Rapid natural killer cell differentiation in a population with near universal human cytomegalovirus infection is attenuated by *NKG2C* deletions.

Martin R.Goodier*, Matthew J. White*, Alansana Darboe*[¶], Carolyn M. Nielsen, Adriana Goncalves[†], Christian Bottomley[§], Sophie E. Moore[∞¶] and Eleanor M. Riley*[‡]

*Department of Immunology and Infection, [†]Department of Clinical Research, [§]Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, [∞]MRC Human Nutrition Research, Cambridge, UK and [¶]MRC International Nutrition Group, MRC Keneba, MRC Unit, The Gambia.

‡ Corresponding Author: Eleanor M Riley

Tel: 44 20 7927 2706

Fax: 44 20 7637 4314

eleanor.riley@lshtm.ac.uk

Keywords: NK cells, age, CD57, NKG2C, HCMV

Running Title: NK cell differentiation in healthy ageing

Abbreviations: NK – natural killer; HCMV – human cytomegalovirus; HCC –High concentration of cytokines

Key Points

- HCMV infection in early life is associated with rapid phenotypic and functional differentiation of NK cells
- Emergence of CD57+ NK cells is attenuated in children lacking *NKG2C*

Abstract

Natural Killer (NK) cells differentiate and mature during the human life course; human cytomegalovirus (HCMV) infection is one known driver of this process. We have explored human NK cell phenotypic and functional maturation in a rural African (Gambian) population with a high prevalence of HCMV. The impact of age on the frequency, absolute number, phenotype and functional capacity of NK cells was monitored in 191 individuals aged from 1 to 49 years. Increasing frequencies of NK cells with age were associated with increased proportions of $CD56^{dim}$ cells expressing the differentiation marker CD57 and expansion of the $NKG2C^{+}$ subset. Frequencies of NK cells responding to exogenous cytokines declined with age in line with a decreased proportion of $CD57^{-}$ cells. These changes coincided with a highly significant drop in anti-HCMV IgG titres by the age of 10 years suggesting that HCMV infection is brought under control as NK cells differentiate (or vice versa). Deletion at the *NKG2C* locus was associated with a gene dose-dependent reduction in proportions of $CD94^{+}$ and $CD57^{+}$ NK cells. Importantly, anti-HCMV IgG titres were significantly elevated in $NKG2C^{-/-}$ children suggesting that lack of expression of NKG2C may be associated with altered control of HCMV in childhood.

Introduction

Natural killer (NK) cells play essential roles in controlling infection and surveillance for damaged, dysfunctional or neoplastic cells[1]. NK cells differentiate during the human life course. $CD56^{bright}$ cells are the least differentiated population of peripheral blood NK cells, expressing c-kit and high levels of the c-type lectin-like receptor CD94/NKG2A, CD62L and natural cytotoxicity receptors(NCR) NKp30 and NKp46, and lacking expression of KIR, CD16 and CD57[2-5]. $CD56^{bright}$ NK cells express cytokine receptors and produce IFN-γ in response to cytokines. By contrast, $CD56^{dim}$ cells express FcγRIII(CD16), express varying levels of CD94/NKG2A, KIR, NCRs and perforin, retain their ability to secrete IFN-γ and have higher cytotoxic capacity[3]. Heterogeneity within the $CD56^{dim}$ subset is associated with acquisition of CD57[2,4,5]. $CD56^{dim}CD57^{-}$ NK cells are phenotypically and functionally similar to $CD56^{bright}$ cells whereas $CD56^{dim}CD57^{+}$ cells produce little IFN-γ, have shorter telomeres and lower proliferative capacity[5,6] but degranulate extensively after crosslinking of CD16[2,5]. Acquisition of CD57 is associated with onset of expression of NKG2C, although the co-dependence of these events and their implications for function are not understood[7,8].

Although the external drivers of NK cell differentiation are incompletely understood, inflammation, associated with infection or loss of immune homeostasis, plays a key role[9]. This view is supported by evidence that the late differentiation marker, CD57, can be induced on NK cells by high concentrations of IL-2[5], that $NKG2C^{+}$ NK cells can be expanded by co-culture with human cytomegalovirus(HCMV) infected fibroblasts[10], that HCMV seropositive individuals have increased frequencies of $NKG2C^{+}$ NK cells[10-13], and that there is rapid expansion of $CD57^{+}NKG2C^{hi}$ NK cells during acute HCMV infection[14] and in individuals infected with Epstein Barr virus(EBV)[7], hantavirus[15], hepatitis viruses[16] and chikungunya virus[17].

Among Caucasians, NK cell maturation is highly age-dependent. Marked phenotypic and functional differences are observed between NK populations in cord blood, in young

children, adults and in elderly individuals[18-22]. Young children have higher frequencies of $CD56^{bright}CD16^{-}$ and $NKG2A^{+}NKG2C^{-}$ NK cells compared to adults and younger adults have higher frequencies of these cells compared to the elderly[18-22]. Moreover, NCR^{+} and $NKG2D^{+}$ NK cells decrease in frequency with increasing age, concomitant with loss of CD62L and acquisition of CD57[2,4,18,22]. NK cell cytokine production decreases with increasing age but cytotoxic responses are conserved[9,20,23]. There is however a lack of data from older children and teenagers.

The extent to which NK cell differentiation is explained by ageing *per se* or by cumulative exposure to infection is unclear. Among allogeneic hematopoetic stem cell transplant recipients, the first wave of repopulating NK cells comprises predominantly $CD56^{bright}$ or $CD56^{dim}CD94^{+}$cells; KIR^{+} and $CD57^{+}$ cells can take up to 1 year to emerge[2,24]. However, among patients who reactivate HCMV after transplantation, $NKG2C^{+}CD57^{+}$ NK cells can be detected within 3 months and the host's pre-transplantation repertoire is fully reconstituted within 6 months suggesting that exposure to infection is a significant determinant of NK cell maturation rates[24-26].

Together, these data suggest that age-related changes in NK cell phenotype and function may be modified by the infection status of the host, and that rates of change across populations may depend upon the prevalence of particular infections. If so, the prevalence of infections such as HCMV may have far-reaching implications for risk of other infections, cancers or autoimmune disease. To begin to address this important aspect of NK cell biology, we have characterised NK cell phenotype and function in an African population characterised by a high burden of infectious disease, including near universal HCMV infection.

Materials and Methods.

Study subjects. This study was approved by the ethical review committees of the Gambia Government/MRC and London School of Hygiene and Tropical Medicine. Subjects were recruited from the villages of Keneba, Manduar and Kantong Kunda in West Kiang district, The Gambia. After giving fully informed consent in accordance with the Declaration of Helsinki, including parental/guardian consent for minors, venous blood samples were collected from 191 individuals aged 1-49 years. Individuals with signs or symptoms of current disease, or known to be pregnant or infected with HIV, were excluded. Plasma was screened for IgG against HCMV(BioKit, Spain), tetanus toxoid(Holzel Diagnostica, Germany), Hepatitis B surface antigen(Diasorin, Italy) and Epstein Barr Virus nuclear antigen(Euroimmun, Germany). Subject characteristics are shown in Table 1.

Peripheral blood mononuclear cell (PBMC) preparation and culture. PBMC were isolated by density gradient centrifugation(Histopaque, Sigma, UK) and analysed *ex vivo* and after 18h culture with cytokines [5ng/ml rhIL-12(Peprotec, London, UK) plus 50ng/ml rhIL-18(R&D systems, Oxford, UK)] or K562 cells (an E:T ratio of 2:1). FITC-conjugated anti-CD107a(BD Biosciences) was added throughout the culture. Brefeldin A and Monensin(BD Biosciences) were added after 15h.

Flow cytometry. PBMC were incubated with combinations of the following monoclonal antibodies: anti-CD3-V500, anti-CD56-PeCy7 and anti-CD94-FITC, anti-NKG2C-PE and anti-NKG2A-APC, anti-CD8-PeCy7, anti-CD57-e450 and anti-CD16-APC-e780 or APC, anti-CD4-PE and anti-CD45RA-APC-H7, anti-CD8-PeCy7, anti-CD27-FITC, anti-CD28-PeCy7 and anti-CCR7-APC,anti-CD45-FITC, anti-CD11c-PE, anti-CD19-PeCy5, anti-CD123-efluor450 and anti-CD14-APCe780, anti-CD107a-FITC, anti-CD25PE and anti-IFN-γ-APC-efluor780 (see supplementary methods). Cells were acquired on a LSRII® flow cytometer using FacsDiva® software. Data analysis was performed using FlowJo®(TreeStar).

NKG2C genotyping. DNA was extracted from whole blood (Wizard genomic DNA extraction kit, Promega, UK) and *NKG2C* genotype was determined by touch-down PCR(Phusion® High Fidelity PCR kits, New England Biolabs, UK)[27]. PCR primers and conditions are described in supplementary methods.

Statistical analysis. Statistical analysis was performed using Statview and Stata version 13.1. Non-linear effects of age were modelled using natural cubic splines in linear regression models; p-values (F-test) and R-squared values were obtained from these models. One way analysis of variance was used to compare responses of individuals of different genotype. Differences between NK cell subsets were compared using Wilcoxon signed rank tests.

Results

High rates of HCMV and EBV infection in the study population. HCMV infection rates are high in Africa and thus, as expected, only 4 of the 191 individuals were HCMV seronegative; seronegatives were aged between 1 and 3 years, suggesting universal HCMV infection within the first 3 years of life (Table 1). Interestingly, anti-HCMV antibody titres were significantly higher among those under 10 years of age than in older individuals suggesting that optimal control of HCMV infection takes some years to develop (Table 1). Epstein Barr virus (EBV) infection was also common, 75% of the cohort being seropositive for EBV nuclear antigen (EBNA). EBNA seropositivity rates were lowest in children ≤ 2 years old and anti-EBNA titres tended to be higher in those under 15 years of age than in older individuals (Table 1).

NK cell numbers and frequencies change with age. NK cell numbers and frequencies, and the distribution of $CD56^{bright}$ and $CD56^{dim}$ subsets (Fig 1A), were analysed by age group (Figure 1). Consistent with previous observations[18-22], the proportion of NK cells among peripheral blood lymphocytes increased significantly with age, reaching a plateau at approximately 15 years (Figure 1B). Within the total NK cell population, the proportion of $CD56^{bright}$ NK cells declined significantly with increasing age (Figure 1C) and the proportion of $CD56^{dim}$ cells increased (Figure 1D) with subset distribution stabilising at approximately 10-12 years (Figure S1). The absolute number of peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cells declined with age, indicating that the increased frequency of $CD56^{dim}$ cells in older individuals was not sufficient to offset the overall decline in NK cell numbers (Figure S1, Table S1).

These early and very marked changes in NK cell phenotype contrasted with more gradual changes in T cell phenotype (Figure S2). Consistent with previous studies[28,29], we observed a steady decline in naïve $CD4^+$ and $CD8^+$ T cell frequencies, with a parallel increase in frequencies of effector memory and central memory T cells. However, in contrast to

published data[28,29], the frequency of TEMRA cells was already high in young children and did not increase further with age, possibly reflecting high levels of antigen exposure in early life. A decline in absolute numbers of all myeloid and lymphoid cell populations was observed throughout life (TablesS2, S3A,B).

Phenotypic differentiation of NK cells is biphasic, and most rapid during the first 5 years of life. We identified 3 distinct populations of $CD56^{dim}$ NK cells: $CD57^-$, $CD57^+$ and those with intermediate CD57 expression ($CD57^{int}$)[6](Figure 2A). The proportion of $CD57^-$ $CD56^{dim}$ NK cells declined significantly with age, mirrored by increasing proportions of $CD57^+$ NK cells; the proportion of $CD57^{int}$ cells was stable, consistent with this being a transitional population (Figure 2B). Strikingly, this was a biphasic rather than a linear process, with the most marked changes in CD57 subset distribution occurring in children aged 5 years or less, with very little change in subset distribution after the age of 10 years (Figure S3 A-C).

The frequency of NK cells expressing CD94, which partners both NKG2A and NKG2C at the cell surface, remained stable throughout life, suggesting that the proportion of NK cells expressing either NKG2A or NKG2C also remains stable (Figures 2C-F, S3D). However, within the $CD94^+$ population, the proportion of $NKG2A^+$ cells decreased with increasing age (Figure 2E, p=0.03, ANOVA) whereas the proportion of $NKG2C^+$ cells increased (Figure 2F, p=0.02, ANOVA). Increasing proportions of $NKG2C^+$ NK cells were offset by decreasing NK cell numbers such that the absolute number of $NKG2C^+$ cells remained stable throughout life (Table S1).

We then assessed whether changes in CD57 expression mirrored changes in NKG2A/NKG2C expression (Figure 3). The proportion of $CD57^-$ cells within the $NKG2A^+$ subset decreased significantly with increasing age, with a reciprocal enrichment of $CD57^{int}$ and $CD57^+$ NK cells (Figure 3A). Nevertheless, the majority of $NKG2A^+$ NK cells remained $CD57^-$ even in older individuals (Figure 3A). By contrast, $NKG2C^+$ NK cells are frequently $CD57^+$ even in children < 2 years and the majority of $NKG2C^+$ NK cells are $CD57^+$ by the age

of 5 years (Figure 3B). The mean fluorescence intensity (MFI) of CD57 expression was very low on NKG2A$^+$ NK cells (at all ages) but increased significantly with increasing age on NKG2C$^+$ cells (Figure 3C, S3G,H) suggesting that NKG2C$^+$ NK cells differentiate rapidly in this cohort (gaining full CD57 expression very early in life) whereas NKG2A$^+$ NK cells differentiate only very slowly. This rapid expansion and differentiation of the NKG2C$^+$ NK cell population is likely a consequence of perinatal HCMV infection. Moreover, anti-HCMV IgG titre was negatively correlated with the frequency of CD57$^+$ NK cells (Figure S4), suggesting that advanced NK cell differentiation may be associated with control of HCMV, or vice versa. EBV serostatus, which has been associated with altered NK cell phenotype in HCMV exposed Europeans[7], had no significant impact on NK cell subset distribution other than a minor increase in CD56^{dim} cell frequency (Figure S5A-G), supporting a recent paper suggesting that acute EBV co-infection has no major impact on NKG2C+CD57+NK cells[30].

Rapid functional maturation of NK cells during childhood in The Gambia. To assess the functional consequences of these phenotypic changes, PBMCs were cultured *in vitro* with K562 target cells or with high concentrations of cytokines (IL-12 and IL-18; HCC); NK cell degranulation (CD107a), CD25 and IFN-γ expression were assessed by flow cytometry (Figure 4). Spontaneous low level degranulation and IFN-γ production was observed amongst unstimulated cells from children below the age of 10 years, perhaps indicating *in vivo* NK cell activation (Figure 4C,I). Incubation with K562 cells increased NK cell degranulation but this did not differ with age (Figure 4D). Conversely, degranulation, upregulation of CD25 and IFN-γ production in response to HCC (Figure 4E,H,K) were all strongly age-related, being significantly higher in children under the age of 10 years than in older individuals (Figure S6).

Spontaneous NK cell degranulation could be attributed to CD56^{dim}CD57$^-$ cells (Figure 5A) whereas spontaneous expression of CD25 and IFN-γ production were restricted to the CD56^{bright} subset (Figure 5B,C). CD107a and CD25 expression were observed in all NK cell

subsets after incubation with K562 cells. Although this did not vary with age, it was higher in $CD57^-$ cells than in $CD57^{int}$ and $CD57^+$ cells (Figure 5D,E) consistent with patterns of expression of the NKp30 activating receptor (which binds B7-H6 on K562 cells)[31,32]. As expected, K562 cells induced little IFN-γ secretion from any NK cell subset (Figure 5F).

$CD57^-$ NK cells (but not $CD57^{int}$ or $CD57^+$ cells) degranulated extensively in response to cytokine stimulation (Figure 5G) and cytokine-induced CD25 expression and IFN-γ production declined with progressive NK cell differentiation, being highest in the $CD56^{bright}$ subset and lowest in the $CD56^{dim}$ $CD57^+$ subset (Figure 5H,I). Although there was a trend for increasing CD107a and CD25 expression with increasing age in $CD57^{int}$ and $CD57^+$ NK cells after cytokine stimulation (Figure 5G,H), this was only significant comparing the very youngest and very oldest age groups ($p<0.01$, ANOVA).

Thus, whilst subtle age-associated changes in NK cell function may be evident within subsets, changing NK cell function with age is primarily due to the changing proportion of cells within subsets.

Impact of *NKG2C* genotype on NK cell numbers and phenotype. Lack of NKG2C expression due to deletion of the *NKG2C* locus has been reported in several populations[27,33-35]. Nineteen of 181 individuals tested here (10.4%) were $NKG2C^{-/-}$ (and lacked surface expression of NKG2C) whereas 68 individuals (37.5%) were heterozygotes, giving a haplotype frequency of 29.3%. $NKG2C^{-/-}$ individuals were distributed evenly across age groups and between the sexes (Table 1).

NKG2C genotype did not affect frequencies of total, $CD56^{bright}$ or $CD56^{dim}$ NK cells although, consistent with published data[34], $NKG2C^{-/-}$ children under 10 years old had lower absolute numbers of NK cells when compared with $NKG2C^{+/-}$ children (Figure S7). However, $NKG2C^{-/-}$ individuals had significantly lower frequencies of $CD56^{dim}$ $CD94^+$ NK cells than did $NKG2C^{+/-}$ and $NKG2C^{+/+}$ individuals (Figure 6A). Absolute numbers of $NKG2A^+$ cells were unaffected by genotype (Figure S8B) whereas absolute numbers of $CD94^+$ cells were

significantly lower among *NKG2C*$^{-/-}$ individuals (Figure S8A). This is consistent with CD94^{+}NKG2A^{+} to CD94^{+}NKG2C^{+} ratios being determined by expansion within the CD94^{+}NKG2C^{+} subset rather than conversion of NKG2A^{+} cells to NKG2C^{+} cells. A significant gene dosage effect was observed with *NKG2C*$^{+/-}$ individuals having intermediate frequencies (Figure 6B) and numbers (Figure S8) of CD94^{+}NKG2A^{+} and CD94^{+}NKG2C^{+} cells. A modest decrease in the MFI for NKG2C expression was observed in *NKG2C*$^{+/-}$ compared to *NKG2C*$^{+/+}$ individuals, although this did not reach statistical significance (Figure S9). *NKG2C*$^{-/-}$ children (under 10 years) had significantly lower frequencies of CD57^{+} NK cells than did heterozygous and homozygous *NKG2C*$^{+}$ children, with a reciprocal increase in both CD57^{-} and CD57^{int} NK cells (Figure 6C). This effect was absent in individuals aged over 10 years.

Finally, to explore whether *NKG2C* genotype might affect control of HCMV, we examined the relationships between age, genotype and anti-HCMV antibody titre (Figure 6D). Anti-HCMV antibody titres were markedly and significantly higher in *NKG2C*$^{-/-}$ than in NKG2C$^{+/+}$ children (under 10 years)(Figure 6D) suggesting that inferior control of HCMV infections in these children may lead to more frequent reactivation and boosting of antibody responses. This effect was not observed in older individuals and appeared to be specific for HCMV since no impact of *NKG2C* genotype was observed on titres of antibodies to childhood vaccine antigens or EBV (Figure S10). One explanation for this is that lack of NKG2C^{+} NK cells may hinder control of HCMV such that the ability to control HCMV viral load (as reflected by anti-HCMV titre) develops more slowly in children who lack NKG2C.

Discussion

It is increasingly appreciated that NK cells are genetically, phenotypically and functionally diverse both at the human population level[36] and within individuals[37]. Moreover, NK cells differentiate through the life course, reflecting the interplay of genes and environment. These adaptations substantially modify NK cell function[20,38,39] and are beginning to be associated with health outcomes[9]. Age is a major determinant of NK cell phenotype and function[18-22] but it is not yet clear whether this is due to primary, age-intrinsic processes or whether age is simply a marker for cumulative environmental exposures. HCMV infection is a major confounder of the association between age and NK cell function[11,26,40] but HCMV status is not reported in many published studies, hindering data interpretation. In order to unpick these issues, detailed phenotypic and functional studies are required across the entire age span and amongst genetically diverse populations in different environments. The data presented here represent the most comprehensive study to date of NK cell phenotype and function from infancy to mature adulthood, the first such study in an African community and the first in a population where confounding by HCMV infection status is minimised due to near universal HCMV infection in infancy.

We previously identified an apparently transitional population of $CD56^{dim}$ NK cells with intermediate CD57 expression ($CD57^{int}$), expressing intermediate levels of CD16, CD62L, IL-12R and IL-18R and with capacity for degranulation, CD25 expression and IFN-γ production between $CD57^-$ and $CD57^+$ NK cells[6]. Here we observe that, whilst frequencies of $CD57^-$ cells decrease and frequencies of $CD57^+$ cells increase with age, a small but persistent population $CD57^{int}$ NK cells is present at all ages, suggesting that differentiation of $CD57^-$ to $CD57^+$ NK cells occurs at a similar rate throughout the life course. If so, age-related changes in $CD57^-$ and $CD57^+$ NK cell frequencies must reflect differential rates of loss or proliferation of these two subsets rather than changing rates of cell conversion. Rates of both apoptosis

and proliferation are reportedly very high in human NK cells[41] but whether these rates differ between $CD57^-$ and $CD57^+$ NK cells is unknown.

One striking observation in this population is the very high frequency of fully differentiated, $CD56^{dim}NKG2C^+CD57^+$ NK cells in very young children: these cells represent up to 50% of all NK cells in 1-2 year olds and up 80% of cells in 6-9 year olds, with the mature adult range(~30-70%) being reached by the age of 10 years. In Europeans, proportions of $CD57^+$ NK cells range from zero at birth (cord blood) to median values of ~50% in adults[20] with values being higher in $HCMV^+$ individuals(30-70%) than in $HCMV^-$ subjects(25-50%)[42]. Although we could not compare our data with a fully age-matched low HCMV prevalence cohort, the frequency of $CD57^+$ NK cells in HCMV seropositive adult Gambians is significantly higher than in an age-matched HCMV seropositive UK cohort, confirming more rapid or extensive NK cell differentiation among Gambians (Figure S11). This may reflect HCMV infection much earlier in life in The Gambia, or higher prevalence of other infections which further expand the $NKG2C^+CD57^+$ NK cell population in $HCMV^+$ individuals[43]. Data on $HCMV^+$ and $HCMV^-$ European children are needed to confirm this.

Interestingly, HCMV and EBV co-infection did not affect NK cell phenotype or function. EBV co-infection has been associated with more extensive NK cell differentiation compared to HCMV alone in some European studies[7] but not in a recent USA study[30] suggesting that perinatal HCMV infection alone is sufficient to drive NK cell differentiation, or that infections other than EBV may also have an impact, in this Gambian cohort. Of note, the biphasic kinetic of NK cell differentiation is not accompanied by a similar biphasic differentiation of T cell populations, consistent with the suggestion that HCMV infection independently affects T cell and NK cell populations[44].

Age-related differences in NK cell function were entirely due to differences in the proportions of $CD57^-$ and $CD57^+$ NK cells. In Caucasian adults, cytokine-induced degranulation, CD25 expression and IFN-γ production all decline with increasing levels of CD57 expression[2,4-6] in

parallel with reduced expression of IL-12 and IL-18 receptors[6] whereas CD57 expression has much less effect on responses receptor crosslinking[6]. This association between CD57 expression and NK cell function also holds true in The Gambia, and in children as young as 3-5 years of age. Since CD57 expression is, to a large extent, driven by HCMV, it appears that infection with HCMV very early in life rapidly skews the entire NK cell population to missing/altered-self/antibody dependent cytotoxicity at the expense of cytokine-driven responses[6,45]. This skewing of NK cell function is much more marked among Gambian adults than among age-matched HCMV seropositive UK adults, again presumably reflecting an earlier age of HCMV infection or increased prevalence of co-infections in The Gambia (Figure S11). Altered NK cell function so early in life could contribute to associations between perinatal HCMV infection, slower growth and increased rates of hospitalisation, as observed in Zambian children[46].

In line with the near universal HCMV infection in infancy in our cohort, and the well documented expansion of $CD57^{+}NKG2C^{+}$ NK cells in HCMV+ individuals[11], frequencies of $NKG2C^{+}$ cells were high in all age groups. Frequencies of $NKG2C^{+}$ NK cells were lower in very young children than in older age groups but adult frequencies were achieved by the age of 6-9 years, suggesting that expansion of the $NKG2C^{+}$ subset begins very quickly after HCMV infection and may continue for some years. This is consistent with data from transplant recipients with acute HCMV infection or HCMV reactivation where frequencies of $NKG2C^{+}$ NK cells increase within a month of infection/reactivation and continue to increase for at least 12 months[14,26,40] and with reports of significantly higher frequencies of $NKG2C^{+}$ NK cells in HCMV+ compared to HCMV- children under the age of 2 years[47].

In Caucasian adults, the $NKG2C^{+}$ NK cells induced by HCMV infection tend to co-express CD57[14]. This was also the case here, although both the frequency of $NKG2C^{+}$ cells expressing CD57 and the median MFI of CD57 expression were lower in children under the age of 2 years than in older individuals. By contrast, CD57 is expressed only at low intensity on $NKG2A^{+}$ NK cells at all ages. These observations are consistent with a model in which

peptides from HCMV UL40 bind to HLA-E, stabilising it at the surface of infected cells where it drives activation, proliferation and differentiation (including expression of CD57) of NK cells expressing NKG2C (the activating receptor on NK cells for HLA-E) whilst simultaneously inhibiting proliferation and differentiation of cells expressing NKG2A (the inhibitory HLA-E receptor)[10,48-50].

Although CD94/NKG2C and CD94/NKG2A are not the only NK cell receptors for HCMV[51,52,53] lack of *NKG2C* was clearly linked to delayed NK cell differentiation and maturation. *NKG2C*$^{+/-}$ heterozygotes had lower frequencies of NKG2C^{+} NK cells than did *NKG2C*$^{+/+}$ individuals (consistent with a previous report[33]) and the frequency and absolute number of CD94^{+} cells was positively associated with *NKG2C* copy number, consistent with the hypothesis that NKG2C^{+} NK cell numbers expand by proliferation rather than by transformation from NKG2A^{+} cells. Importantly, however, a high proportion of CD56^{dim} NK cells in *NKG2C*$^{-/-}$ individuals lack expression of CD94/ NKG2A as well as CD94/NKG2C, raising questions about which other receptors might be expressed on these cells in order to maintain NK cell homeostasis and HCMV latency. HCMV reactivation in recipients of *NKG2C*$^{-/-}$ stem cells drives differentiation of functional KIR+NKG2A^{-} NK cells[53] suggesting that activating KIR may compensate for lack of CD94/NKG2C. Stable expansions of KIR^{+} NKG2A^{-}NKG2C^{-} NK cells have also been observed in HCMV seropositive adults[54].

Consistent with activation and expansion of NKG2C^{+} cells prior to their acquisition of CD57, proportions of CD57^{+} NK cells were significantly lower in *NKG2C*$^{-/-}$ subjects than in those with one or more copies of *NKG2C*, in particular for children aged under 10 years. The magnitude of this effect is remarkable, achieving statistical significance despite the rather small number of *NKG2C*$^{-/-}$ subjects, and likely to be highly biologically relevant. It would be interesting to know whether delayed NK cell differentiation in HCMV-infected *NKG2C*$^{-/-}$ subjects is seen in other populations and whether it confers any survival advantage or whether this is offset by impaired control of HCMV (as implied by the significantly higher anti-HCMV antibody titres). These studies will need to be large enough to achieve statistical

power, which will depend on both the prevalence of the *NKG2C*-null haplotype and of HCMV. The 29.3% haplotype frequency of the *NKG2C* deletion in our African cohort is higher than that recorded elsewhere[33,35,55]. Whether the frequency of this haplotype is linked to current or historic intensities of HCMV infection might also merit further investigation.

Despite our study cohort being almost uniformly HCMV seropositive, considerable heterogeneity is observed in NK cell phenotype and function within each age group. Whilst some of this is heterogeneity may be genetically determined and/or stochastic[37], exposure to infections in addition to HCMV may also affect NK cell maturation[15,17,7,56]. Further studies are needed to determine whether this is simply a cytokine driven expansion, and thus likely to occur in response to many acute inflammatory stimuli, or whether some pathogens express specific ligands for $CD57^{+}NKG2C^{+}$ NK cells.

In summary, our study has revealed rapid phenotypic and functional differentiation of peripheral NK cells in a population with extremely high rates of perinatal HCMV infection. Intriguingly, NK cell phenotype seems to be highly dependent on the expression of NKG2C, reaffirming the notion that signalling via NKG2C is causally linked to NK cell differentiation. Further studies are now warranted to evaluate the impact of early HCMV infection on the ability of NK cells to contribute to protection from other infections throughout the life course.

Acknowledgements

We would like to thank Professors Andy Hall and Andrew Prentice for facilitating the initiation of this project, Kerry Jones for logistical and technical support, MRC fieldworkers Sulayman Bah, Wally Kamara and Lamin Jatta for recruiting subjects, Bai Lamin Dondeh for database management, staff of the MRC International Nutrition Group and MRC Gambia (Keneba) for their support, and the villagers of Keneba, Manduar and Kantong Kunda for participating in the study. We would also like to thank Professor Beate Kampmann and colleagues (MRC Gambia, Fajara) for advice and access to laboratory facilities. This study was funded by the UK Medical Research Council (G1000808).

Authorship

Contribution: M.R.G. designed research, performed experiments, analysed and interpreted data and wrote the manuscript; M.J.W. designed and performed research and analysed and interpreted data. A.D. designed and performed research; C.M.N. performed experiments and analysed data._A.G. performed research; C.B. analysed data; S.M. designed research and coordinated recruitment of study subjects and E.M.R. designed research, supervised data collection, directed data analysis and wrote the manuscript.

Conflict-of-interest disclosure: The authors have no competing financial interests to declare.

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Figure Legends

Figure 1. Age-related changes in NK cell frequencies (**A).** Natural killer cells were identified within PBMC after gating on singlets and viable lymphocytes. $CD56^{+}CD3^{-}$NK cells were then subsequently gated into $CD56^{bright}$ and $CD56^{dim}$ subsets. Frequencies of (**B**) all NK cells, (**C**) $CD56^{bright}$, and (**D**) $CD56^{dim}$ NK cells, are shown for each age group. Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Asterisks represent significant trends across the entire cohort (* $p<0.05$, *** $p<0.001$, F-test).

Figure 2. Age related changes infrequencies of CD57-and c-type lectin-like receptor-expressing NK cell subsets.A.$CD56^{dim}$ cells were gated into $CD57^{-}$, $CD57^{intermediate}$ and $CD57^{+}$ subsets. The $CD57^{-}$ population was gated using an isotype matched control reagent and the $CD57^{+}$ gate was set at an MFI of 3000.**B.** Frequency distribution by age group of $CD57^{-}$, $CD57^{int}$ and $CD57^{+}$ subsets within the $CD56^{dim}$ NK cell population. Asterisks denote statistically significant trends for changes in NK cell subset frequency by age (*** $p<0.001$, F-test). **C,E.** Gating strategy for $CD94^{+}NKG2A^{+}$ and $CD94^{+}NKG2C^{+}$ cells within the $CD56^{dim}$ NK cell subset. Frequencies of $CD94^{+}$(**D**), $NKG2A^{+}$ and $NKG2C^{+}$(**F**) NK cells by age group. Asterisks denote statistically significant differences in frequencies of $NKG2A^{+}$ and $NKG2C^{+}$ cells by age group (* $p<0.05$, ANOVA).Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Age groups are as shown in Figure 1.

Figure 3. CD57 is preferentially expressed on $NKG2C^{+}$ NK cells. $CD56^{dim}$ NK cells were gated as in Figure 1A and the frequency of $CD57^{-}$, $CD57^{int}$ and $CD57^{+}$ cells is shown within (**A**) CD94/$NKG2A^{+}$ or (**B**) CD94/$NKG2C^{+}$ NK cells, by age group. **C.** Mean fluorescence intensity (MFI) for CD57 expression on $NKG2A^{+}$ and $NKG2C^{+}$ NK cells by age group. Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Asterisks denote statistically significant trends

by age within each subset (** $p < 0.01$;*** $p<0.001$, F-test). Age groups are as shown in Figure 1.

Figure 4. Age-associated changes in NK cell function. Example flow cytometry plots are shown for $CD3^-$ lymphocytes from a 1 year old **(A)** and a 22 year old **(B)**, cultured in medium alone (upper panels) or stimulated with high concentrations of IL-12+ IL-18 (HCC, lower panels) and assayed for degranulation (CD107a), CD25 and IFN-γ expression. **C-K:** NK cells were assayed for degranulation (**C-E**), CD25 (**F-H**) or IFN-γ (**I-K**) expression after *in vitro* culture in medium alone (**C,F,I**) or with K562 target cells (**D,G,J**) or IL-12 + IL-18 (HCC; **E,H,K**). Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Asterisks denote significant age related trends for frequencies of NK cells expressing CD107a, CD25 or IFN-γ(** $p < 0.01$;*** $p<0.001$, F-test).

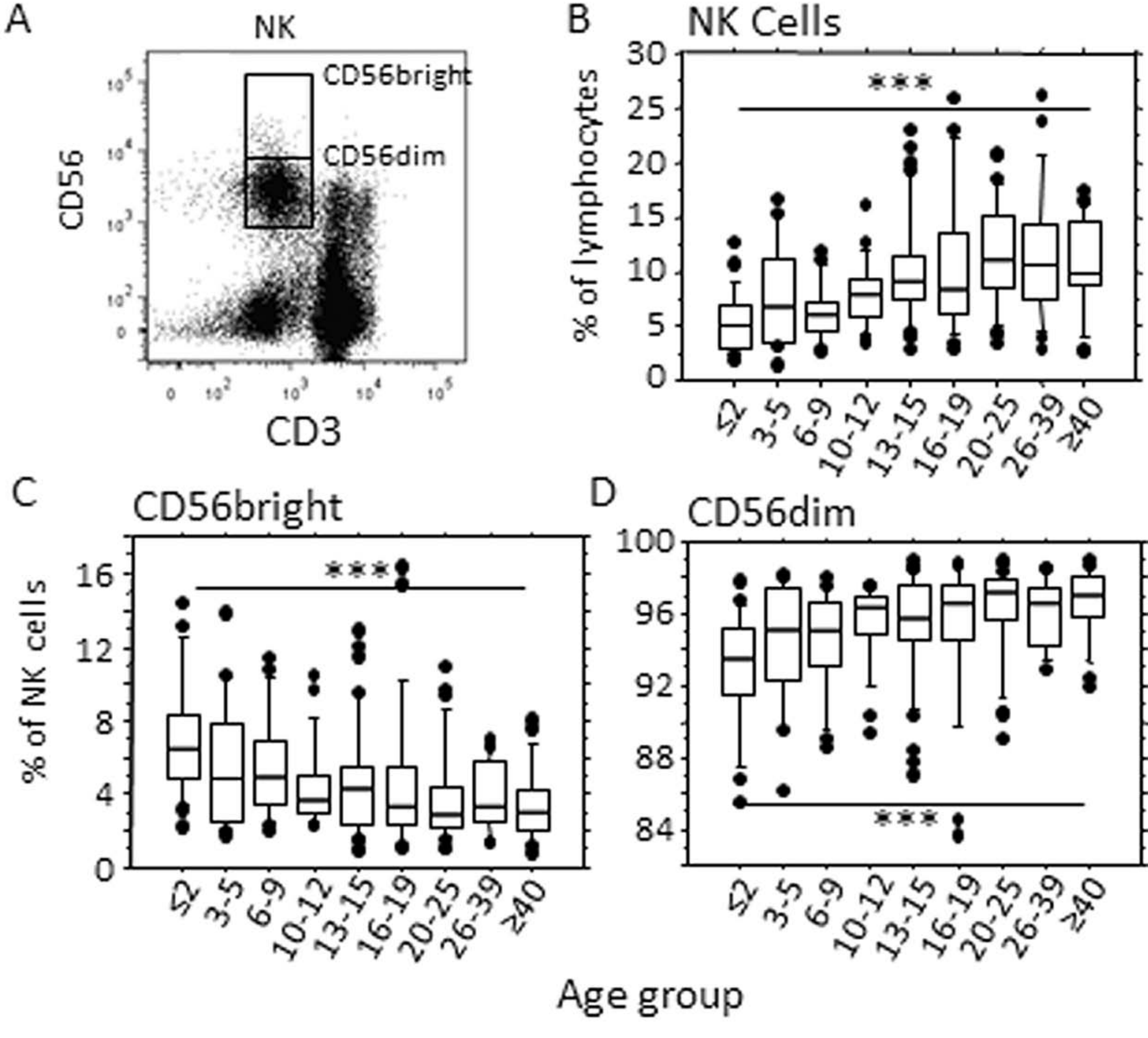
Figure 5. NK cell function reflects CD57 expression, irrespective of age. Bright ($CD56^{bright}CD57^-$), CD57- ($CD56^{dim}CD57^-$),CD57int ($CD56^{dim}CD57^{int}$) and CD57+ ($CD56^{dim}CD57^+$) NK cell subsets were analysed for CD107a (**A,D,G**),CD25 (**B,E,H**) or IFN-γ (**C,F,I**) after *in vitro* culture in medium alone (**A-C**), with K562 target cells (**D-F**) or with IL-12 + IL-18 (HCC; **G-I**).Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. There were no significant age-related trends in response within any of the subsets. Asterisks denote statistically significant differences between $CD57^-$, $CD57^{int}$ and $CD57^+$subsets. ($p<0.001$ for all comparisons, Wilcoxon-signed rank).

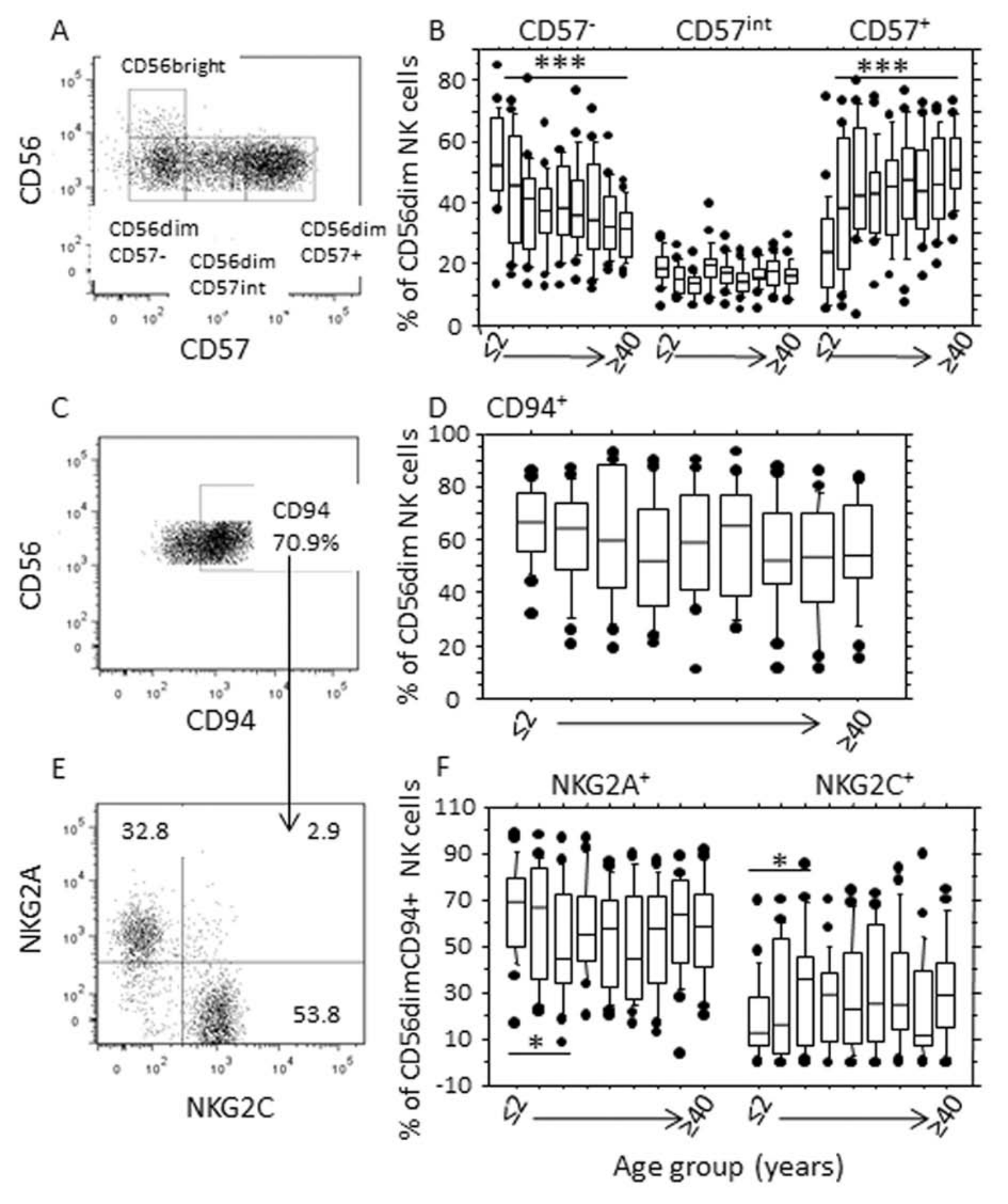
Figure 6. Effect of *NKG2C* genotype on NK cell maturation phenotype and HCMV antibody titre. A. Frequency of $CD94^+$ cells within the $CD56^{dim}$ NK cell population in individuals with zero (*NKG2C*$^{-/-}$), one (*NKG2C*$^{+/-}$) or two (*NKG2C*$^{+/+}$) copies of the *NKG2C* gene. **B.** Impact of *NKG2C* genotype on the frequencies of $CD94^+$ NK cells expressing either $NKG2A^+(NKG2C^-)$ or $NKG2C^+(NKG2A^-)$ cells. **C.** Impact of *NKG2C* genotype on the

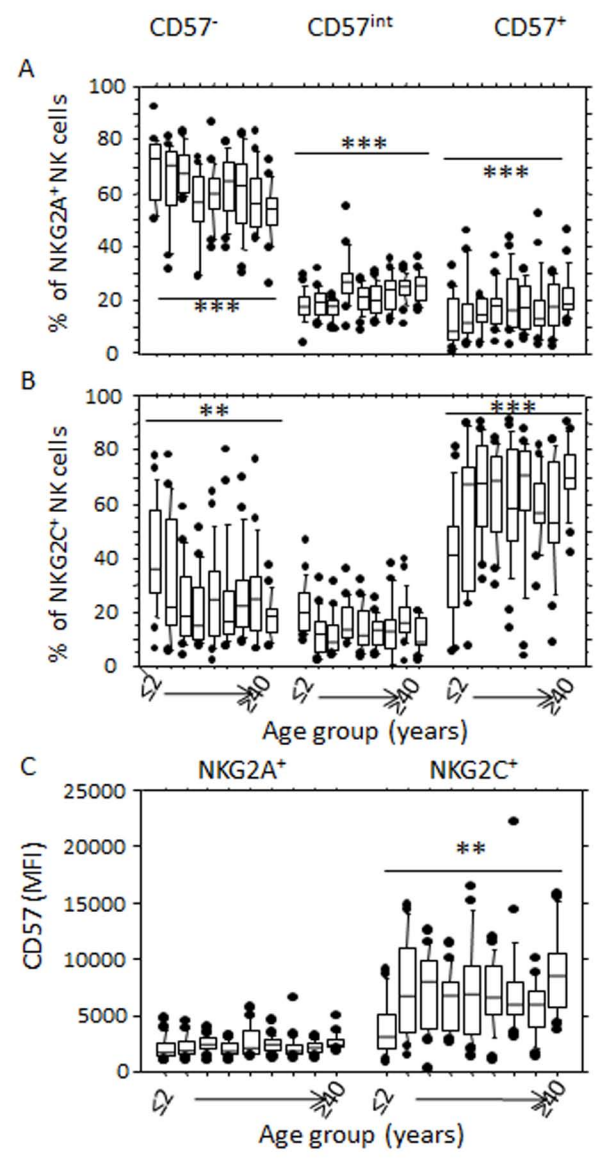
frequency of $CD57^-$, $CD57^{int}$ and $CD57^+$ NK cells in subjects<10 and ≥ 10 years of age. **D.** Anti-HCMV antibody titres by age (years) and *NKG2C*$^-$genotype. Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Asterisks denote statistically significant differences between genotypes for all comparisons shown ($p<0.05$, *** $p< 0.001$, ANOVA).

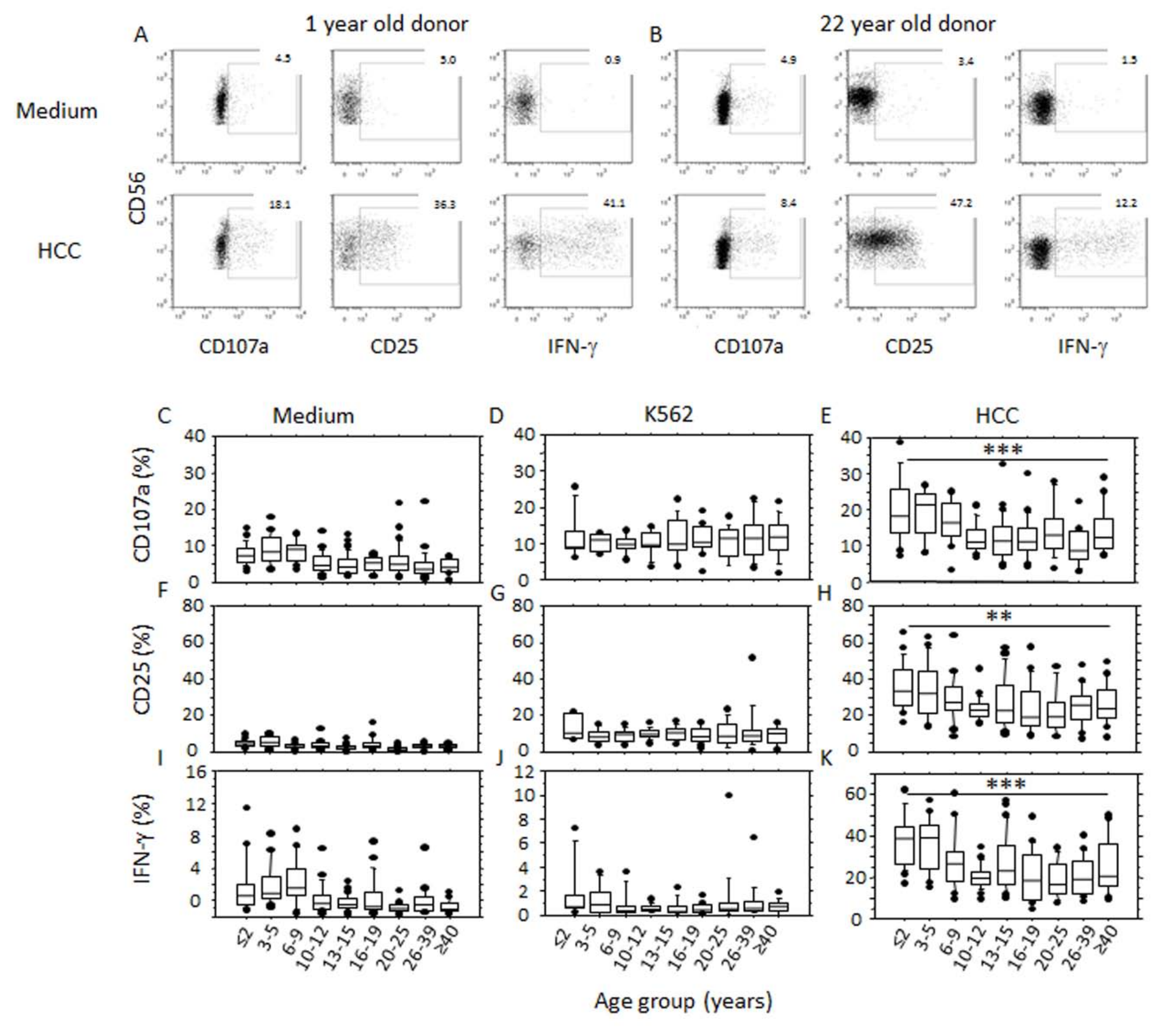
Age Group	n (Male/Female)	HCMV IgG$^+$ n (%)	HCMV IgG Titre (IU/ml) Median (range)	EBNA IgG$^+$ n (%)	EBNA IgG Titre (IU/ml) Median (range)	*NKG2C* genotype[5] n (%)		
						+/+	+/-	-/-
1-2	23 (9/14)	20 (86.9)	487.5 (81.8-845.2)[1]	12 (52.2)	107.0 (48.5-178.6)	11 (47.8)	10 (43.4)	2 (8.7)
3-5	19 (6/13)	18 (94.7)	288.4 (80.9-1681.8)	13 (68.4)	134.0[3] (32.5-328.7)	7 (37.8)	10 (52.6)	2 (10.5)
6-9	18 (11/7)	18 (100)	361.1 (89.2-2200.2)[2]	16 (88.9)	103.6 (33.1-219.7)	8 (47.0)	7 (41.2)	2 (11.8)
10-12	20 (10/10)	20 (100)	215.4 (43.4-1693.6)	18 (90.0)	119.3[4] (37.2-359.5)	8 (44.4)	8 (44.4)	2 (11.1)
13-15	23 (10/13)	23 (100)	252.6 (51.5 -1057.9)	16 (70.0)	114.6 (29.7-193.4)	11 (47.8)	10 (43.4)	2 (8.7)
16-19	23 (11/12)	23 (100)	177.6 (61.2-678.1)	18 (78.2)	99.9 (23.9-195.2)	10 (47.6)	8 (38.1)	3 (14.3)
20-25	22 (11/11)	22 (100)	252.5 (81.5-828.4)	19 (86.4)	93.9 (27.6-171.7)	11 (52.4)	8 (38.1)	2 (9.5)
26-39	22 (13/9)	22 (100)	165.9 (39.0-968.4)	19 (86.4)	88.8 (24.9-272.7)	14 (73.7)	3 (15.7)	2 (10.5)
40-49	21 (10/11)	21 (100)	191.2 (53.5-735.2)	13 (61.9)	73.4 (24.0-183.2)	14 (70.0)	4 20.0)	2 (10.0)
Total	191 (91/100)	187 (97.9)	252.6 (39-2200.1)	145 (75.9)	101.8 (23.9-359.5)	94 (51.9)	68 (37.6)	19 (10.5)

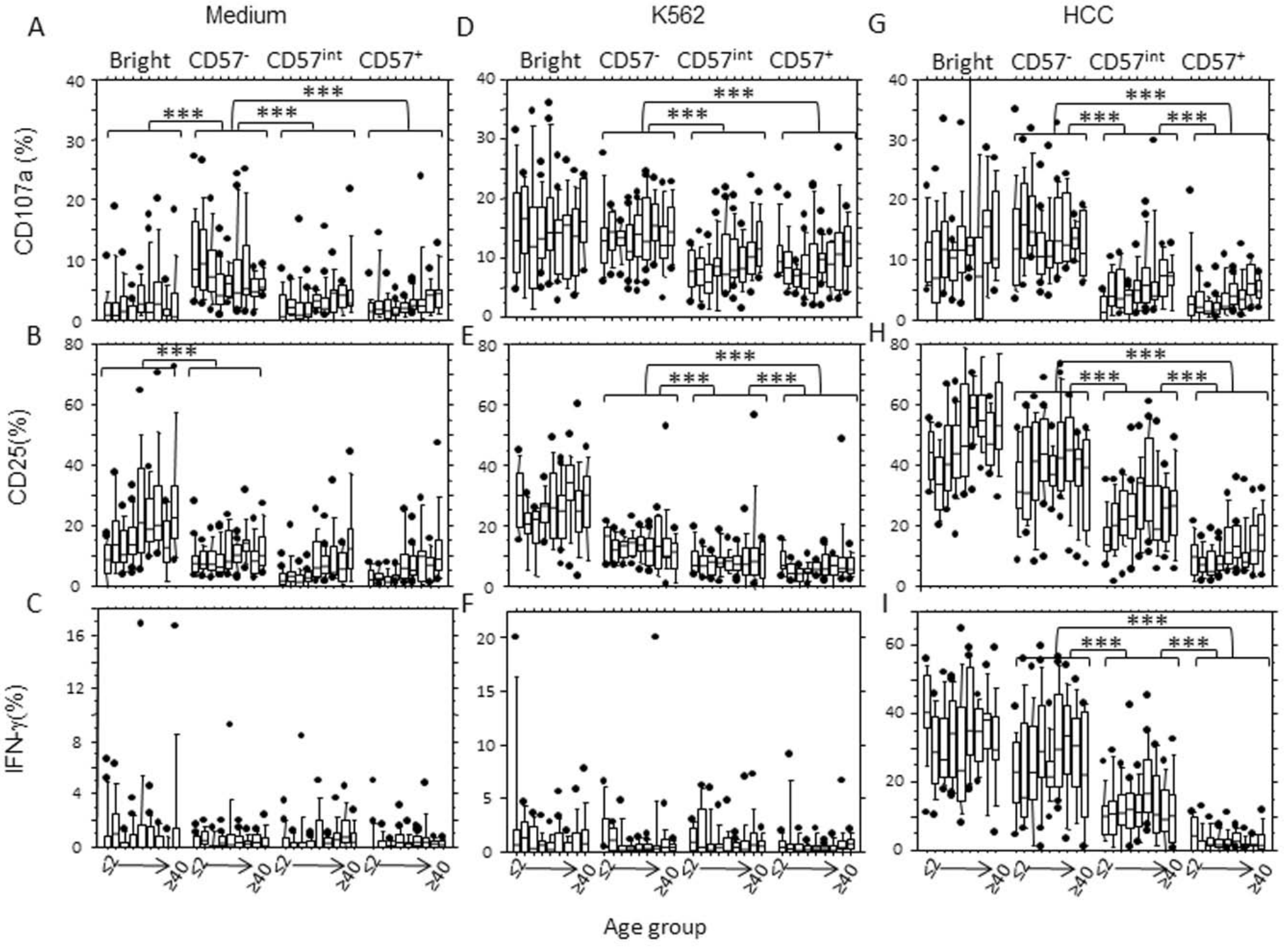
Table 1. Cohort characteristics. [1]Significantly higher anti-HCMV IgG titres compared to 16-19 year olds and all groups over 26 years old, $p<0.05$, ANOVA.[2]Significantly higher anti-HCMV IgG titres compared to all groups over 16 years old, $p< 0.01$, ANOVA.[3]Significantly elevated anti-EBNA IgG titres compared to all groups over 16 years old. [4]Significantly elevated anti-EBNA IgG titres compared to all groups over 20 years old. [5]NKG2C genotypes were obtained from a total of 181 individuals.


A
NK
CD56bright
CD56dim
CD56
CD3
B
NK Cells
% of lymphocytes
***
C
CD56bright
% of NK cells
***
D
CD56dim
***
≤2
3-5
6-9
10-12
13-15
16-19
20-25
26-39
≥40
Age group


A
CD56bright
CD56
CD56dim
CD57-
CD56dim
CD57int
CD56dim
CD57+
CD57
B
CD57-
CD57int
CD57+
***
***
% of CD56dim NK cells
≤2
≥40
C
CD56
CD94
70.9%
CD94
D
CD94+
% of CD56dim NK cells
E
32.8
2.9
53.8
NKG2A
NKG2C
F
NKG2A+
NKG2C+
*
*
% of CD56dimCD94+ NK cells
Age group (years)


CD57-
CD57int
CD57+
A
% of NKG2A+ NK cells
100
80
60
40
20
0
***
***
***
B
% of NKG2C+ NK cells
**
***
≤2
≥40
Age group (years)
C
NKG2A+
NKG2C+
CD57 (MFI)
25000
20000
15000
10000
5000
0
**
≤2
≥40
Age group (years)


A
1 year old donor
B
22 year old donor
Medium
HCC
CD56
4.5
5.0
0.9
18.1
36.3
41.1
4.9
3.4
1.5
8.4
47.2
12.2
CD107a
CD25
IFN-γ
CD107a
CD25
IFN-γ
C
Medium
D
K562
E
HCC
CD107a (%)
F
G
H
CD25 (%)
I
J
K
IFN-γ (%)
***
**
***
≤2
3-5
6-9
10-12
13-15
16-19
20-25
26-39
≥40
Age group (years)


A
Medium
D
K562
G
HCC
Bright
CD57-
CD57int
CD57+
CD107a (%)
B
CD25(%)
E
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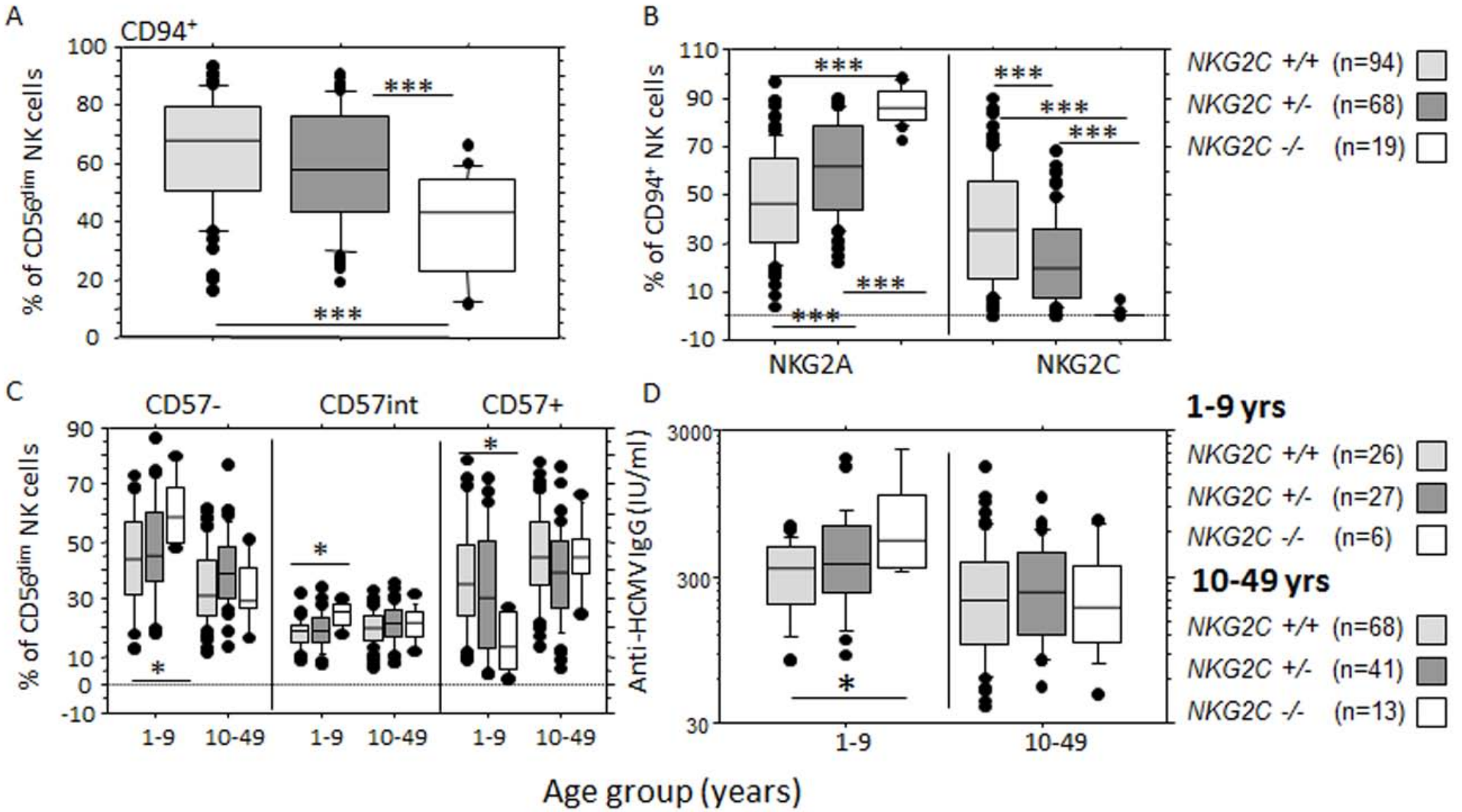
C
IFN-γ(%)
F
I
***
≤40
≥40
Age group


A
CD94+
% of CD56dim NK cells
100
80
60
40
20
0
***
***
B
% of CD94+ NK cells
110
90
70
50
30
10
-10
***
***
***
***
***
***
NKG2A
NKG2C
NKG2C +/+ (n=94)
NKG2C +/- (n=68)
NKG2C -/- (n=19)
C
CD57-
CD57int
CD57+
% of CD56dim NK cells
90
70
50
30
10
0
-10
*
*
*
*
1-9
10-49
1-9
10-49
1-9
10-49
D
Anti-HCMV IgG (IU/ml)
3000
300
30
*
1-9
10-49
1-9 yrs
NKG2C +/+ (n=26)
NKG2C +/- (n=27)
NKG2C -/- (n=6)
10-49 yrs
NKG2C +/+ (n=68)
NKG2C +/- (n=41)
NKG2C -/- (n=13)
Age group (years)

Supplementary Methods

Flow Cytometric analysis. For *ex vivo* analysis cells were incubated with the following monoclonal antibodies: NK cell panel: anti-CD3-V500, anti-CD56-PeCy7 and anti-CD94-FITC (all from BD Biosciences), anti-NKG2C-PE and anti-NKG2A-APC (both from R&D systems), anti-CD8-PeCy7, anti-CD57-e450 and anti-CD16-APC-e780 (all from eBioscience, Oxford, UK); T cell panel: anti-CD3-V500, anti-CD4-PE and anti-CD45RA-APC-H7 (BD Biosciences), anti-CD8-PeCy7, anti-CD27-FITC, anti-CD28-PeCy7 and anti-CD57-e450 (eBioscience) and anti-CCR7-APC (R&D systems); Lineage panel: anti-CD3-V500, anti-CD56-PeCy7 and anti-CD40-APC (BD Biosciences), anti-CD45-FITC, anti-CD11c-PE, anti-CD19-PeCy5, anti-CD123-e450 and anti-CD14-APCe780 (eBioscience). Phenotypic analysis of *in vitro* stimulated cells was performed with the following monoclonal antibodies: anti-CD3-V500, anti-CD56-PeCy7, anti-CD107a-FITC (all from BD Biosciences), anti-CD57-e450, anti-CD16-APC and anti-CD25-PE (all from eBioscience). After staining for cell surface markers, cells were fixed and permeablised (Cytofix-Cytoperm, BD Biosciences) and washed in Perm-wash buffer (BD Biosciences) and either measured directly or stained with anti-IFN-γ-APC-efluor780-conjugated antibody (eBioscience).

NKG2C deletion PCR conditions (see also reference 27, main text)**.** The primers for detection of wild type NKG2C genotypes were NKG2C200_F (5'-AGTGTGGATCTTCAATGATA-3') and NKG2C200_R (5'-TTTAGTAATTGTGTGCATCCT-3'), yielding a 200bp product. The primers for detecting the NKG2C deletion were BREAK411_F (5'ACTCGGATTTCTATTTGATGC3') and BREAK411_R (5'ACAAGTGATGTATAAGAAAAAG3'), yielding a 411bp product. PCR was performed with initial denaturation at 95°C for 3 min, followed by 10 cycles of denaturation at 94°C for 30s, annealing for 30s from 65°C to 55°C (65°C on the first cycle and minus one degree per cycle) and extension at 72°C for 30s, followed by 26 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s. PCR products were separated and identified using agarose gel electrophoresis.

Age group	$CD3^{-}CD56^{+}$	$CD56^{br}$ $CD16^{-}$	$CD56^{dim}CD16^{+}$	$CD94^{+}$	$NKG2A^{+}$	$NKG2C^{+}$	$CD57^{+}$
≤2	359 (54-1587)[1]	17 (5-89)	343 (117-1614)	291 (46-1463)	217 (44-777)	40 (0-905)	97 (4-1226)
3-5	188 (80-839)	14 (4-41)	253 (84-924)	158 (41-752)	122 (51-487)	40 (0-351)	69 (19-588)
6-9	96 (24-669)	8 (3-15)	150 (32-327)	81 (14-245)	44 (13-286)	41 (0-239)	36 (5-495)
10-12	154 (40-656)	6.5 (1.7-24)	163 (51-818)	110 (24-571)	59 (22-235)	27 (0-271)	64 (14-393)
13-15	227 (65-584)	9 (3-45)	280 (90-759)	129 (54-403)	59 (20-274)	35 (0-344)	80 (20-349)
16-19	150 (28-774)	6 (3-22)	193 (50-924)	121 (17-671)	43 (20-327)	39 (0-343)	68 (8-529)
20-25	179 (53-364)	6 (2-18)	201 (28-374)	143 (23-323	52 (26-185)	35 (0-259)	89 (9-288)
26-39	110 (20-161)	6 (1-17)	177 (65-544)	89 (10-213)	44 (17-132)	12 (0-82)	60 (16-126)
40-49	115 (24-249)	5 (1-12)	168 (36-408)	99 (20-217)	43 (13-134)	36 (0-128)	73 (13-189)

Table S1: Changes in absolute numbers of NK cell subsets in different age groups.

[1] Cells/µl blood, median (range).

Age group	$CD45^+$ Leukocytes	$CD3^+$ T cells	$CD19^+$ B cells	$CD3^-CD56^+$ NK cells	$CD14^+$ Monocytes	Lin-$CD11c^+$ $CD123^-$ mDC	Lin-$CD11c^-$ $CD123^+$pDC
≤2	9133 (3266-20933)[1]	5878 (2350-14737)	1705 (555-4189)	359 (54-1587)	151 (55-314)	71 (15-181)	22 (3-73)
3-5	6000 (3333-13067)	4116 (2470-9748)	1060 (436-2163)	188 (80-839)	99 (45-242)	31 (7-140)	14 (3-75)
6-9	2333 (1111-3889)	1713 (843-2901)	381 (147-723)	96 (24-669)	44 (15-89)	14 (6-36)	8 (2-19)
10-12	2400 (1533-5667)	1701 (843-2901)	437 (224-984)	154 (40-656)	35 (15-140)	22 (8-62)	11 (3-52)
13-15	2900 (1900-5250)	2248 (1440-4058)	424 (224-1209)	162 (67-497)	59 (18-222)	17 (10-74)	14 (1-79)
16-19	2342 (1500-3600)	1751 (987-2432)	341 (145-751)	143 (28-774)	47 (20-114)	21 (3-42)	11 (3-42)
20-25	2074 (1167-3852)	1609 (644-2827)	254 (99-597)	179 (53-354)	43 (14-214)	22 (4-39)	7 (1-39)
26-39	1567 (1000-3833)	1221 (719-3350)	221 (79-412)	110 (30-161)	42 (9-147)	15 (9-37)	5 (1-21)
40-49	1778 (778-2778)	1312 (538-2036)	229 (74-627)	115 (24-249)	48 (15-155)	17 (8-40)	5 (1-18)

Table S2. Absolute numbers of lymphoid and myeloid lineage cells in different age groups.

[1]Cells/µl blood, median (range).

Age group	$CD3^+CD4^+$ T cells	$CD4^+$ Naive	$CD4^+$ TCM	$CD4^+$ TEM	$CD4^+$ TEMRA	$CD4^+$ $CD28^-$ $CD57^-$
≤2	3381 (1437-9315)[1]	1923 (963-7201)	197 (37-369)	682 (206-1673)	177 (78-535)	921 (343-3270)
3-5	2461 (1187-4508)	1646 (708-2692)	104 (50-426)	493 (296-2317)	88 (56-188)	497 (195-1871)
6-9	1056 (469-1727)	620 (283-1150)	58 (18-152)	263 (98-719)	32 (11-162)	285 (89-527)
10-12	1012 (583-2144)	564 (291-1181)	60 (17-194)	344 (162-1000)	43 (8-106)	230 (26-925)
13-15	1140 (748-2010)	622 (255-931)	58 (14-240)	441 (216-955)	45 (17-82)	356 (69-1154)
16-19	1064 (608-1638)	557 (235-1172)	52 (16-168)	379 (109-523)	29 (9-155)	290 (31-415)
20-25	946 (394-1695)	417 (186-827)	46 (20-173)	360 (90-693)	39 (8-73)	287 (109-591)
26-39	732 (425-1760)	322 (76-931)	49 (28-160)	302 (160-752)	25 (10-86)	241 (98-954)
40-49	702 (333-1478)	250 (55-881)	52 (17-196)	335 (113-718)	25 (9-81)	198 (83-528)

Table S3A. Change in $CD4^+$T cell memory and senescence with age.

[1] Cells/µl blood, median (range).

Age group	$CD3^+CD8^+$ T cells	$CD8^+$ Naive	$CD8^+$ TCM	$CD8^+$ TEM	$CD8^+$ TEMRA	$CD8^+$ $CD28^-$ $CD57^-$
≤2	1730 (494-3917) [1]	1245 (270-2890)	30 (6-394)	389 (89-1135)	167 (31-938)	408 (146-1747)
3-5	977 (582-3659)	746 (353-2023)	24 (13-203)	217 (150-1881)	84 (25-498)	245 (86-1518)
6-9	474 (242-1085)	306 (176-640)	14 (7-183)	134 (41-338)	43 (5-127)	115 (25-449)
10-12	466 (230-1467)	311 (144-880)	18 (5-249)	169 (72-698)	61 (6-186)	124 (8-646)
13-15	553 (210-1969)	349 (132-1234)	23 (3-142)	231 (42-776)	74 (6-324)	172 (16-1164)
16-19	448 (176-806)	314 (110-594)	19 (3-118)	171 (29-260)	46 (4-123)	101 (8-230)
20-25	399 (102-1132)	259 (77-706)	16 (2-132)	170 (23-413)	44 (5-275)	112 (28-648)
26-39	313 (109-1192)	215 (41-705)	12 (4-66)	131 (76-519)	30 (5-211)	102 (34-664)
40-49	338 (118-575)	183 (52-365)	15 (5-60)	143 (47-373)	24 (1-137)	82 (29-326)

Table S3B. Change in $CD8^+$ T-cell memory and senescence with age.

[1] Cells/µl blood, median (range).

Supplementary figure legends

Figure S1. Changes in NK cell and NK cell subset distribution with age across the entire cohort. Linear regression models were fitted as a natural cubic spline to the data in which age was included. **A-C.** Percentages of NK cells and $CD56^{bright}$ and $CD56^{dim}$ subsets corresponding to figure 1(and as gated in figure 1A). **B-F.** Absolute numbers of $CD3^{-}CD56^{+}$ and $CD56^{bright}$ and $CD56^{dim}$ subsets. R^2 and p values (F-test) are shown.

Figure S2. Changes in T cell maturation with age. **A.** T cell differentiation status was assessed after gating on CD4 or CD8 and subsequently on naïve (TN), central memory (TCM), effector memory (TEM) and terminally differentiated effector memory (TEMRA) using a combination of CD45RA and CCR7. Frequencies of naïve and memory subsets are shown in age stratified groups for $CD4^{+}$ (**B-E**) and $CD8^{+}$ T cells (**F-I**). Horizontal bars represent median values, boxes extend from the 25^{th} to the 75^{th} percentile and whiskers represent the 95^{th} percentiles. Asterisks represent significant trends across the entire cohort (* $p<0.05$, *** $p<0.001$, F-test).

Figure S3. Changes in NK cell maturation and c-type lectin-like receptor defined NK cell subsets with age across the entire cohort. A-C. Models of changes in frequencies of CD57-defined subsets corresponding to data shown in figure 2 (and as gated in figure 2A). **D-F.** Changes in the frequencies of CD94, NKG2A and NKG2C expressing NK cells, (as gated in figure 2C and E). **G,H**. Changes in MFI of CD57 on $NKG2A^{+}$ and $NKG2C^{+}$ NK cells (corresponding to data shown in figure 2 F,G). R^2 and p values (F-test) are shown.

Figure S4. Frequency of mature CD57+ cells is inversely correlated with HCMV IgG titre. A-C. A Positive correlation of HCMV IgG tite is observed with the % of CD57- NK cells **(A)** and a negative correlation with the % of CD57+ NK cells **(C)** whereas no significant effect is observed for the CD57intermediate subset **(B)**. R^2 and p values (F-test) are shown.

Figure S5. Limited impact of EBV serostatus on NK cell subsets. The frequencies of total NK cells (**A**) CD56bright (**B**) CD56dim (**C**) CD94 (**D**) NKG2A (**E**) NKG2C (**F**) and the Mean Fluorescence Intensity MFI) for CD57 (**G**) and frequencies of CD57 defined subsets (**H**) were compered in EBNA seronegative and seropositive individuals. *p<0.01.

Figure S6. Changes in IL-12 + IL-18 induced NK cell function with age across the entire cohort. A. CD107a, **B.** CD25 and **C.** IFN-γ expression in gated $CD3^{-}CD56^{+}$ NK cells. (Models correspond to data shown in figure 5 E,H,K). R^2 and p values (F-test) are shown.

Figure S7. NKG2C genotype affects frequencies and absolute numbers of NK, $CD56^{bright}$ and $CD56^{dim}$ cells. Frequencies and absolute numbers of NK cells across the entire cohort **(A, B)** and (**C,D)** in groups stratified according to age (<10 and 10-49 years old). Frequencies and (**F, H)** absolute numbers of $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Figure S8. Impact of NKG2C genotype on absolute numbers of NK cell subsets. A. $CD94^{+}$ NK cells, **B.** $NKG2A^{+}$ or $NKG2C^{+}$ NK cells and **C.** CD57 defined NK cell subsets are shown for all study subjects. **D-F.** Effect of NKG2C genotype on study subjects grouped according to ages <10 or 10-49 years old; **D.** $CD94^{+}$ NK cells, **E.** $NKG2A^{+}$ or $NKG2C^{+}$ NK cells or **F.** CD57-defined subsets. * p<0.05.

Figure S9. Expression levels of NKG2C in *$NKG2C^{+/-}$* and +/+ individuals. The mean fluorescence intensity is shown for NKG2C in **(A)** all +/+ and +/- study subjects and **(B)** in individuals grouped into age strata (<10 and 10-49 years old).

Figure S10. NKG2C genotype does not affect antibody titres to vaccine antigens. No significant impact of genotype is observed on titres of antibody against Tetanus Toxoid **(A)** or Hepatitis B surface antigen **(B)** and Epstein-Barr Virus nuclear antigen **(C)** in children (<10 years old) or in adults (10-49 years old).

Figure S11. Elevated frequencies of c-type lectin-like receptor positive NK cell subsets and reduced NK cell functional capacity in Gambian compared to UK Adults. The frequencies of $CD57^+$ (**A**), $NKG2A^+$ (**B**) and $NKG2C^+$ (**C**) within CD56dim NK cells are compared Gambian adults (n=65) and HCMV seronegative (n=78) and seropositive UK adults (n=43) aged between 20 and 49 years. Frequencies of cells expressing CD107a (**D**), CD25 (**E**) and IFN-γ (**F**) were determined within total NK cells. ** $p<0.01$, *** $p<0.001$, $p<0.0001$.

Figure S1

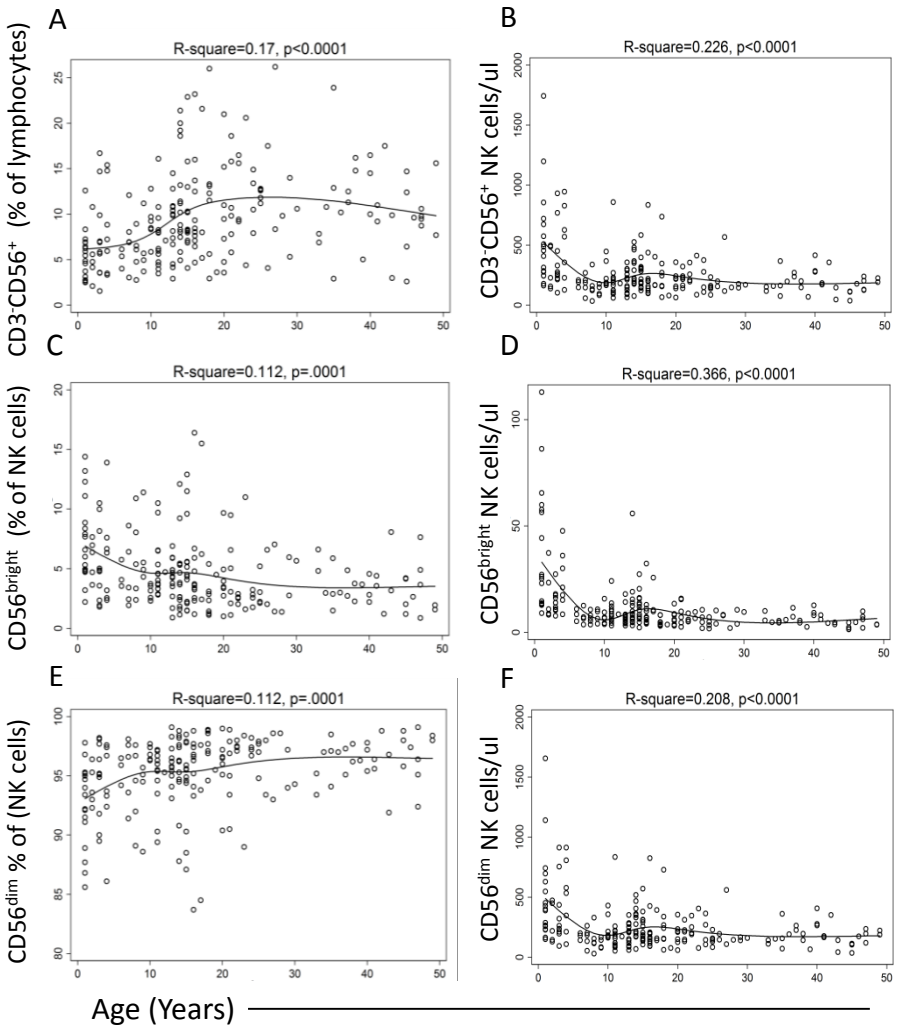

A
R-square=0.17, p<0.0001
CD3-CD56+ (% of lymphocytes)
B
R-square=0.226, p<0.0001
CD3-CD56+ NK cells/ul
C
R-square=0.112, p=.0001
CD56bright (% of NK cells)
D
R-square=0.366, p<0.0001
CD56bright NK cells/ul
E
R-square=0.112, p=.0001
CD56dim % of (NK cells)
F
R-square=0.208, p<0.0001
CD56dim NK cells/ul
Age (Years)

Figure S2

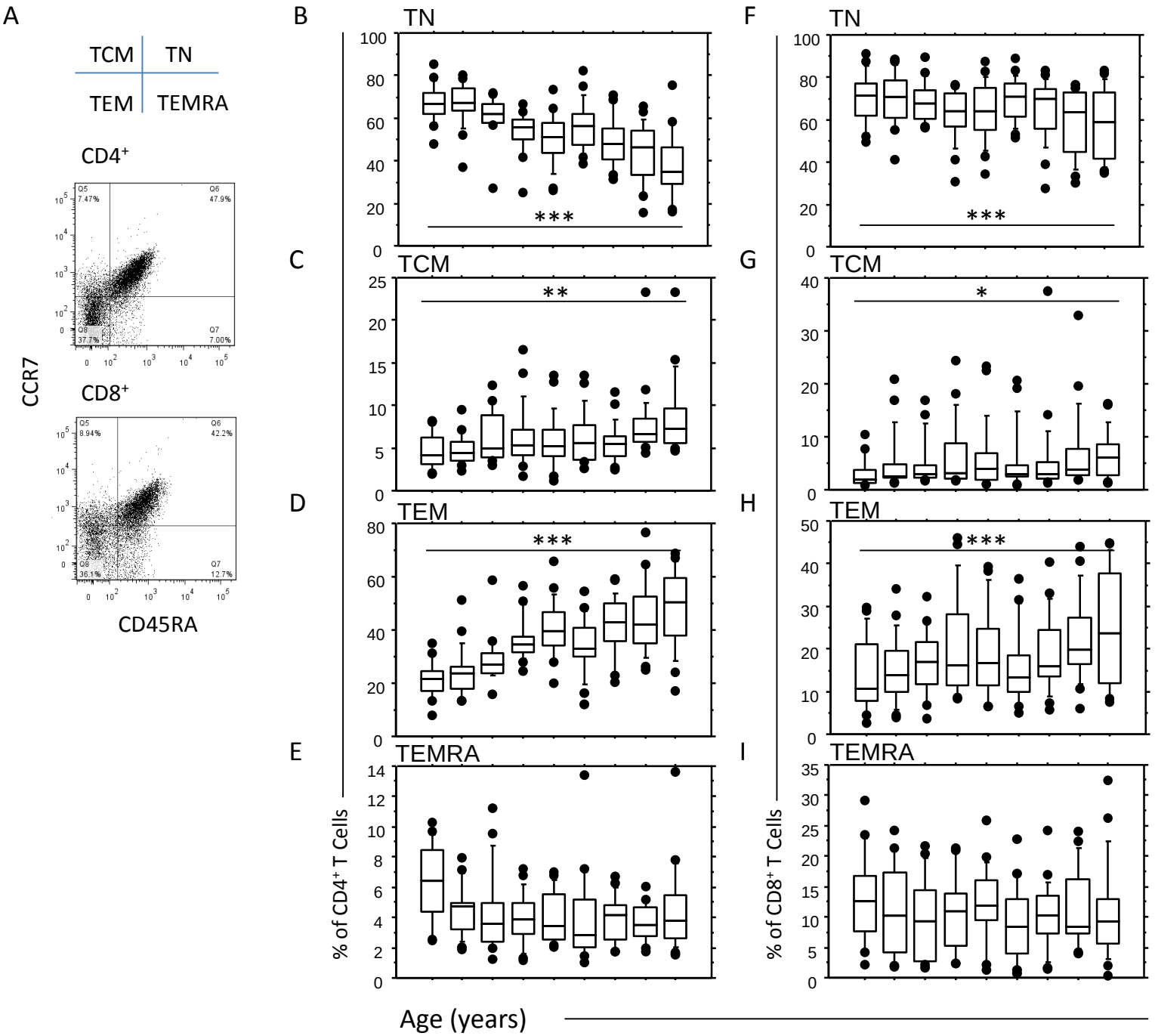

A
TCM
TN
TEM
TEMRA
CD4+
CD8+
CCR7
CD45RA
B
TN
C
TCM
D
TEM
E
TEMRA
F
TN
G
TCM
H
TEM
I
TEMRA
% of CD4+ T Cells
% of CD8+ T Cells
Age (years)

Figure S3

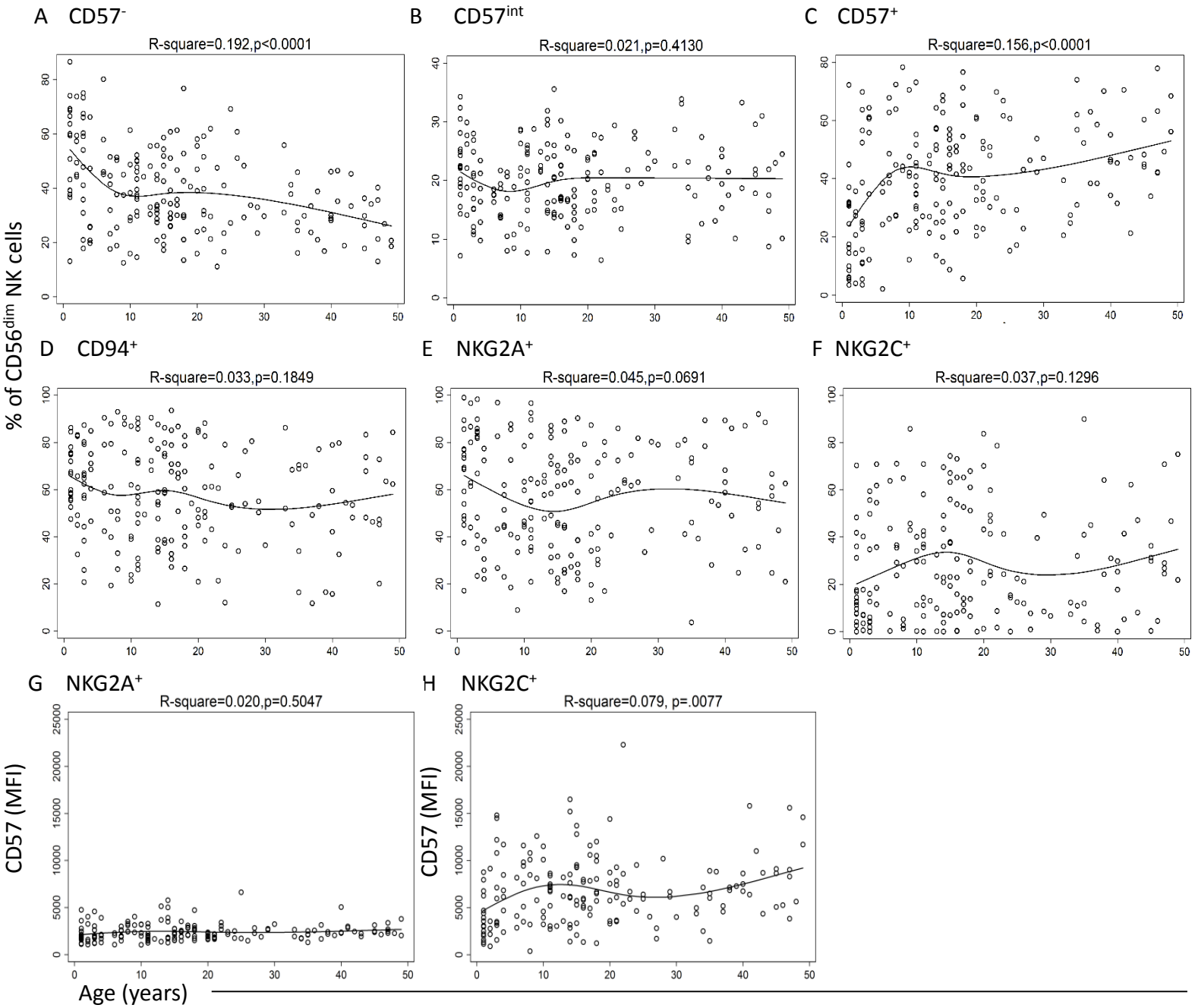

A CD57-
R-square=0.192,p<0.0001
B CD57int
R-square=0.021,p=0.4130
C CD57+
R-square=0.156,p<0.0001
% of CD56dim NK cells
D CD94+
R-square=0.033,p=0.1849
E NKG2A+
R-square=0.045,p=0.0691
F NKG2C+
R-square=0.037,p=0.1296
G NKG2A+
R-square=0.020,p=0.5047
H NKG2C+
R-square=0.079, p=.0077
CD57 (MFI)
CD57 (MFI)
Age (years)

Figure S4

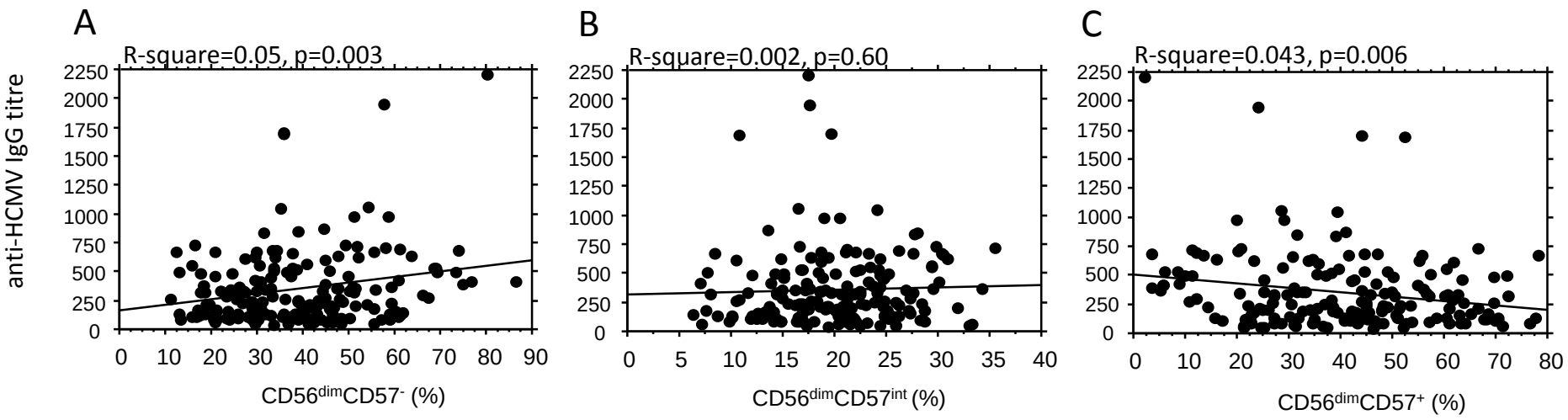

A
R-square=0.05, p=0.003
B
R-square=0.002, p=0.60
C
R-square=0.043, p=0.006
anti-HCMV IgG titre
CD56dimCD57- (%)
CD56dimCD57int (%)
CD56dimCD57+ (%)

Figure S5

Impact of EBV serostatus on NK cell phenotypic markers

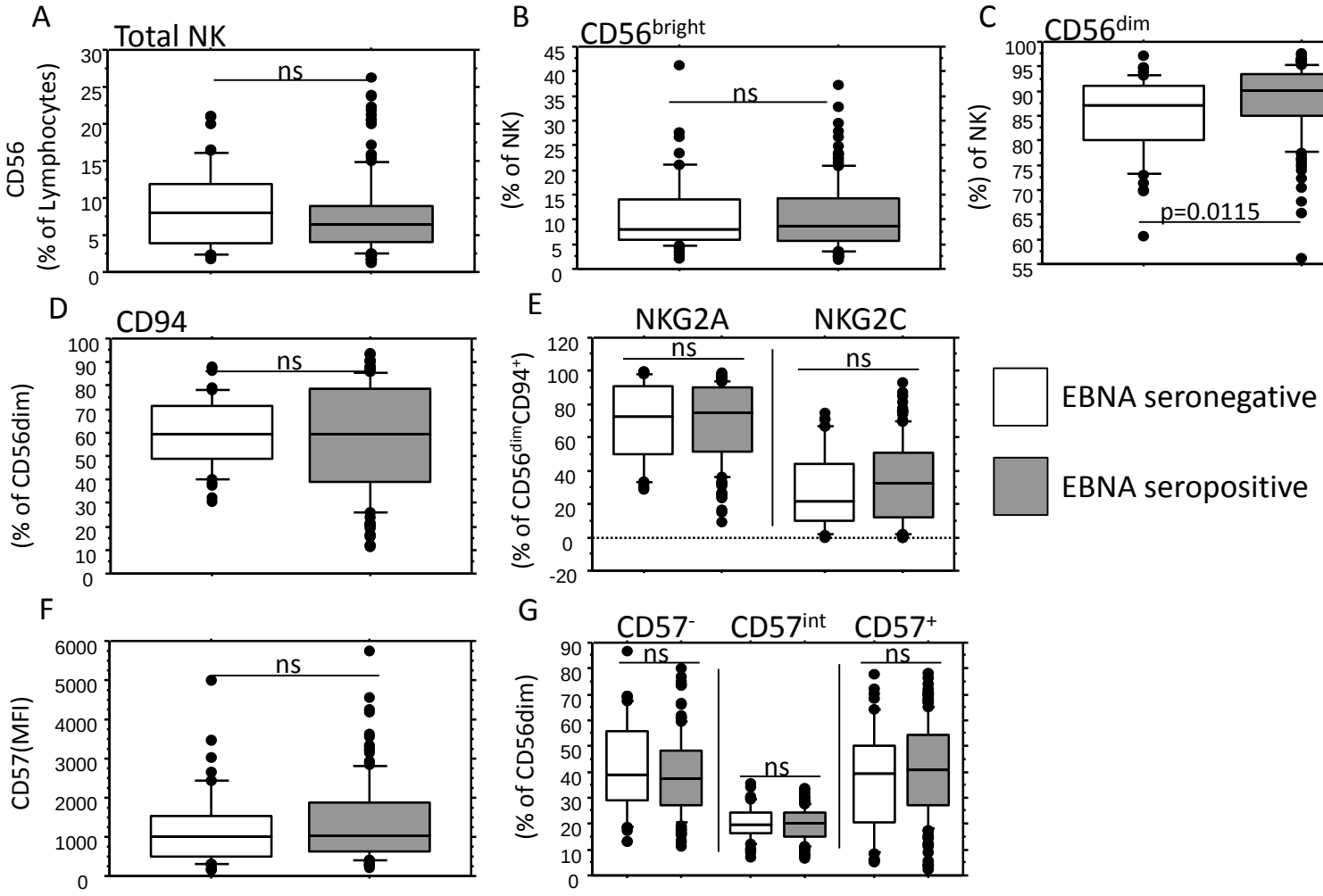


Figure S6

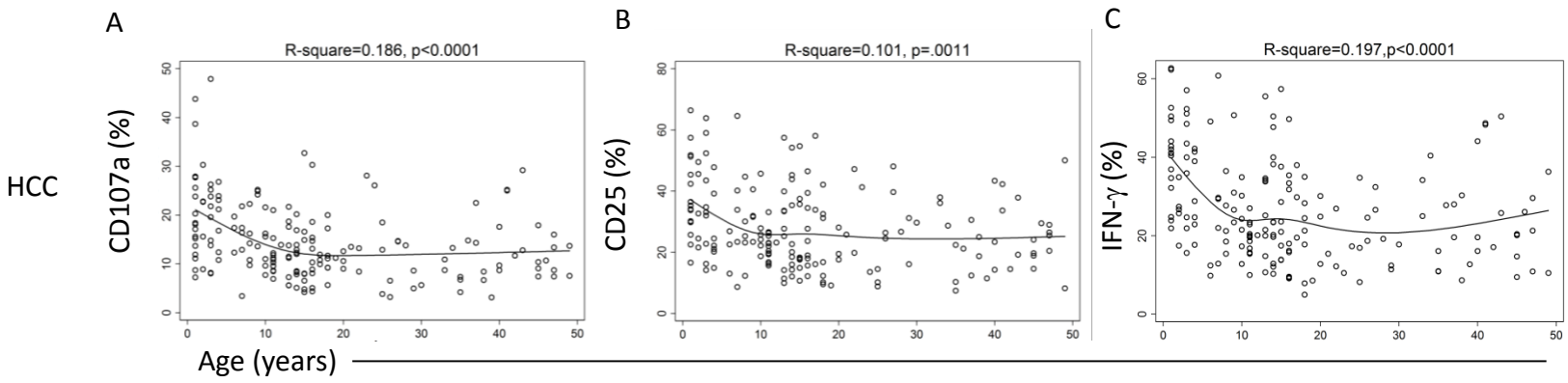

HCC
A
R-square=0.186, p<0.0001
CD107a (%)
B
R-square=0.101, p=.0011
CD25 (%)
C
R-square=0.197,p<0.0001
IFN-γ (%)
Age (years)

Figure S7

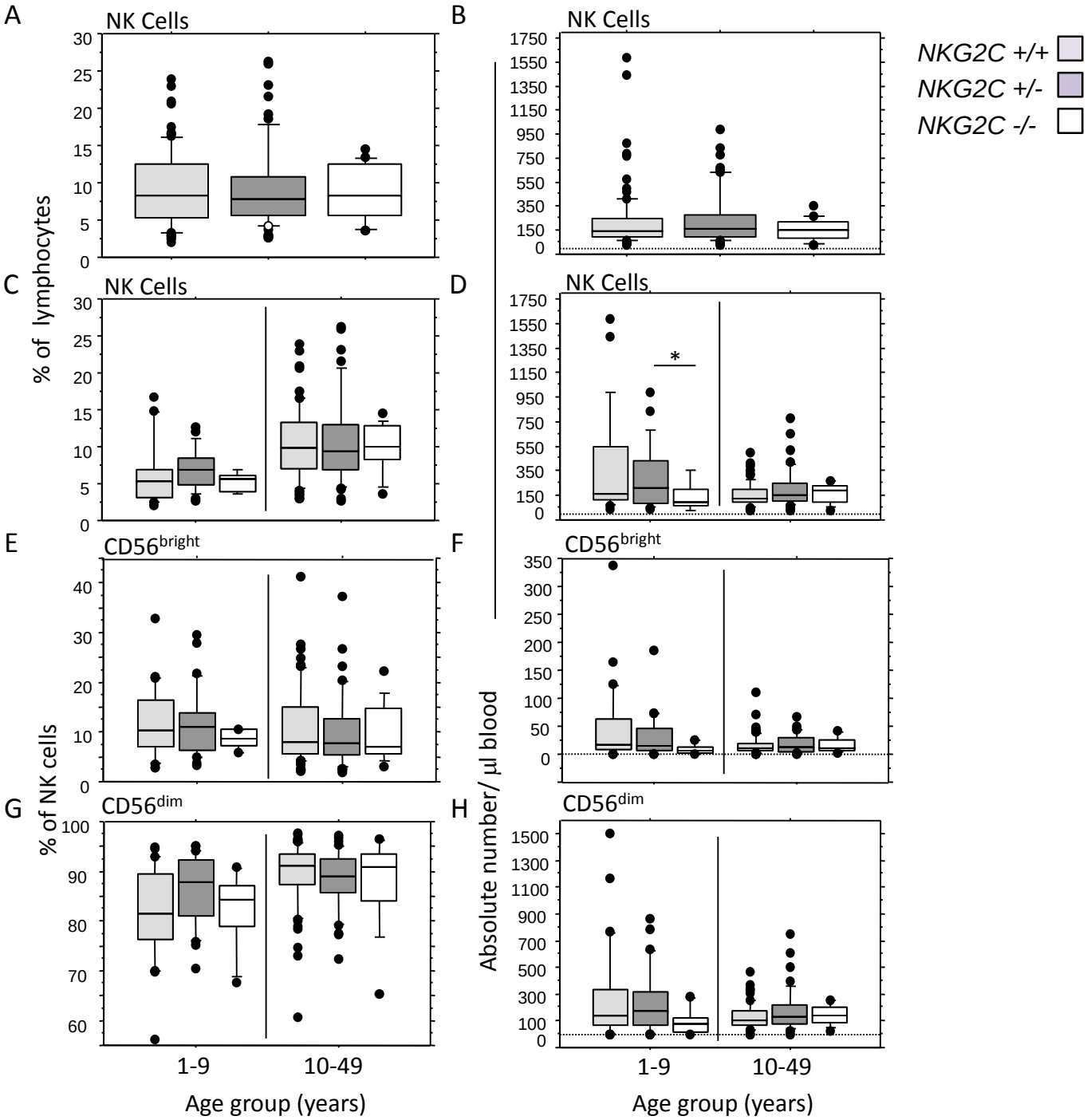

A
NK Cells
B
NK Cells
C
NK Cells
D
NK Cells
*
E
CD56bright
F
CD56bright
G
CD56dim
H
CD56dim
% of lymphocytes
% of NK cells
Absolute number/ µl blood
1-9
10-49
Age group (years)
NKG2C +/+
NKG2C +/-
NKG2C -/-

Figure S8

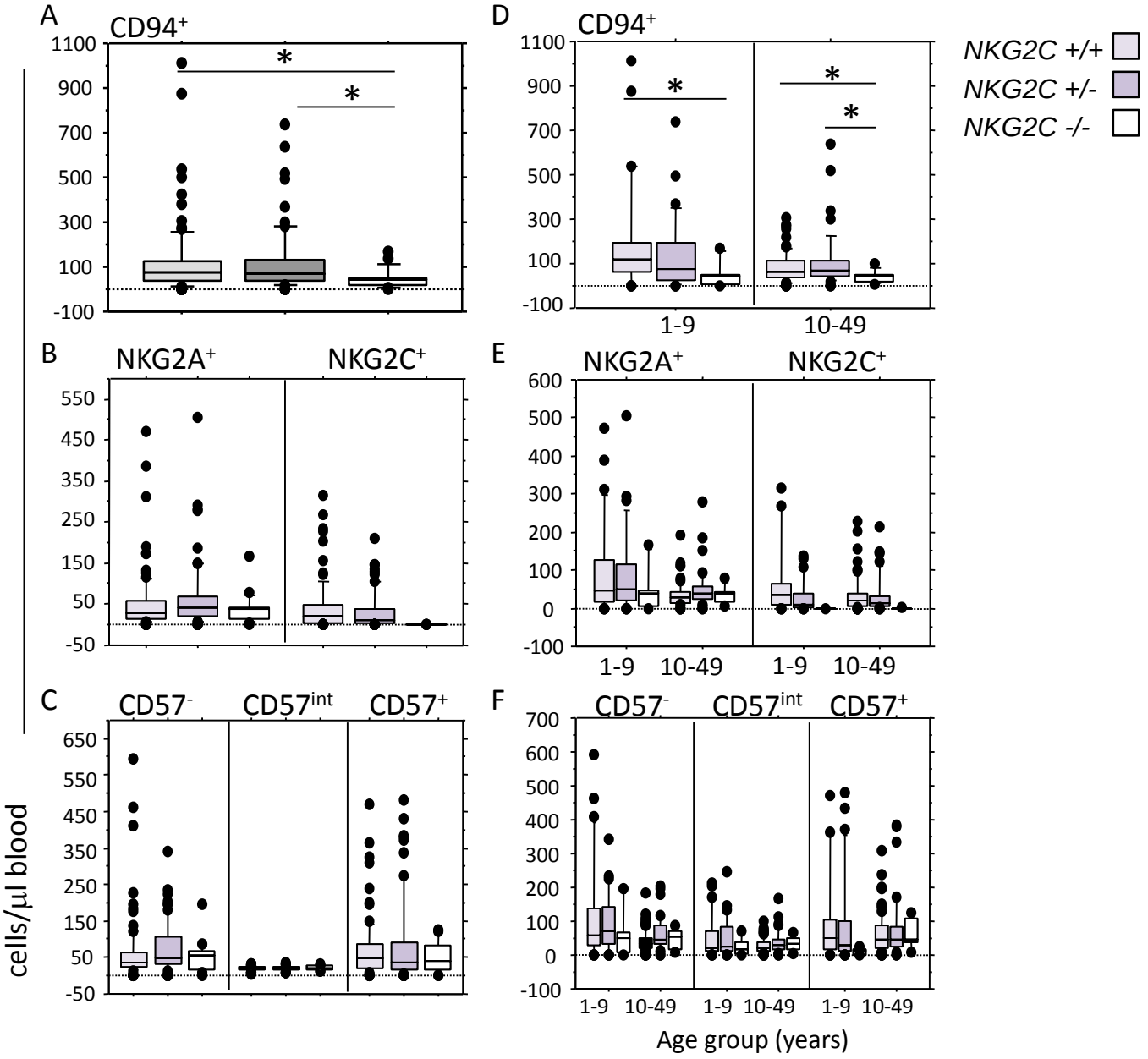

A
CD94+
1100
900
700
500
300
100
-100
*
*
B
NKG2A+
NKG2C+
550
450
350
250
150
50
-50
C
CD57-
CD57int
CD57+
650
550
450
350
250
150
50
-50
cells/µl blood
D
CD94+
1100
900
700
500
300
100
-100
*
*
*
*
1-9
10-49
E
NKG2A+
NKG2C+
600
500
400
300
200
100
0
-100
1-9
10-49
1-9
10-49
F
CD57-
CD57int
CD57+
700
600
500
400
300
200
100
0
-100
1-9
10-49
1-9
10-49
1-9
10-49
Age group (years)
NKG2C +/+
NKG2C +/-
NKG2C -/-

Figure S9

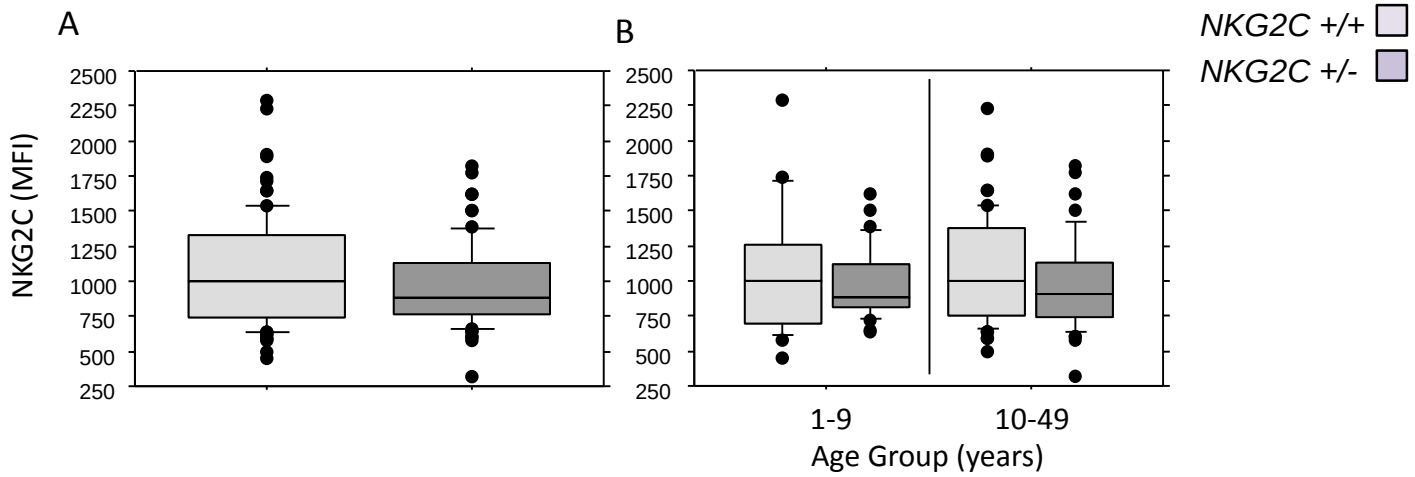

A
B
NKG2C (MFI)
2500
2250
2000
1750
1500
1250
1000
750
500
250
1-9
10-49
Age Group (years)
NKG2C +/+
NKG2C +/-

Figure S10

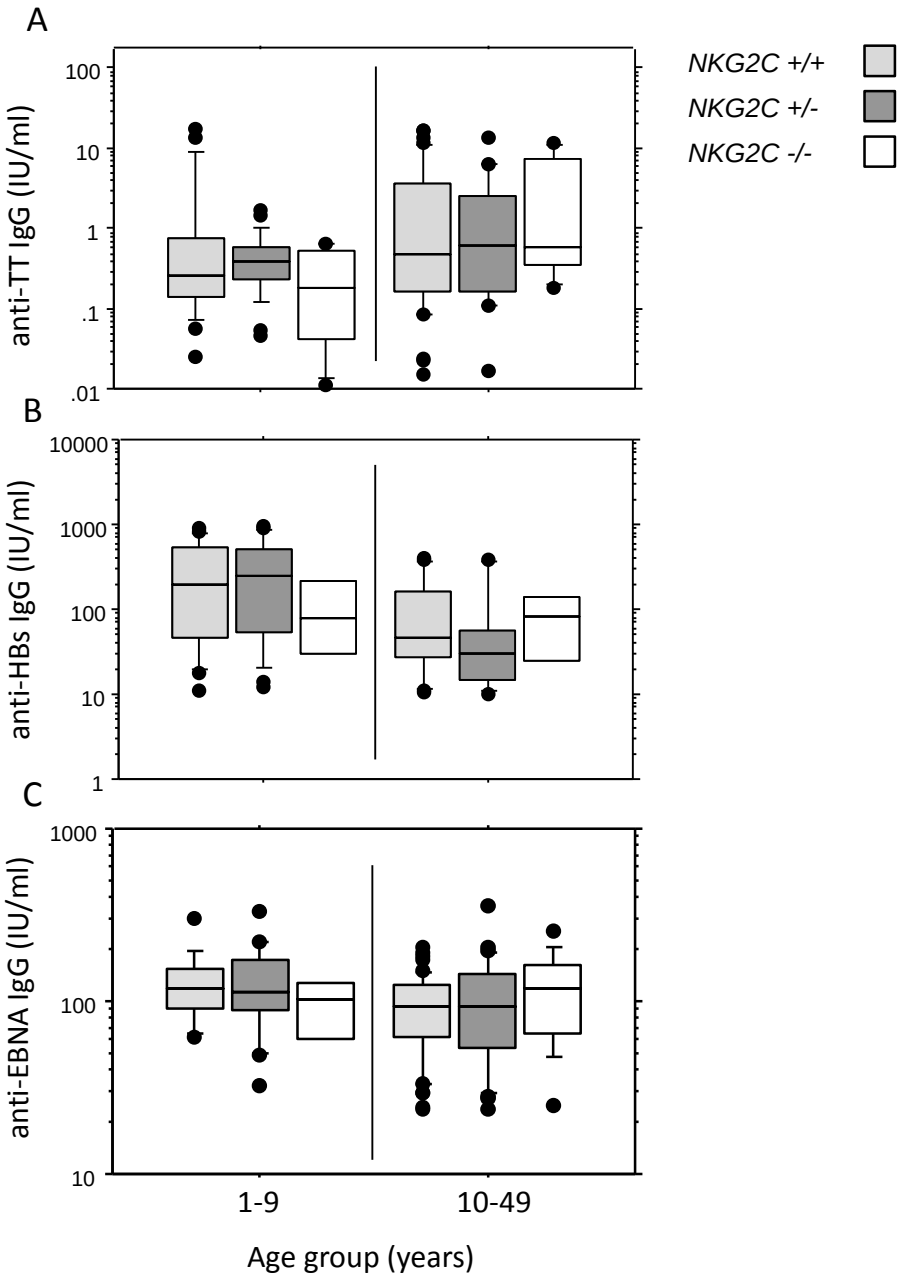
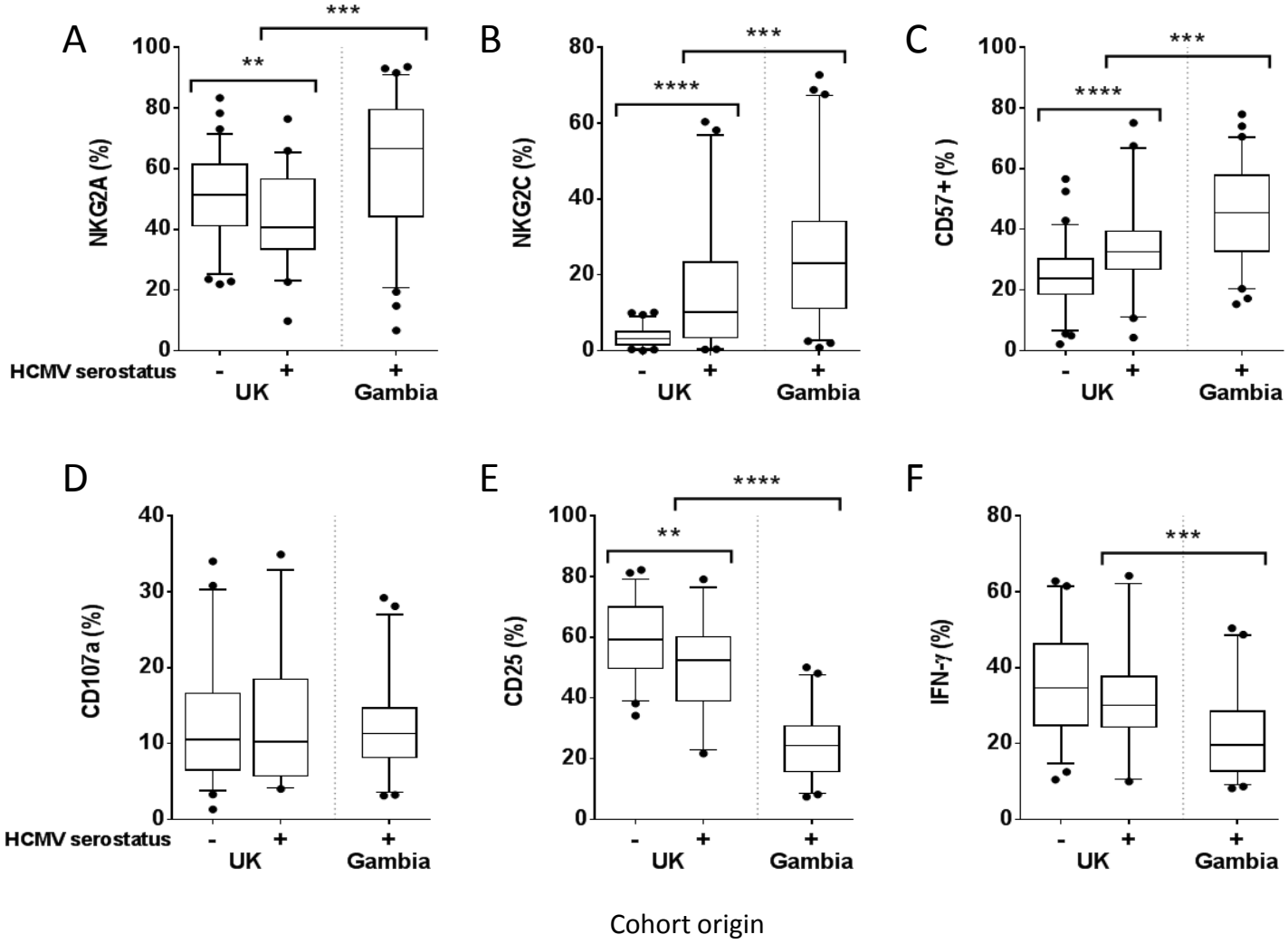

A
B
C
anti-TT IgG (IU/ml)
anti-HBs IgG (IU/ml)
anti-EBNA IgG (IU/ml)
100
10
1
.1
.01
10000
1000
100
10
1
1000
100
10
1-9
10-49
Age group (years)
NKG2C +/+
NKG2C +/-
NKG2C -/-

Figure S11


A
NKG2A (%)
100
80
60
40
20
0
**
***
HCMV serostatus
-
+
+
UK
Gambia
B
NKG2C (%)
80
60
40
20
0
****
***
C
CD57+ (%)
100
80
60
40
20
0
****
***
D
CD107a (%)
40
30
20
10
0
E
CD25 (%)
100
80
60
40
20
0
**
****
F
IFN-γ (%)
80
60
40
20
0
***
Cohort origin