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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

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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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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 manufactureŕs 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

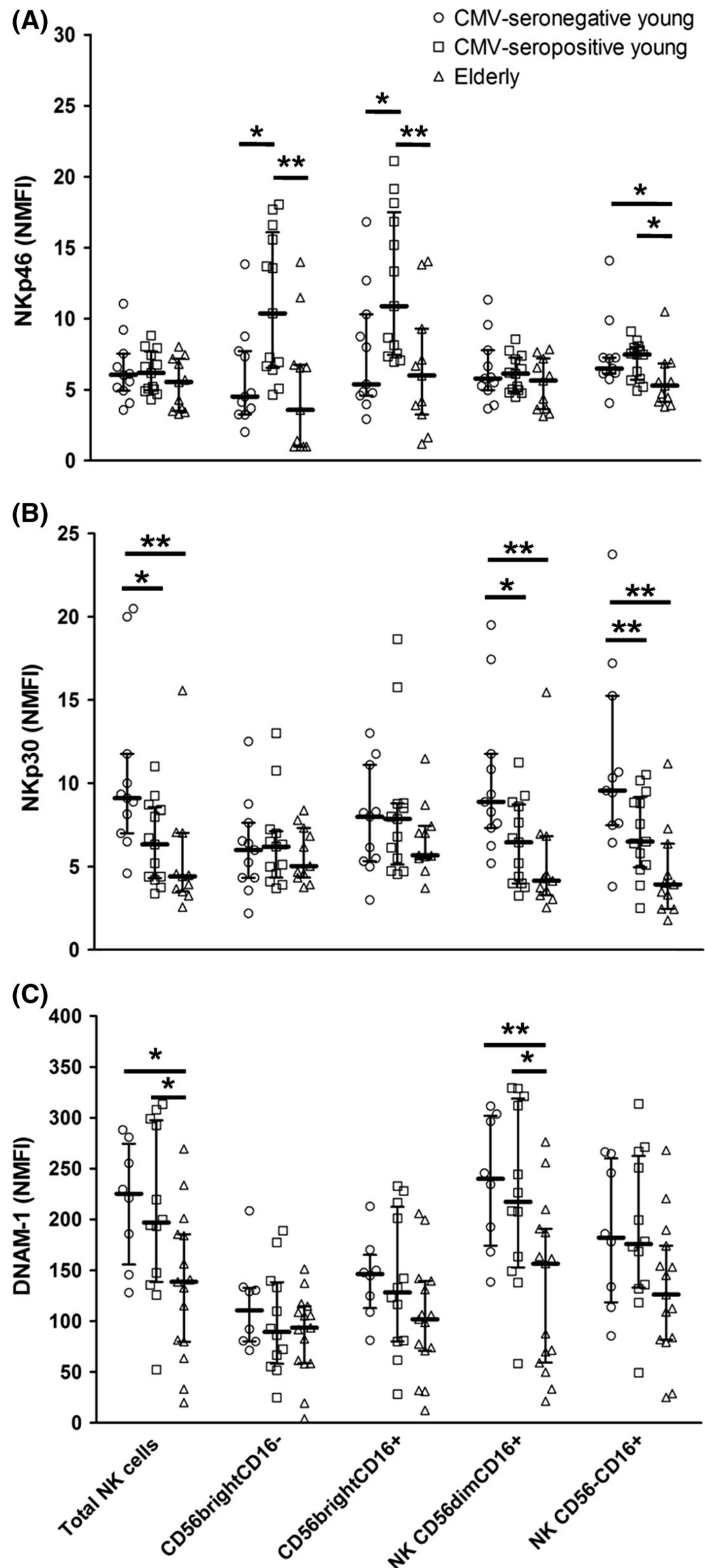
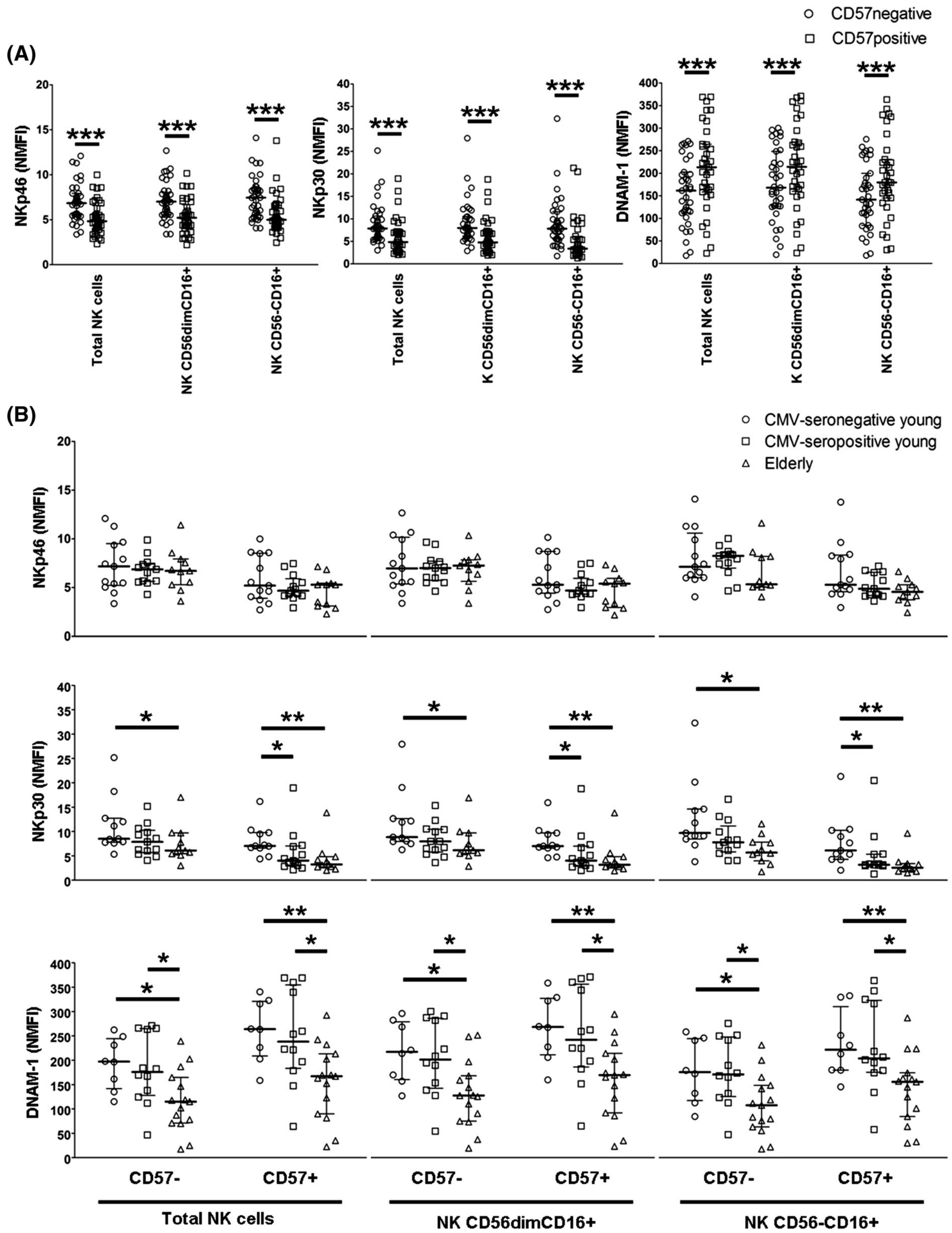


Fig. 1 Expression of NKp46, NKp30 and DNAM-1 on NK cells from young and elderly healthy donors. Expression of NKp46, NKp30 and DNAM-1, measured as normalized median fluorescent intensity (NMFI), was determined on the different NK cell subpopulations from three groups of donors: CMV-seronegative young (*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+
(B)
CMV-seronegative young
CMV-seropositive young
Elderly
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).

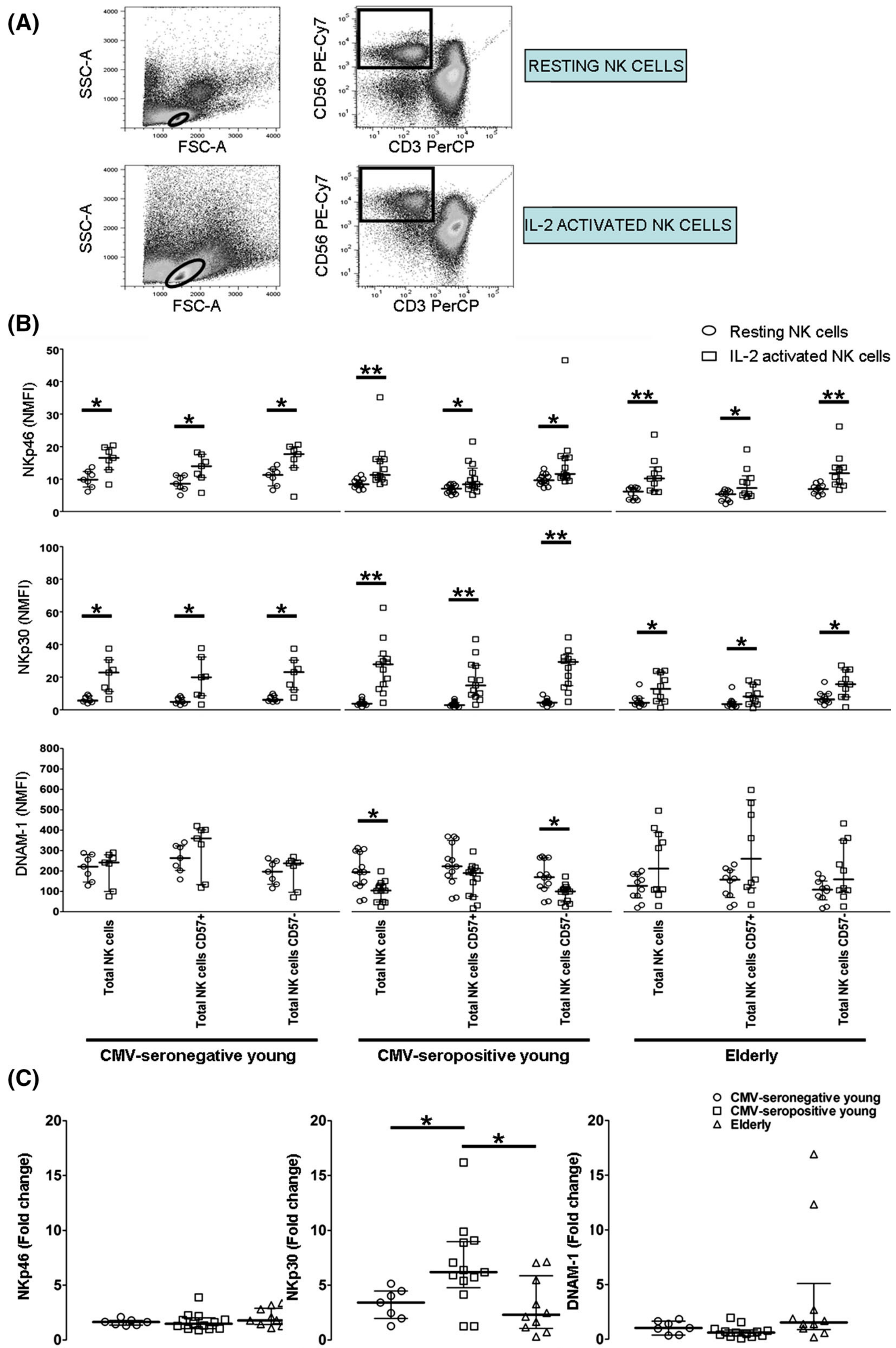
In this work we have analyzed the effect of CMV-seropositivity and ageing on the expression of NK activating receptors on NK cell subsets defined by the expression of CD56, CD16 and CD57.

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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