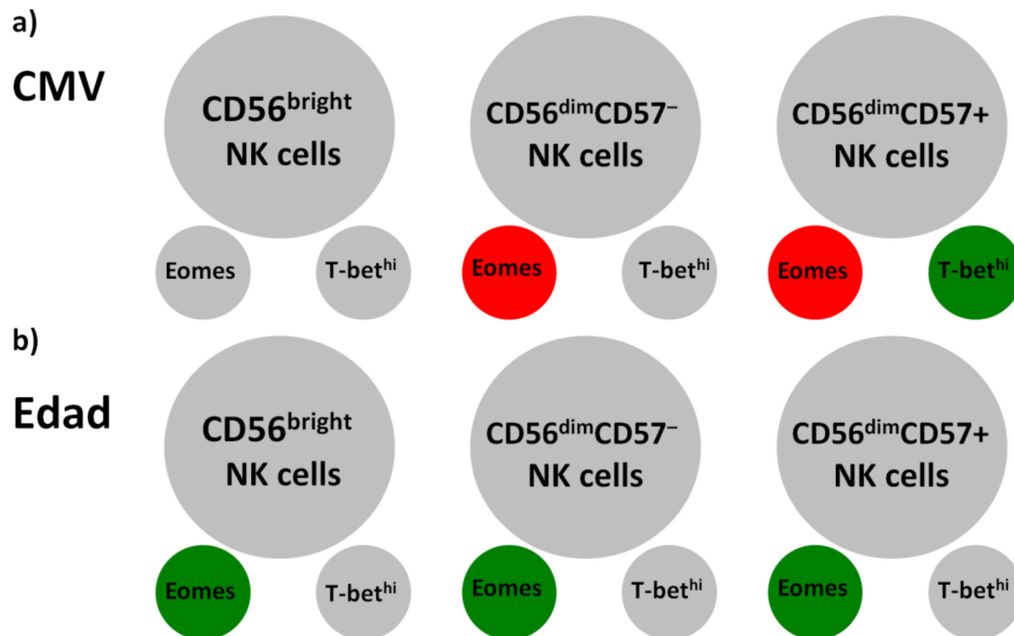


Figura 7. Expresión de Eomes y T-bet^{hi} en las distintas subpoblaciones de células NK en función al a) CMV (jóv. CMV– vs jóv. CMV+, y mediana edad CMV– vs mediana edad CMV+) y b) a la Edad. (jóv. mediana edad y ancianos CMV–/+). En Eomes y T-bet^{hi} los circulos en color rojo expresa incremento y en color verde disminución, círculos en gris no expresan cambios.

Expresión de receptores NK en pacientes con leucemia mieloide crónica (LMC) tratados con inhibidores de tirosina quinasa (TKI) y su relación con el envejecimiento

En tercer lugar, una vez descrita en las secciones anteriores la importancia de la edad y el CMV en el fenotipo, diferenciación y función de las subpoblaciones de células NK en individuos sanos, realizamos el estudio de la expresión de los receptores NK, en pacientes con leucemia mieloide crónica (LMC) tratados con inhibidores de tirosina quinasa (TKI), y su relación con la edad. Aunque el estudio se diseñó para incluir la estratificación de los pacientes según la variable del seroestatus para CMV, los resultados nos mostraron que la totalidad de los pacientes de LMC resultaron ser seropositivos para CMV, por lo que no pudimos incluir esta variable en el estudio. Existen evidencias previas que demuestran que

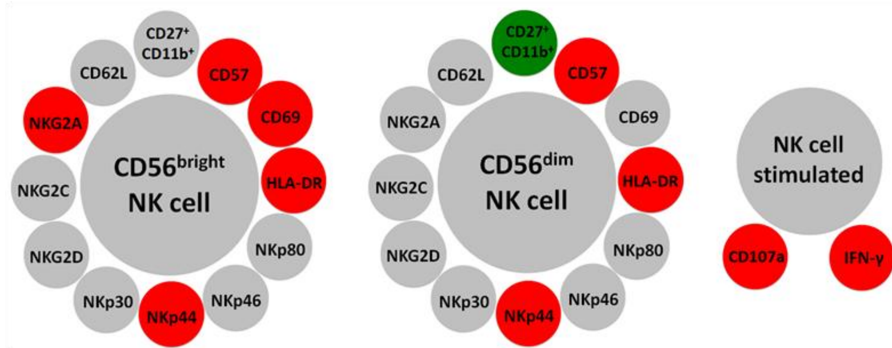
LMC causa un profundo impacto sobre el fenotipo y función de las células NK con un deterioro progresivo de la función NK a lo largo de la evolución de la enfermedad y alteraciones en la expresión de receptores activadores e inhibidores [64, 113]. También existen evidencias de que el envejecimiento es un mal factor pronóstico tanto para la supervivencia como para la respuesta al tratamiento en pacientes con LMC.

Los resultados de nuestro estudio en mostraron en los controles sanos un aumento en los marcadores de las células NK, tales como HLA-DR, NKp44,CD69 y del porcentaje de CD107a e IFN-γ bajo estimulación con línea celular K562, en ancianos comparados con controles de mediana edad. Este aumento probablemente es la consecuencia de la inflamación asociada al envejecimiento conocida como Inflamm-aging. Inflamm-aging es un estado inflamatorio de bajo grado en el envejecimiento, asociado a un estímulo antigénico crónico y altos niveles de estrés, que favorece el aumento de citoquinas proinflamatorias como IL-6, IL-8 y TNF-α [134, 135]. Nuestros resultados también revelaron un descenso significativo en la expresión de receptores NK y marcadores de activación como NKp80, HLA-DR, CD69, NKp44, en células NK $CD56^{bright}$ y $CD56^{dim}$y también una reducción de NKp30, NKG2D, NKG2A en $CD56^{bright}$ en pacientes ancianos con LMC tratados con TKI cuando se comparó con controles sanos. Asimismo, pacientes de mediana edad con LMC tratados con TKI comparados con sus controles mostraron un descenso de HLA-DR, CD69, NKG2A, y NKp46 en células $CD56^{dim}$y de NKp80 en células $CD56^{bright}$. En células $CD56^{dim}$ el marcador de diferenciación CD57 y el receptor activador NKp44 de pacientes con LMC tratados con TKI, se encuentran elevados en ambos grupos de edad, lo que indica la activación de estas células NK maduras en respuesta al tratamiento con TKI. A pesar de los datos que muestran una disminución de los receptores NK al momento del diagnóstico de la LMC [112-114] nuestro estudio confirma que las células NK de pacientes con LMC en tratamiento con TKI, pueden expresar receptores activadores como NKp30, NKp46, y NKp80, aunque en algunos casos, sus niveles son más bajos que los encontrados en los controles sanos emparejados según la edad. Estos cambios en los receptores, pueden ser la consecuencia de la interacción de las células NK con los blastos de origen mieloide circulantes que presentan una sobreexposición de sus ligandos [110, 117]. La expresión de NKG2D, que media en parte la respuesta antileucémica a través de los ligandos MICA/B de los blastos de la LMC, se encuentra bien conservada en pacientes con LMC en tratamiento con TKI, a excepción de

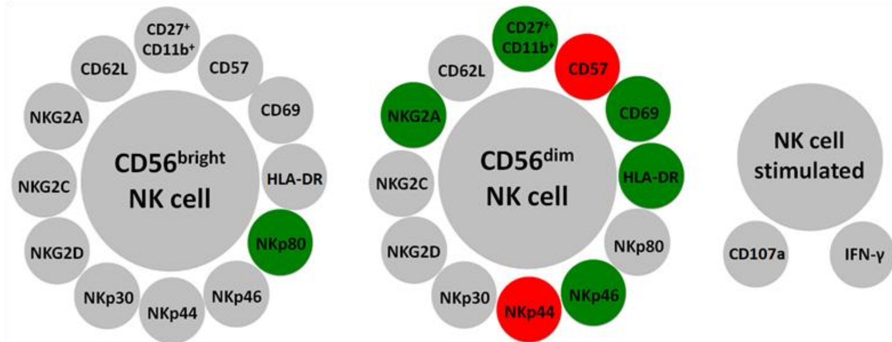
las células CD56^{bright} de pacientes ancianos en los que se halla disminuida, lo que confirman estudios previos que describen que tras el tratamiento con TKI la expresión de NKG2D se restaura a niveles normales en comparación con el momento del diagnóstico(115, 116). En pacientes que muestran una respuesta citogenética y molecular completa, se observa que aquellos que tienen una mayor cantidad de células NK maduras y que recuperan la expresión de sus receptores activadores, controlan la enfermedad y mantienen la remisión de la enfermedad tras el cese de tratamiento con TKI (106, 115, 136). La expresión elevada de CD107a en ancianos sanos comparada con pacientes con LMC tratados con TKI de la misma edad, probablemente se asocie con las alteraciones observadas en ancianos debido al Inflamm-aging. Finalmente el efecto de la edad sobre los pacientes con LMC tratados con TKI reflejó una disminución en la expresión de los receptores NKp44 y CD69 en pacientes de edad avanzada en comparación con pacientes de mediana edad tratados con TKI. Todos los demás receptores del estudio incluyendo el ensayo de degranulación CD107a y la producción de IFN-γ no arrojaron diferencias significativas, lo que nos indica que la edad no es una limitación para la recuperación de las células después del tratamiento con TKI.

Estos resultados demuestran la influencia del CMV, la edad y la LMC, sobre el fenotipo y función de las células NK. El uso de citoquinas y agentes inmonumoduladores para potenciar la actividad funcional de las células NK, especialmente en individuos de edad avanzada, puede ser una alternativa interesante en futuros ensayos clínicos y abre la posibilidad para una mejora en la remisión eficaz a largo plazo después del cese del tratamiento con TKI en pacientes con LMC.

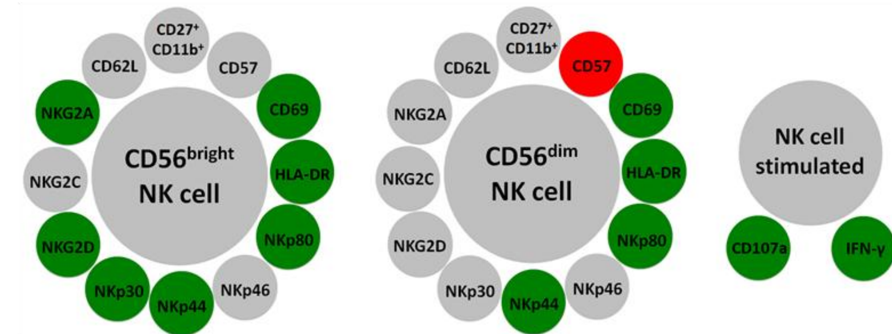
A Age effect on NK cell compartment from healthy individuals: Middle age vs Old



B NK cell compartment in Middle age CML patients vs healthy individuals



C NK cell compartment in Old age CML patients vs healthy individuals



D Age effect on NK cell compartment from CML patients : Middle age vs O

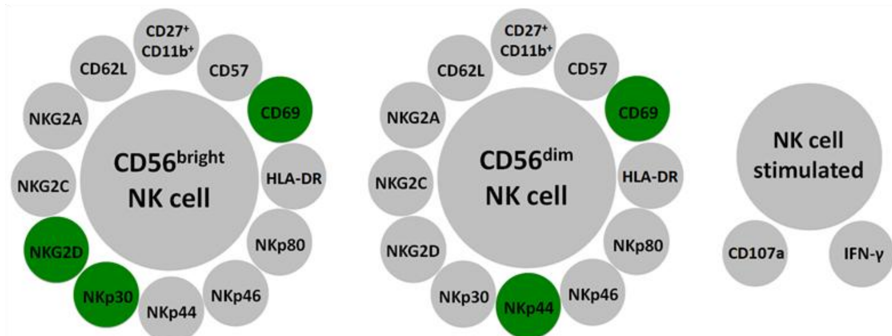


Figura 9. Principales diferencias en células NK en reposo y estimuladas con la línea celular K562 de acuerdo con la Edad y la LMC. Los círculos representan subpoblaciones de células NK que expresan los marcadores de membrana en células CD56bright y CD56dim en reposo y la expresión de CD107a e IFN-γ en células NK estimulada con K562. Se usó color rojo para el aumento de la expresión y color verde para la disminución en la expresión de los marcadores en células NK de personas sanas de mediana edad en comparación con individuos ancianos sanos (A), de pacientes con LMC en comparación con donantes sanos de la misma edad (B, C), o en pacientes de mediana edad con LMC en comparación con pacientes ancianos con LMC (D).

6. Conclusiones

1. Tanto el serostatus para CMV en donantes jóvenes sanos como la edad se asocia con diferencias en la expresión de los receptores activadores NKp46, NKp30 y DNAM-1 en diferentes subpoblaciones de células NK definidas por la expresión de CD56, CD16 y CD57. Mientras que la baja expresión de DNAM-1 se asocia con la edad, la expresión disminuida de NKp30 se relaciona con la seropositividad al CMV. El tratamiento in vitro con IL-2 incrementa la expresión de los receptores NKp30 y NKp46 en las diferentes subpoblaciones estudiadas. Estos resultados enfatizan la importancia de la determinación del estado serológico para CMV en estudios dirigidos a analizar la respuesta inmune en ancianos y abren la posibilidad de modular el deterioro de las células NK en ancianos mediante el uso de citoquinas como la IL-2. Esta conclusión se desprende del artículo **"Expression of NKp30, NKp46 and DNAM-1 activating receptors on resting and IL-2 activated NK cells from healthy donors according to CMV-serostatus and age".** Biogerontology. 2015, 16:671–683, DOI: 10.1007/s10522-015-9581-0.

2. La seropositividad CMV y la edad afectan de forma diferente a las subploblaciones NK definidas por la expresión de los marcadores CD300, CD57 y CD161 y por la expresión de los factores de transcripción Eomes y T-bet en subpoblaciones NK. En células NK $CD56^{dim}$ existe un incremento de la subpoblación $CD300a^{+}CD57^{+}CD161^{-}$ asociado a la seropositividad por CMV y un descenso en la subpoblación $CD300a^{+}CD57^{-}CD161^{+}$ asociado tanto a la seropositividad CMV como a la edad. Mientras que la seropositividad por CMV se asocia a una mayor expresión de Eomes en células NK $CD56^{dim}$ de jóvenes, su expresión es menor en las células NK $CD56^{dim}$ de los grupos de mediana edad y ancianos CMV seropositivos. La expresión de estos factores de transcripción permite definir estadios de diferenciación NK. Así la expresión de Eomes es más alta en la subpoblación $CD56^{bright}$ y disminuye gradualmente en las poblaciones $CD56^{dim}CD57^{-}$ y $CD56^{dim}CD57^{+}$, mientras que la expresión de T-bet es menor en la población $CD56^{bright}$ y aumenta gradualmente en las poblaciones $CD56^{dim}CD57^{-}$ y $CD56^{dim}CD57^{+}$ en los diferentes grupos estudiados. Estas alteraciones pueden estar implicadas en el deterioro funcional de las células NK asociado a la edad. Esta conclusión se desprende del artículo **"Effect of CMV and aging on the differential expression of CD300a, CD161, T-bet, and Eomes on NK cell subsets".** Frontiers in Immunology. Nov. 07; 2016, DOI: 10.3389/fimmu.2016.00476.

3. A pesar del efecto perjudicial del envejecimiento en el pronóstico de la LMC, y de las alteraciones en el fenotipo y función de las células NK de estos pacientes, incluso tras el tratamiento con TKI, nuestros resultados demuestran que los receptores de activación de las células NK pueden expresarse en pacientes con LMC de mediana edad y ancianos tratados con TKI, lo que indica que la edad no es una limitación para la recuperación de las células NK en individuos en tratamiento con TKI y pone en evidencia la importancia del estudio de agentes inmunomoduladores que permitan revertir la inmunosenescencia NK en estos pacientes. Esta conclusión se desprende del artículo "**Effect of age on NK cell compartment in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors"** Frontiers in Immunology. Nov. 08; 2018, DOI: 10.3389/fimmu.2018.02587

Conclusions

1. In healthy young donors, age and CMV seropositivity are associated with differences in the expression of activating receptors NKp46, NKp30 and DNAM-1 in different subpopulations of NK cells defined by the expression of CD56, CD16 and CD57 markers. A lower expression of DNAM-1 is associated with age, whereas a decreased expression of NKp30 is related to CMV seropositivity. In vitro treatment with IL-2 increases the expression of NKp30 and NKp46 receptors in the different subpopulations studied. These results emphasize the importance of the determination of serological status for CMV in the elderly and open the possibility of modulating the deterioration of NK cells in the elderly through the use of cytokines such as IL-2. This conclusion was drawn from the article **"Expression of NKp30, NKp46 and DNAM-1 activating receptors on resting and IL-2 activated NK cells from healthy donors according to CMV-serostatus and age".** Biogerontology. 2015, 16:671–683, DOI: 10.1007/s10522-015-9581-0.

2. CMV latent infection is associated with significant changes in the expression of NK receptors, including the inhibitory receptors CD300a and CD161. T-bet and Eomes are differentially expressed on NK cell subsets defined by the expression of CD56 and CD57, and its expression is affected by CMV latent infection and aging, which can be involved in the age-associated changes observed in the differentiation and function of NK cells. This conclusion was drawn from the article **"Effect of CMV and aging on the differential expression of CD300a, CD161, T-bet, and Eomes on NK cell subsets".** Frontiers in Immunology. Nov. 07; 2016, DOI: 10.3389/fimmu.2016.00476.

3. Despite the deleterious effect of aging in CML prognosis and the alterations in the phenotype and function of NK cells of these patients, even after treatment with TKI, our results showed that activating NK cell receptors can be expressed in both middle-aged and elderly TKI-treated CML patients, indicating that age is not a limitation for the recovery of NK cells in individuals treated with TKI and also highlights the importance of studying the immunomodulatory agents that allow to reverse the immunosenescence of NK cells in these patients. This conclusion was drawn from the article "**Effect of age on NK cell compartment in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors"** Frontiers in Immunology. Nov. 08; 2018, DOI: 10.3389/fimmu.2018.02587

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8. Otras Aportaciones

- **Differential effect of cytomegalovirus infection with age on the expression of CD57, CD300a, and CD161 on T-cell subpopulations.** Fakhri Hassouneh, **Nelson Lopez-Sejas**, Carmen Campos, Beatriz Sanchez-Correa, Raquel Tarazona, Rafael Solana and Alejandra Pera. Frontiers in Immunology, Jun 2, 2017. doi: 10.3389/fimmu.2017.00649

- **Effect of Cytomegalovirus (CMV) and Ageing on T-Bet and Eomes Expression on T-Cell Subsets**. Fakhri Hassouneh, **Nelson Lopez-Sejas**, Carmen Campos, Beatriz Sanchez-Correa, Raquel Tarazona, Alejandra Pera, and Rafael Solana. Int J Mol Sci, Jun 29, 2017. doi: 10.3390/ijms18071391

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