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Coordinated expansion of both memory T cells and NK cells in response to CMV infection in humans

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NK cells are key players in the fight against persistent viruses. Human cytomegalovirus (HCMV) infection is associated with the presence of a population of CD16$^+$ CD56^{dim} NKG2C$^+$ NK cells in both acutely and latently infected individuals. Here, we studied the nature of these terminally differentiated NK cells in different human populations infected with HCMV: healthy donors stratified by age, thymectomized individuals, pregnant women suffering from primary CMV infection, and lung transplant patients. Both CD16$^+$ CD56^{dim} NK- and CD8 T-cell phenotypes as well as functional capacities were determined and stratified according to age and/or CMV event. Similarly to T-cell responsiveness, we observe an accumulation over time of NKG2C$^+$ NK cells, which preferentially expressed CD57. This accumulation is particularly prominent in elderly and amplified further by CMV infection. Latent HCMV infection (without replication) is sufficient for NKG2C$^+$ CD57$^+$ NK cells to persist in healthy individuals but is not necessarily required in old age. Collectively, the present work supports the emerging concept that CMV shapes both innate and adaptive immunity in humans.

Keywords: Aging · Human · NK cells · T lymphocytes · Viral infection

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Introduction

Natural killer (NK) cells are a subset of lymphocytes comprising 5–20% of peripheral blood mononuclear cells (PBMCs) in humans [1]. NK cells participate in the innate immune response and play important roles in tumor surveillance and defense against viral infections [2]. Based on the expression of the CD56 and the CD16 surface markers, three NK-cell subsets have been characterized in humans: CD56^{dim}CD16$^+$ cells represent approximately 90% of circulating NK cells and are considered the mature mainly cytotoxic NK subset; CD56^{bright}CD16$^{neg/dim}$ NK cells constitute approximately 10% and are considered immature with a

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cytokine-mediated immune-modulatory role [3]. Furthermore, a scarce subset of NK cells, devoid of CD56 expression and displaying a reduced functional capacity, has been identified in healthy controls [4] and in chronic viral infections [5–7].

The contribution of NK cells to the antiviral immune response has been extensively studied in murine viral infections, demonstrating that NK cells impede viral replication [8, 9]. In humans, rare NK-cell deficiencies have established the critical role of functional NK cells in the control of human viral infections, in particular toward herpesviruses [10, 11].

Human cytomegalovirus (HCMV) chronically infects 50–90% of humans. HCMV is usually acquired early in life, and most immunocompetent hosts are asymptomatic. However, HCMV can cause severe disease in immunocompromised individuals, such as HIV-seropositive patients and transplant recipients on immunosuppressive therapy, resulting from the reactivation of latent infection. Furthermore, maternal HCMV reactivation or primary infection (PI) during pregnancy can result in the infection of the fetus. HCMV is the most common cause of congenital infection and is associated with long-term sequelae [12].

An effective defense against HCMV requires the participation of innate and adaptive immunity with a central role for NK cells and T lymphocytes. Although primary HCMV infection of immunocompetent subjects is usually subclinical, chronic infection is associated with an age-related deterioration of the immune system, in particular with (i) the accumulation of late differentiated and oligoclonal $CD8^+$ T-cell populations (referred to as memory inflation) [13, 14], (ii) an exhaustion of immune resources [15], and (iii) the development of an "immune risk phenotype" that is predictive of early mortality in the elderly [16, 17].

CMV also has an impact on NK cells. In MCMV infection, a specific population of NK cells expands, contracts after viral control, and generates long-lived "memory" NK cells expressing Ly49H that are more protective during a second encounter with MCMV [9, 18]. Looking at the impact of other herpesviruses to induce late differentiated NK cells either during the acute phase of symptomatic EBV-induced mononucleosis [19] or during recurrent HSV-2 infection [20], recent studies did not find any expansion of NK cells expressing the activating receptor NKG2C. Other publications have described the expansion of $NKG2C^+$ NK cells following infections with Hantavirus [21] and Chikungunya virus [22], but only in patients chronically infected with HCMV. These findings are in line with several studies that consistently describe the expansion of $NKG2C^+$ NK cells in response to HCMV [23–28].

Considering that HCMV infection contributes to age-associated changes in adaptive immunity and that aging also affects the distribution of NK cells [29, 30], we have investigated the possible contribution of HCMV at the different phases of infection to the age-associated alterations in NK-cell subsets. For this purpose, we used patient samples from different clinical settings where both age and CMV events could be dissected: (i) healthy volunteers of different age (long-term carriers of HCMV or uninfected), (ii) CMV-seronegative women who acquire primary HCMV-infection during pregnancy, (iii) lung transplant adults who suffer or not from HCMV reactivations posttransplantation, and (iv) young adults thymectomized in early life for whom adaptive immunity exhibits signs of premature immune aging. By looking at both innate and adaptative HCMV-induced responses simultaneously, we have drawn a parallel between the impact of HCMV on the composition of highly differentiated T-cell and NK-cell subsets in human.

Results

Effect of age on human NK-cell properties

We analyzed the phenotype of $CD16^+$ $CD56^{dim}$ NK cells in more than a hundred healthy donors stratified according to their age. Subject information (age, gender, and HCMV status) is provided in Table 1. While we observed a big heterogeneity in the $CD16^+$ $CD56^{dim}$ absolute counts in each group, no statistical differences were observed between young, middle-aged, and elderly adults in concordance with previous observations [29, 31]. We thus divided the $CD16^+CD56^{dim}$ NK-cell population into three distinct subsets based on the expression of NKG2A, NKG2C, and CD57, as illustrated in Figure 1A, and referred to as: early ($NKG2A^+$ $NKG2C^-$), intermediate ($NKG2A^-$ $NKG2C^-$), late ($NKG2A^-$ $NKG2C^+$). Expression of CD57 refines each population into $CD57^-$ and $CD57^+$ subsets. $CD16^+$ $CD56^{dim}$ $NKG2A^-$ $NKG2C^+$ $CD57^+$ is referred to as terminally differentiated NK cells. We found that phenotypic changes occur with aging. Indeed, the composition of the intermediate population $NKG2A^-$ $NKG2C^-$ balances toward a more differentiated pattern with a majority of cells expressing CD57 in old age (Fig. 1C and D; $NKG2A^-$ $NKG2C^-$ $CD57^-$ Kruskal–Wallis: $p < 0.0001$ and $NKG2A^-$ $NKG2C^-$ $CD57^+$ Kruskal–Wallis: $p = 0.003$). Furthermore, both the frequency (Fig. 1) and absolute cell numbers (Supporting Information Fig. 1) of the two most differentiated NK-cell subsets (total $NKG2A^-$ $NKG2C^+$ and $NKG2A^-$ $NKG2C^+$ $CD57^+$; Figs. 1B and D; Supporting Information 1A and C, respectively) were significantly higher in older people (Kruskal–Wallis: $p < 0.0001$).

Having established that NK-cell distribution was different in older compared to young people, we were next interested in monitoring functional alterations. We assessed the potential capacity to produce IFN-γ and to degranulate (i.e. CD107a expression upregulation) of NK cells by incubating PBMC from young, middle-aged and old individuals, with the class I negative cell line K562 (Fig. 2A). Cells from elderly were equally potent to produce IFN-γ and to degranulate as their younger subjects suggesting that NK cells from elderly maintain robust functional properties. Of note, functionality of NK cells from elderly resulted from the capacity, equally shared between early and late-differentiated NK cells, to both secrete IFN-γ and degranulate (Fig. 2B and C).

Effect of HCMV infection on human NK-cell properties

Higher frequency of terminally differentiated NK cells ($NKG2C^+$ $CD57^+$) in elderly adults is intriguing as this unique population

Table 1. Characteristics of donors examined in this study

Groups	*n*	Age (years)	Gender (% of male)	CMV status (% of seropositivity)	nCD3$^-$ CD16$^+$ CD56$^+$ (cells/μL)
Young (Y)	30	22.19 (18.62–25)	30.3	43.7	181.5 (1.92–1691)
Middle age (M)	30	34.5 (26–55)	37.5	59.4	206.7 (37.5–2725)
Old (O)	60	84 (75.4–99)	40	58.4	168.5 (45.5–593.7)
PI during pregnancy (IM)	10	31.5 (23–41)	0	100	n.d
Healthy donor (HD)	10	36 (23–55)	0	50	n.d
LTP	17	33.95 (21–60)	70.6	35.3	n.d
Thymectomized patients (Yatec)	61	23 (18–29)	72.2	54.4	224.2 (77.6–839.7)

of mature NK cells, defined with distinct function [32] was recently reported in association with HCMV infection [23, 24]. We therefore analyzed the distribution of the different subsets in individuals stratified according to HCMV status (Fig 3A–C). HCMV-seronegative elderly had an increase of late NKG2C$^+$ or late NKG2C$^+$CD57$^+$ cells compared to younger HCMV-seronegative individuals (Kruskal–Wallis: $p = 0.001$ and $p = 0.001$, respectively). Similarly, HCMV-seropositive elderly had also an increase of late NKG2C$^+$ or late NKG2C$^+$CD57$^+$ cells compared to younger HCMV-seropositive individuals (Kruskal–Wallis: $p = 0.003$ and $p = 0.002$, respectively). Moreover, HCMV-seropositive individuals exhibited a significant increase of NKG2C$^+$ and NKG2C$^+$CD57$^+$ cells compared to their age-matched HCMV-seronegative counterparts throughout life (Fig. 3A and C). Overall, our results show that the increased frequency of NK cells with a late differentiation phenotype is particularly evident in advanced age (Kruskal–Wallis: $p = 0.001$ for HCMV-seronegative donors) and amplified independently by HCMV infection (Kruskal–Wallis: $p = 0.002$ for HCMV-seropositive donors).

In order to further inform on the timing of differentiated NK-cell expansion in HCMV infection, we analyzed these cells in pregnant women, who encountered HCMV for the first time during pregnancy. This primary infection (PI) event led to the development of late CD57$^+$ NK-cell subsets compared to healthy age-matched HCMV-seronegative donors (Fig. 3D; HD− versus PI, $p = 0.01$ and $p = 0.02$, respectively). Of note, the expansion of late NK cells in seroconverting pregnant women reached the same levels as in chronically HCMV-infected donors (Fig. 3D, p = NS between HD+ and PI).

In order to assess the role of HCMV persistence and/or replication in shaping NK-cell subset distribution, we studied a clinical setting with frequent HCMV reactivations. HCMV infection is the most common viral complication after solid organ transplantation [33, 34] providing a unique model to study the consequences of HCMV acute infection in humans. NK-cell phenotypes were analyzed in PBMCs isolated from 17 lung-transplant recipients classified into three groups according to their CMV donor/recipient (D/R) serostatus: no CMV infection (D$^-$/R$^-$ patients; $n = 3$), chronic CMV infection (D$^-$/R$^+$; $n = 3$ and D$^+$/R$^+$ patients; $n = 3$), and primary CMV infection (D$^+$/R$^-$ patients $n = 8$). While the main NK-cell subsets were characterized by early and intermediate differentiated phenotypes (Fig. 4A), once again, we observed a higher proportion of NK cells with a more differentiated phenotype in the context of HCMV infection (i.e. in D$^-$/R$^+$; D$^+$/R$^+$; D$^+$/R$^-$ patients), (Fig. 4A). To avoid confounding effects from the recipients who encountered HCMV previously, we focused on D$^+$/R$^-$ patients and found that the frequency of NKG2C$^+$ CD57$^+$ NK cells was increased in individuals reactivating HCMV compared to individuals without evidence of viral reactivation (Fig. 4B). This demonstrates that both HCMV antigenemia and replication imprint NK-cell diversity. In order to decipher their respective impact, we looked at the kinetics of viremia and of the associated frequencies of late differentiated CD57$^+$ NK cells in patients exhibiting several episodes of active HCMV replication (Fig. 4C). About two-thirds of our lung transplant recipients reactivated HCMV after transplantation. Recipients were monitored for HCMV reactivation by PCR and placed on antiviral therapy when HCMV viremia was detected. After 2–4 weeks of antiviral therapy, HCMV viremia was virtually undetectable in the blood (and in bronchoalveolar lavages when tested). During the 24 months of follow-up, we did not find recurrent expansions of the late differentiated CD57$^+$ NK cells despite several episodes of viremia, nor a contraction phase after resolution of viral replication, as exemplified by kinetics of three different patients (Fig. 4C). These findings indicated that an accumulation of late differentiated CD57$^+$ NK cells took place over time (Fig. 4C and Supporting Information Fig. 2) and that their expansion (observed both in blood and in bronchoalveolar lavages; Supporting Information Fig. 3) is not sufficient to protect against subsequent HCMV reactivation. Interestingly, NKG2C$^+$ CD57$^+$ NK cells were barely detectable in patients who did not exhibit HCMV viremia. NKG2C$^+$ CD57$^+$ NK cells were more frequent in recipients exposed chronically to HCMV ($p = 0.01$), and even greater in those same recipients who experienced active HCMV replication (Fig. 4D; $p = 0.0006$). This hierarchy translated into a positive correlation between the number of HCMV reactivations and the frequency of this particular terminally differentiated NK cells (Fig. 4E; $r = 0.79$, $p = 0.0002$).

Because HCMV reactivation results in a more mature NK-cell phenotype, we investigated the effect of HCMV viremia on NK-cell function. We measured IFN-γ production and CD107a expression on NK cells after incubation with K562 cells (Supporting

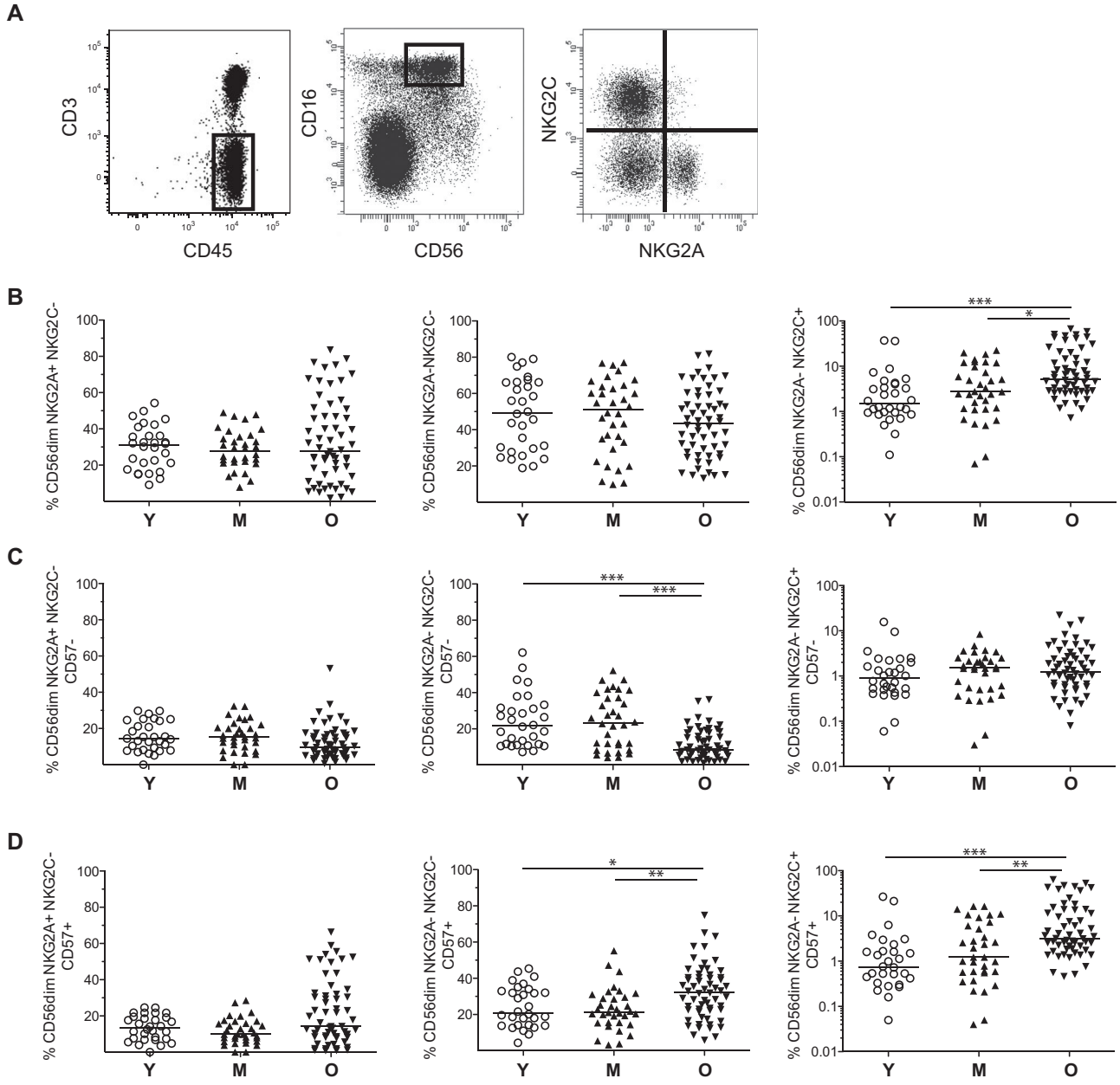


Figure 1. Evolution of human NK-cell phenotype with aging. (A) Gating strategy to determine the differentiation profile of NK-cell compartment, looking at the expression of CD16, CD56, NKG2A, and NKG2C. (B–D) The frequency of NKG2A and NKG2C markers on (B) $CD16^{+}CD56^{dim}$, (C) $CD16^{+}CD56^{dim}$ $CD57^{-}$, and (D) $CD16^{+}CD56^{dim}$ $CD57^{+}$ subsets among Young (white circle; $n = 30$), Middle age (black upward triangle; $n = 30$) and Old individuals (black downward triangle; $n = 60$) were evaluated by flow cytometry. Each symbol represents an individual donor and horizontal bars indicate the median. p values were calculated using the Mann–Whitney test for group comparisons. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$.

Information Fig. 4A). $CD56^{+}$ NK cells were able to respond against MHC class I-negative cellular target K562 (Supporting Information Fig. 4B and C). Focusing on $NKG2C^{+}$ NK cells, there was no detectable difference in CD107a expression between recipients who reactivate HCMV compared to the ones who did not (Fig. 4F; $p = 0.6$). However, $NKG2C^{+}$ NK cells from seropositive patients who reactivated HCMV tended to produce more IFN-γ compared to patients without HCMV viremia (Fig. 4F; $p = 0.1$) in concordance with the findings of Foley et al. [28]

Interplay between terminally differentiated NK cells and CD8+ T cells in humans

Expansion of mature $NKG2C^{+}$ $CD57^{+}$ NK cells following HCMV reactivation with potent function and their continuous persistence in the presence of HCMV antigens suggest that NK cells may exhibit memory-like properties, a characteristic usually restricted to adaptive immune responses. To address this, we compared the frequencies of late differentiated $CD57^{+}$ NK cells and memory $CD8^{+}$ T cells

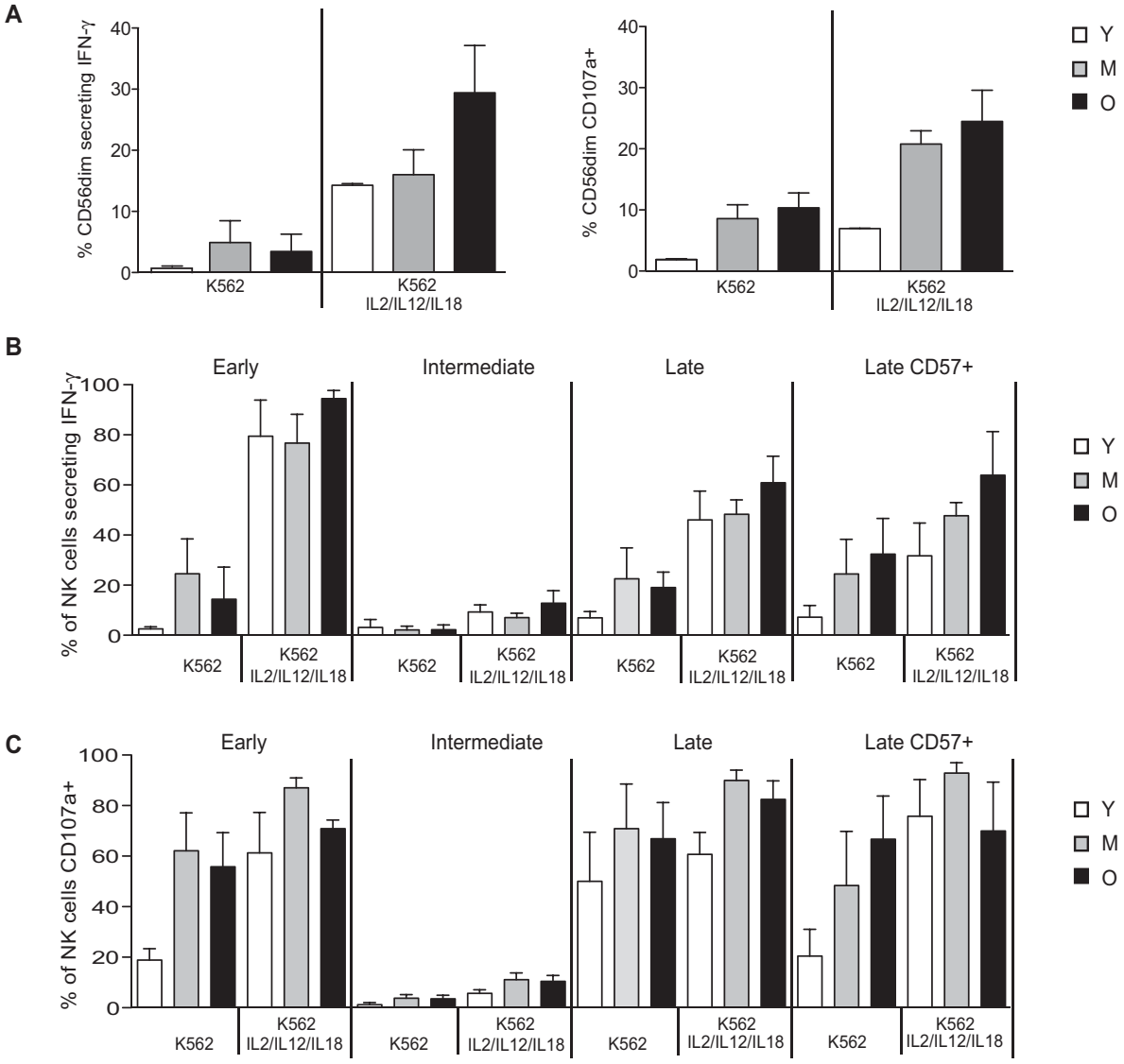


Figure 2. Human NK-cell function delineated by age of individual. (A) The percentage of $CD56^{dim}$ NK cells exhibiting functionality through the secretion of IFN-γ (left) or through degranulation capacity (CD107a marker, right) was determined by flow cytometry. (B, C) The percentage of early ($CD56^{dim}$ $NKG2A^{+}NKG2C^{-}$), intermediate ($CD56^{dim}$ $NKG2A^{-}NKG2C^{-}$), late ($CD56^{dim}$ $NKG2A^{-}NKG2C^{+}$), and terminally differentiated NK cells ($CD56^{dim}$ $NKG2A^{-}$ $NKG2C^{+}CD57^{+}$) (B) secreting IFN-γ or (C) expressing CD107a were also determined by flow cytometry. PBMCs from Young (white bar; $n = 3$), Middle age (gray bar; $n = 5$) and old individuals (black bar; $n = 5$) were stimulated for 6 h with K562 target cells or with K562 target cells in presence of cytokines (IL-2/IL-12/IL-18). Data are shown as the mean + SEM of the indicated number of donors.

based on donor HCMV serostatus. Figure 5A shows the markers used to identify $CD8^{+}$ memory T cells (CD27/ CD45RA/CD57) as well as their capacity to secrete IFN-γ and TNF-α upon stimulation with HCMV antigens. As expected, we observed that HCMV-seropositive donors present a higher count (and percentage, data not shown) of terminally differentiated $CD8^{+}$ $CD57^{+}$ memory T cells than their uninfected counterparts in all groups of individuals tested (Fig. 5B). Similarly, HCMV-seropositive control groups have a higher distribution of late $CD57^{+}$ NK cells compared to HCMV-seronegative groups (Fig. 5C). Noteworthy, the inclusion of young adults thymectomized during early childhood (Yatec) who represent a model of premature immune aging [35, 36] offers a unique opportunity to determine if chronic infections, such as HCMV, imprint the adaptive and innate immune system. In this context, we previously observed that Yatec exhibits immune parameters reminiscent of premature immune aging in terms of reduced naïve T-cell counts and oligoclonal memory $CD8^{+}$ T-cell repertoire and that this immune risk phenotype was accentuated when individuals were HCMV seropositive [15]. Thus, in this particular context where T-cell immunity is severely challenged, we investigated if NK cells would relay adaptive immunity. Functional assays revealed an elevated frequency of IFN-γ and CD107a

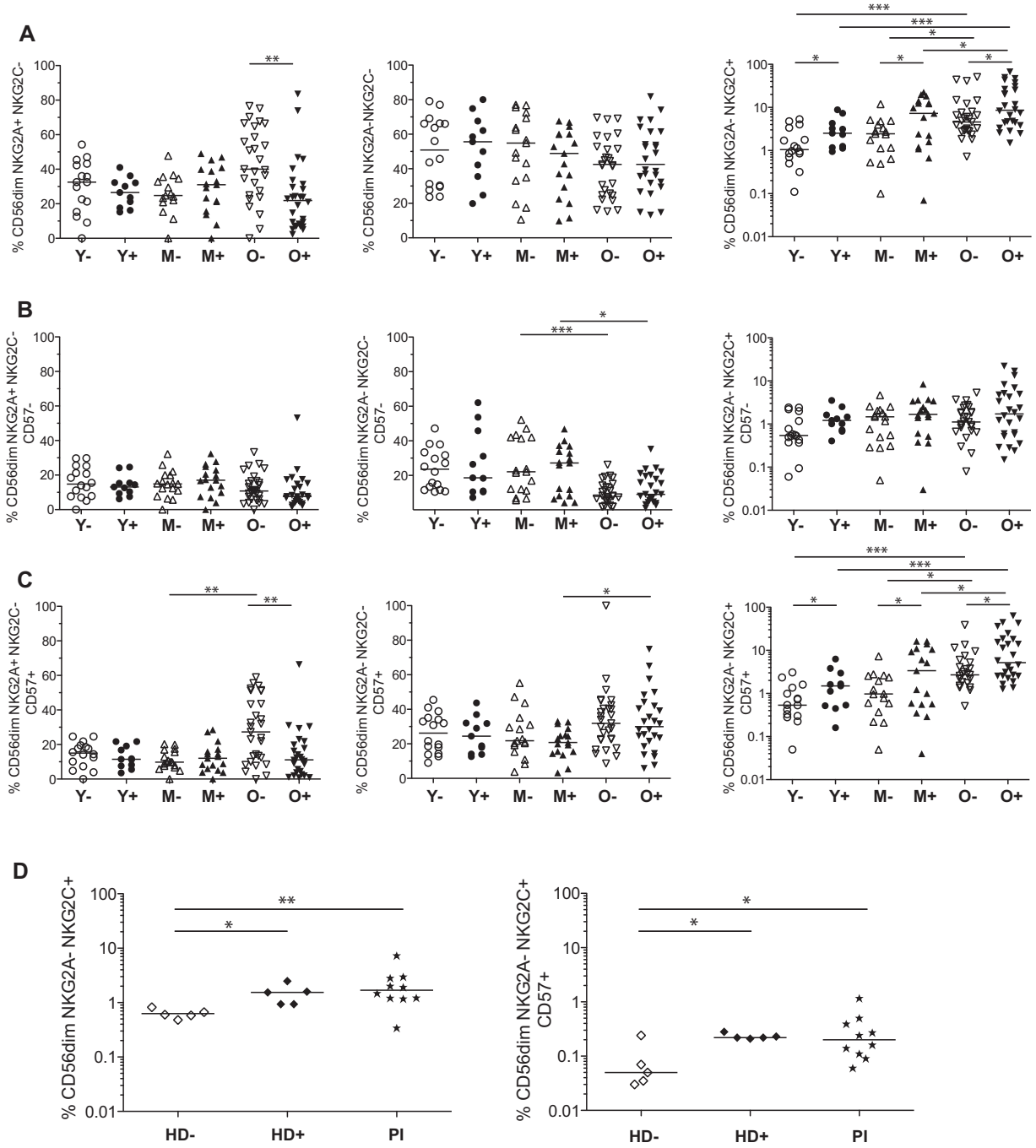
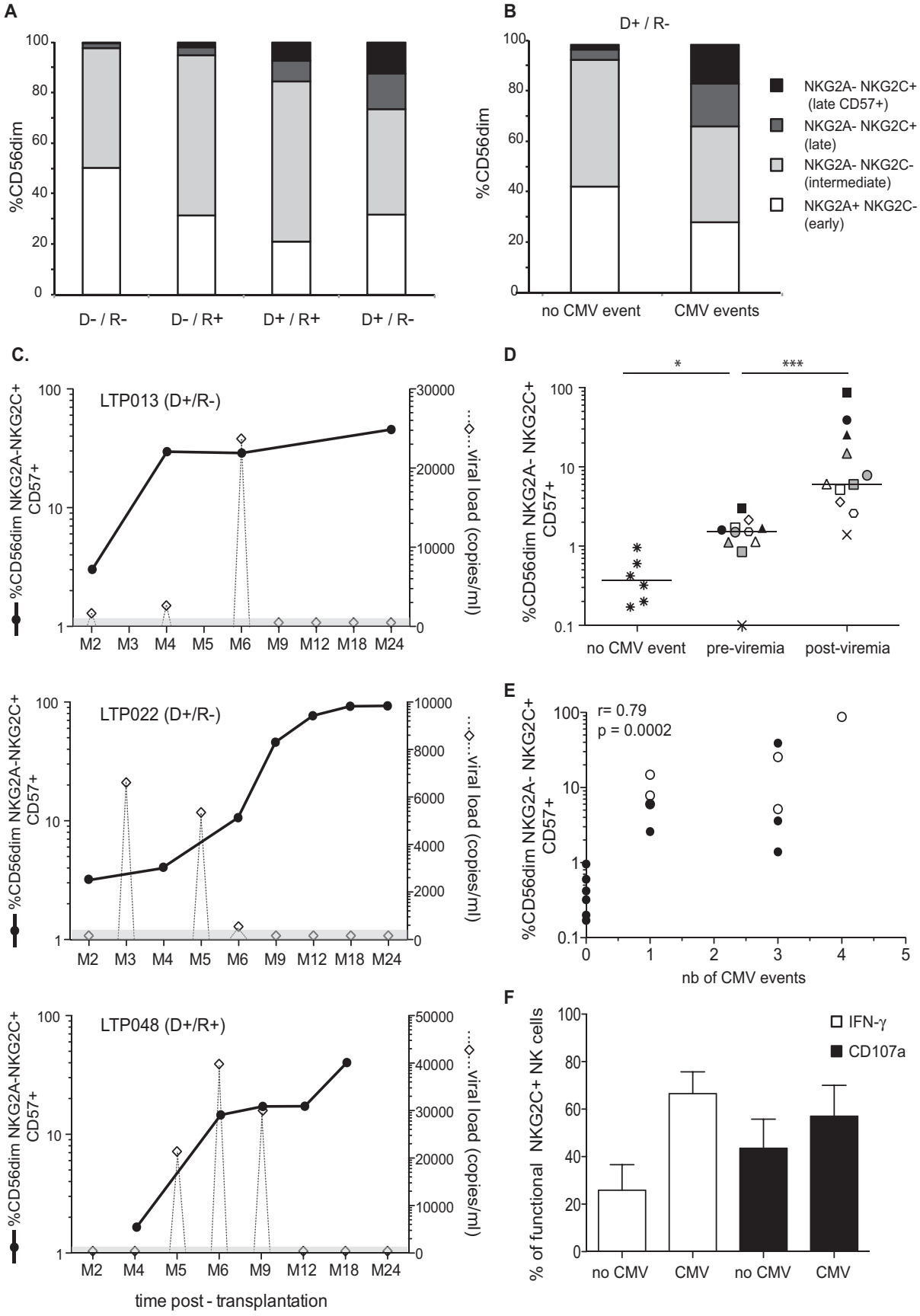


Figure 3. Evolution of NK-cell phenotype according to HCMV status. (A–C) The expression of NKG2A and NKG2C markers on (A) total $CD16^{+}CD56^{dim}$, (B) $CD16^{+}CD56^{dim}$ $CD57^{-}$, and (C) $CD16^{+}CD56^{dim}$ $CD57^{+}$ subsets among HCMV-seronegative Young (Y−: white circle; $n = 17$), HCMV-seropositive Young (Y+: black circle; $n = 13$), HCMV-seronegative Middle age (M−: white upward triangle; $n = 12$), HCMV-seropositive Middle age (M+: black upward triangle; $n = 18$), HCMV-seronegative Old (O−: white downward triangle; $n = 25$) and HCMV-seropositive Old individuals (O+: black downward triangle; $n = 30$) was determined by flow cytometry. (D) The frequency of $CD16^{+}CD56^{dim}$ $NKG2A^{-}$ $NKG2C^{+}$ (left) and $CD16^{+}CD56^{dim}$ NKG2A- $NKG2C^{+}$ $CD57^{+}$ (right) observed ex vivo in pregnant women suffering from primary HCMV infection (PI: stars; $n = 10$). For cohort comparison, subsets frequencies from age-matched controls (five HCMV-seronegative or five HCMV chronically infected women) are indicated (HD− and HD+, respectively). Each symbol represents an individual donor and horizontal bars indicate the median. p values were calculated using the Mann–Whitney test for group comparisons. $^{*}p<0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$.



positive NK cells in response to K562 alone (Supporting Information Fig. 5A), demonstrating that NK cells from Yatec are more prone to be functional without cytokines sensitization with IL-2/IL-12/IL-18. Cytokines sensitization amplifies particularly the response of late CD57$^+$ NK cells (Supporting Information Fig. 5B and C). Of note, terminally differentiated NK cells from HCMV-seropositive Yatec were amplified compared to their age-matched control groups, suggesting that both innate and adaptive immunity were highly shaped by HCMV infection (Fig. 5B and C; Y+ versus Yatec+, $p = 0.04$).

CD8$^+$ T cells expressing CD57 were reported to possess shorter telomeres than CD57$^-$ cells and express an effector memory phenotype [37, 38]. It is possible that NK cells expressing CD57 might also represent NK cells clonally expanded upon encounter with HCMV. Interestingly, we observed a correlation between the frequency of terminally differentiated CD8$^+$ T cells (which encompass HCMV-specific CD8$^+$ T cells) and the frequency of late differentiated CD57$^+$ NK cells (Fig. 5D; $r = 0.49$, $p < 0.0001$). By looking at each group of donors, we observed a dichotomy between HCMV-seropositive and HCMV-seronegative individuals leading to a high frequency of both terminally differentiated T and NK cells only in young HCMV carriers (healthy or thymectomized) (Supporting Information Fig. 6A,B, and D). By contrast, high frequency of late NKG2C$^+$CD57$^+$ NK cells can be found in elderly independently of HCMV status (Supporting Information Fig. 6C). Furthermore, we also detected an association between HCMV-specific T-cell responsiveness and the frequency of late CD57$^+$ NKG2C$^+$ NK cells (Fig. 5E; $r = 0.34$, $p = 0.01$) suggesting that innate and adaptive immunity are complementary to control HCMV latent replication.

Discussion

To date, antigen-specific NK-cell memory has been documented in mice [18, 39, 40]. In human, the memory properties of NK cells have not yet been formally demonstrated [41, 42]. Here, we show that functional NKG2C$^+$ CD57$^+$ NK cells expand in the context of aging and HCMV infection. We demonstrate that NKG2C$^+$ NK cells persist and can reach a high frequency in lung transplant patients (LTP) even after viral control. The preferential acquisition of CD57 on NK cells expressing NKG2C in HCMV-infected hosts suggests that antigen exposure may drive NK-cell differentiation as previously described [23, 32, 43]. Moreover, NKG2C$^+$ NK cells expand preferentially following in vitro coculture with HCMV-infected fibroblast [44] or in solid-organ transplant recipients experiencing HCMV reactivation [24]. Collectively these different studies support that NKG2C expansion is driven by the recognition of HCMV-infected cells, but the exact nature of the ligand remains unknown. The fact that a high frequency of terminally differentiated NK cells can be found in HCMV-seronegative elderly suggest that parameters related to aging (other than HCMV) could imprint the peripheral repertoire. Mechanisms could include common factors between HCMV infection and immune aging such as immune senescence, proinflammatory environment, and increased homeostatic turnover.

Following transplantation using adult grafts containing mature NK cells from HCMV-seropositive donors, posttransplant HCMV reactivation represents a secondary expansion of HCMV-primed NK memory like cells. Despite the variation of viremia occurrence between our LTP, we did not observe discrete peaks of late differentiated NK cells, but rather a continuous expansion of this compartment. This is reminiscent of the CD8$^+$ T-cell responses that are described to accumulate over time [13, 14, 45]. In contrast to other viral chronic infections, HCMV-specific T cells persist at elevated frequencies, reaching up to 50% of peripheral CD8$^+$ T cells [13]. Therefore, in the same way, the accumulation of NKG2C$^+$ CD57$^+$ NK cells could participate to the control of recurrent HCMV replication. Indeed, we found a positive correlation between the level of HCMV specific T cells and the frequency of NKG2C$^+$ CD57$^+$ late differentiated NK cells enabling us to draw a parallel between NK- and T-cell responsiveness against HCMV. A number of similarities between T and NK compartment can be described: (i) HCMV is driving phenotypic differentiation [46, 47], (ii) although HCMV-specific cells are differentiated, they are fully functional [48–50], and (iii) late differentiated cells persist and even inflate overtime despite CD57 expression [13, 14, 45].

Interestingly, the different papers illustrating the shaping of NK cell subsets with aging [29, 30] did not take into account the impact of HCMV in the studied population despite the elevated HCMV-seroprevalence found in elderly. Here, we show, for the first time in human that the acquisition of a more mature

Figure 4. Impact of HCMV events on NK-cell distribution in LTP (A) Phenotypic analysis of NK cells in the different donor/recipient combinations defined with HCMV serostatus (three D$^-$/R$^-$; three D$^-$/R$^-$; three D$^-$/R$^-$; eight D$^-$/R$^-$). NK differentiation phenotypes are described as follow: early (NKG2A$^+$NKG2C$^-$; in white), intermediate (NKG2A$^-$NKG2C$^-$; in light gray), late (NKG2A$^-$NKG2C$^+$; in dark gray), and terminally differentiated NK cells (late CD57$^+$ NKG2A$^-$NKG2C$^+$; in black). (B) Focus on the D$^-$/R$^-$ combination distinguishing between the cases where no HCMV event was described ($n = 3$) versus the patients where HCMV replication occurred during the transplantation follow-up ($n = 5$). (C) Three representative kinetics of the frequency of terminally differentiated NK cells (left axis) and viremia (right axis) during the follow-up of transplanted patients (LTP013, LTP022 and LTP048; month 2–24). Gray zone represents the limit of detection for HCMV viral load (165 copies/mL). (D) Frequency of terminally differentiated NK cells observed in 17 transplanted patients who did not experience HCMV events ($n = 6$) versus patients who were detectable for HCMV viral load ($n = 11$). Symbols represent individual donors and horizontal bars indicate the median. p values were calculated using the Mann–Whitney test for group comparisons or using the Wilcoxon paired test for comparison between pre- and postviremia. Each symbol represents a patient $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. (E) Positive correlation between the number of HCMV events (detectable HCMV viral load) and the amplification of the terminally differentiated NK-cell compartment. D$^-$/R$^-$ pairs are illustrated with a white circle; the other D/R pairs are represented in black circles. (F) Functionality of late NKG2C$^+$ NK cells from LTP ($n = 10$), assessed by their secretion of IFN-γ (white bars) and by their degranulation capacity (CD107a marker; black bars) stratified by the occurrence of HCMV replication. In these experiments, PBMC from LTP were stimulated for 6 h with K562 target cells in presence of IL-2/IL-12/IL-18. Data are shown as mean + SEM.

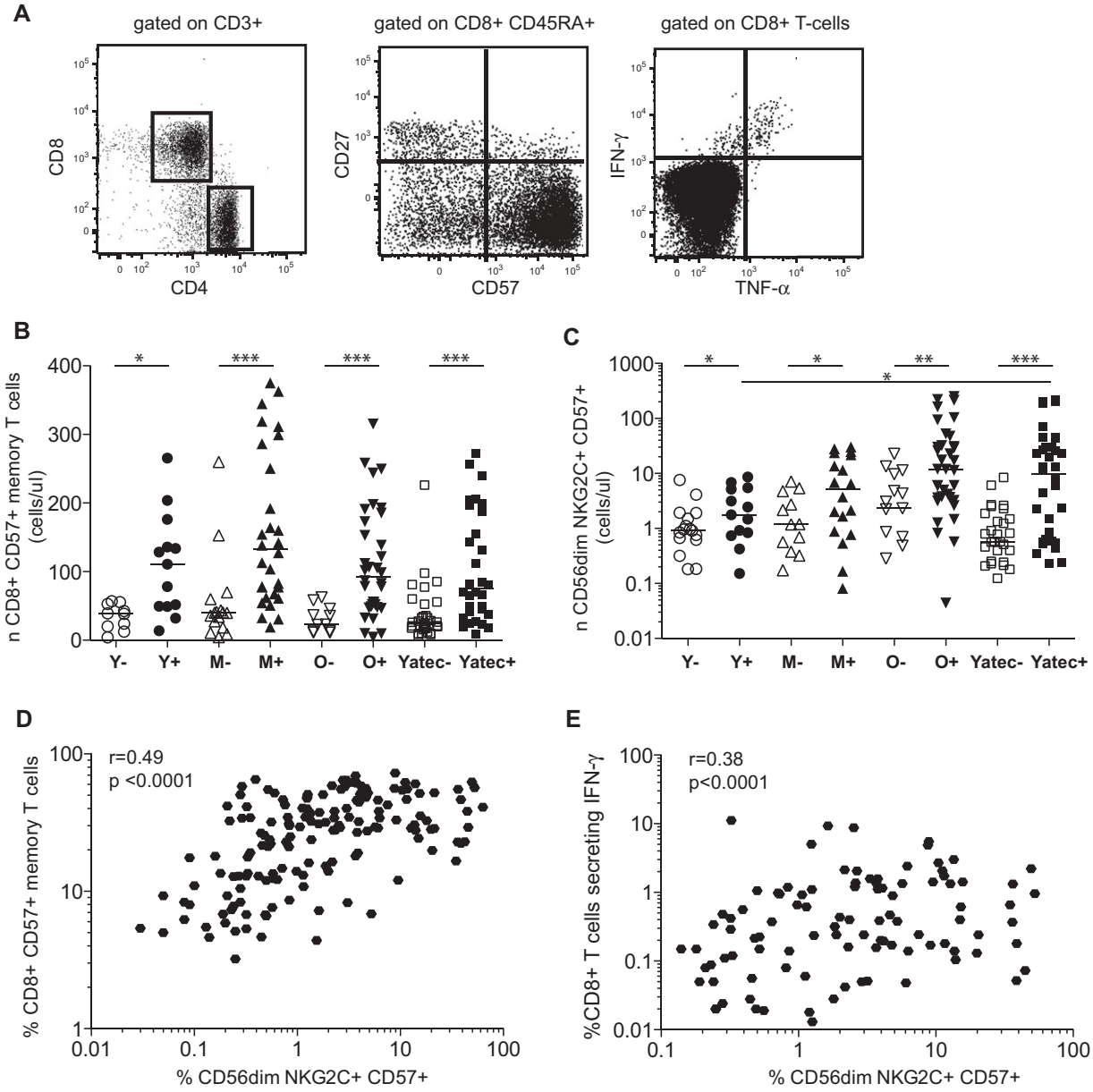


Figure 5. Parallels between CD8$^+$ T cells and NK cells (A) Representative FACS profile showing the expression of CD27/CD57 phenotypic markers on CD8$^+$ T cells as well as their secretion capacity for IFN-γ and TNF-α after overnight stimulation of PBMCs with overlapping HCMV-specific peptides. (B) Cell count of senescent CD57$^+$ memory CD8$^+$ T cells among HCMV-seronegative Young (Y−: white circle; $n = 10$), HCMV-seropositive Young (Y+: black circle; $n = 13$), HCMV-seronegative Middle age (M−: white upward triangle; $n = 12$), HCMV-seropositive Middle age (M+: black upward triangle; $n = 18$), HCMV-seronegative Old (O−: white downward triangle; $n = 13$), HCMV-seropositive Old individuals (O+: black downward triangle; $n = 32$), HCMV-seronegative Yatec (Yatec−: white square; $n = 27$) and HCMV-seropositive Yatec (Yatec+: black square; $n = 30$). (C) Cell count of late differentiated NK cells (CD56^{dim} NKG2C$^+$ CD57$^+$) according to HCMV status in Young (Y− versus Y+), in Middle age (M− versus M+), in Old (O− versus O+) and in Yatec (Yatec− versus Yatec+). Symbols represent individual donors and horizontal bars indicate the median. P values were calculated using the Mann–Whitney test for group comparisons. $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.001$. (D) Correlation between the frequencies of senescent memory CD8$^+$ T cells and late differentiated NKG2C$^+$ CD57$^+$ NK cells. (E) Correlation between the frequency of HCMV-specific CD8$^+$ T cells, assessed by IFN-γ secretion, and the frequency of late differentiated NK CD57$^+$ cells. Correlation coefficients were derived from Spearman analysis.

phenotype within NK cells with aging is not restricted to HCMV-seropositive individuals.

NK cells can profoundly influence the quality and magnitude of T- and B-cell responses by [51, 52], (i) activating or killing antigen-presenting cells and regulatory T cells, (ii) modulating the proliferation and effector functions of cytotoxic T cells, (iii) by skewing helper T-cell polarization, and (iv) by enhancing B-cell activation and isotype switching. Contrarily, there is so far no knowledge about the complementary nature of NK cells and their capacity to counter balance loss of adaptive immunity. It

was therefore of particular interest to follow how Yatec would cope with HCMV infection. We previously demonstrated that, in Yatec, the maintenance of a strong cellular response toward HCMV and a reduced capacity to produce new lymphocytes, can lead to a premature exhaustion of immune resources, in particular with the loss of the naïve compartment in favor of the accumulation of terminally differentiated oligoclonal $CD8^+$ T cells mimicking the situation observed in elderly [15]. In this informative context where adaptive immunity is altered, we hypothesize that NK cells may contribute positively to the control of HCMV latency. This is in line with our observation that HCMV resulted in a marked expansion of $NKG2C^+$ $CD57^+$ NK cells in Yatec. This suggests that NK cells have the potential to contribute alongside memory T- and B-cell responses during subsequent encounters with HCMV [26, 53]. Therefore, both innate and adaptive immunity are solicited during viral infections, such as HCMV. It has yet to be discovered how innate and adaptive immunity coordinate their fight against infection.

Materials and methods

Study subjects

Blood samples from 30 young (median = 22.19 years), 30 middle-aged (median = 34.5 years), and 60 elderly (median = 84 years) healthy adults, were obtained for this study (Table 1). We also studied Yatec ($n = 61$; median = 23 years). Yatec had complete removal of the thymus within 15 days after birth during open-heart surgery due to transposition of great vessels [15]. Pregnant women with primary HCMV infection were also studied ($n = 10$; median = 31.5 years). Diagnosis of primary HCMV infection was made on average at 14 ± 8 (mean $\pm$ SD) weeks of gestation and was based on a documented seroconversion or high titers of anti-HCMV IgM with increasing titers of anti-HCMV IgG when serostatus at the beginning of pregnancy had not been documented. LTP ($n = 17$) were also included in the study (median = 34 years). Posttransplant immunosuppressive treatment consisted of adapted doses of corticosteroid, calcineurin inhibitor, and a purine synthesis inhibitor, with or without depleting antibodies. Blood samples were obtained sequentially at months 2, 4, 6, 9, 12, 18, or 24 following transplantation. Regular monitoring of HCMV replication episodes was performed by quantitative PCR; viremia denoted positive virus-specific PCR in total blood from these patients. For each individual, PBMCs (isolated by density gradient centrifugation) were cryopreserved until use. HCMV serology was performed on plasma samples using a Mastazyme-CMV serology kit (Mast Diagnostics, Merseyside, UK), according to the manufacturer's recommendations. To rule out false-negative serology, we also assess specific T-cell-mediated response to HCMV immunodominant antigens by flow cytometry.

Ethics statement

All participants provided written informed consent. The study was approved by the Comité de Protection des Personnes of the Pitié Salpétrière Hospital, Paris and by the Comité d'Ethique of the Hôpital Erasme, Brussels.

Flow cytometry analysis and functional assessment

From fresh EDTA blood, absolute counts (cells per microliter) were determined using CYTO-STAT tetraCHROME kits on a FC500 cytometer (Beckman Coulter) and analyzed with Flow-Count Single Platform Method (Beckman Coulter). Directly conjugated and unconjugated antibodies were obtained from the following vendors: BD Biosciences (San Jose, CA): CD4 (HV500), CD8 (APC-Cy7), TNF-α (PE-Cy7), IFN-α (AF700), CD40L (PE), IL-2 (APC), CD16 (APC-H7), CD56 (PE-Cy7); Beckman Coulter (Pasadena, CA): CD3 (ECD), CD45 (KO), NKG2A (APC), CD45RA (ECD); BioLegend (San Diego, CA): CD57 (PB), CD3 (BV650); CD8 (BV650), CD27 (AF700); R&D systems (Abingdon, UK): NKG2C (PE); Milteny Biotec (Bergisch Gladbach, Germany): CD107a (VB). Staining for cell surface markers was performed with standard method as previously described [54]. Cells were analyzed on a Fortessa flow cytometer (Becton Dickinson). Data were analyzed using FlowJo v8.2 (Tree Star, Inc) and DIVA softwares (BD Biosciences).

To assess functional capacity of HCMV-specific $CD8^+$ T cells, PBMC were stimulated with 15 amino acid long synthetic peptides (5 μM) overlapping by ten amino acids and spanning the two HCMV proteins, pp65 and IE1. After 1 h, the secretion inhibitor brefeldin A (5 μg/mL; Sigma-Aldrich) was added and the incubation was continued overnight at 37°C in a 5% CO_2 atm. Cytofix/Cytoperm™ (BD Biosciences) was used to fix/permeabilize the cells prior to staining for intracellular IFN-γ and TNF-α. The limit of detection for cytokine secretion was 0.01% in $CD8^+$ T-cell populations based on the nonspecific production of effector molecules detected in unstimulated cells.

To assess NK cells functional capacity, frozen PBMC were cultured overnight at 37°C with IL-2, IL-12, and IL-18 (at 100 U/mL; 10 ng/mL; 100 ng/mL, respectively) and then incubated with the human erythroleukemia class I negative cell line K562 (E:T at 1:1) for 6 h as previously described [22]. Brefeldin and monensin were added after 1 h of incubation. Cells were thereafter stained for cell-surface markers including CD3, CD16, CD56, CD57, NKG2A, NKG2C, and CD107a, fixed/permeabilized and then stained for intracellular IFN-γ expression. Functional measures were corrected by subtracting background signals obtained by analyzing the same samples incubated in medium alone. Exhaustive phenotypic and functional analysis of NK cells were conducted with a beta version of the "FunkyCells ToolBox" software (www.FunkyCells.com) developed by Dr. Martin Larsen (INSERM U1135, Paris, France).

Statistical analysis

Univariate statistical analysis was performed using GraphPad prism software. Groups were compared using the nonparametric Kruskal–Wallis or Mann–Whitney tests. Wilcoxon paired test was used on lung transplant recipients for comparison between the different time points from the same patient. p values < 0.05 were considered significant.

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Abbreviations: **HCMV**: human cytomegalovirus · **LTP**: lung transplant patients · **PI**: primary infection · **Yatec**: young adults thymectomized during early childhood

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