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g-NK cells from umbilical cord blood are phenotypically and functionally different than g-NK cells from peripheral blood

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ABSTRACT

FcRγ-deficient natural killer (NK) cells, designated as g-NK cells, exhibit enhanced antibody-dependent cellular cytotoxicity (ADCC) capacity and increased IFN-γ and TNF-α production, rendering them promising for antiviral and antitumor responses. g-NK cells from peripheral blood (PB) are often associated with prior human cytomegalovirus (HCMV) infection. However, the prevalence, phenotype, and function of g-NK cells in umbilical cord blood (UCB-g-NK) remain unclear. Here, we demonstrate significant phenotypical differences between UCB-g-NK and PB-g-NK cells. Unlike PB-g-NK cells, UCB-g-NK cells did not show heightened cytokine production upon CD16 engagement, in contrast to the conventional NK (c-NK) cell counterparts. Interestingly, following *in vitro* activation, UCB-g-NK cells also exhibited elevated levels of IFN-γ production, particularly when co-cultured with HCMV and plasma from g-NK$^+$ adults. Furthermore, g-NK$^+$ plasma from PB even facilitated the *in vitro* expansion of UCB-g-NK cells. These findings underscore the phenotypic and functional heterogeneity of g-NK cells based on their origin and demonstrate that components within g-NK$^+$ plasma may directly contribute to the acquisition of an adult phenotype by the "immature" UCB-g-NK cells.

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ADCC; g-NK$^+$ plasma; PB-g-NK; UCB-g-NK

1. Introduction

Natural killer (NK) cells, as cytotoxic innate lymphocytes, rapidly respond to malignancy or viral infection without prior antigen (Ag) sensitization.[1] Congenital NK cell deficiency can lead to complex infections, including difficulties in controlling herpesviruses like human cytomegalovirus (HCMV).[2] Adoptive expanded NK (eNK) cell infusion can reduce HCMV infection incidence.[3] Despite lifelong HCMV infection, many individuals remain asymptomatic.[4] Emerging evidences challenge traditional immune memory paradigms, revealing adaptive features within innate immune cells, exemplified by adaptive NK or FcRγ-deficient NK (referred to as g-NK) cells from prolonged HCMV infection.[5]

In humans, adaptive NK cells represent a heterogeneous concept, encompassing subsets characterized by NKG2C$^+$ and g-NK, which show substantial overlap.[6] The selection of human adaptive NK clones with versatile functions could be driven by NKG2C or other unidentified receptors due to limited gene rearrangement capacity, and possibly by the formation of a convergent inflammatory memory signature and epigenetic memory repertoire.[7] These subsets comprise g-NK cells expressing higher CD57 and lower NKG2A, SYK, PLZF, DAB2, and EAT2[5,8] g-NK cells also express self-specific KIRs for education and clonal-like expansion.[9–11] Compared to conventional NK (c-NK) cells, g-NK cells demonstrate superior responsiveness to CD16 activation.[5,12] They also exhibit antibody (Ab)-dependent expansion and response to HCMV stimulation, indicating potential engagement of the CD16 pathway in HCMV recognition.[11]

g-NK cells are primarily observed in peripheral blood (PB) or liver resident NK cells,[12,13] with limited reports in umbilical cord blood (UCB). Intrauterine transmission can lead to congenital HCMV infection, with a transmission rate of 50% in women with primary HCMV infection during pregnancy.[14] Vertical transmission and HCMV seropositivity in UCB may lead to the generation of UCB-g-NK cells. Baek et al. investigated 13 UCB samples that are anti-CMV IgG$^+$/IgM$^-$, and only one sample show 33% of g-NK cells.[15] However, there remains an insufficiency of functional investigations concerning UCB-g-NK and comprehensive reports on the phenotypic and functional disparities between PB-g-NK and UCB-g-NK cells.

Here, we conducted a comparative analysis of PB-g-NK and UCB-g-NK cells, focusing on their distribution, phenotype, cytokine production ability, and expression of cytotoxic molecules. Our findings unveiled distinctive traits of UCB-g-NK cells compared to classical PB-g-NK cells, especially the pattern of CD16 pathway response. Our findings indicate that the heightened CD16 pathway response resulting from FcRγ deficiency is contingent on the origin of NK cells, and g-NK cells from different

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sources potentially exhibit unique intracellular signaling events or thresholds in response to CD16 pathway activation.

2. Materials and methods

2.1. Ethical statement

The human blood samples employed were exclusively for scientific research. Before commencing the experiments, necessary approvals were secured from the French National Ethics Committee (Approval No. BB-0033–00031 for UCB samples and Approval No. 21PLER2018–0069 for PB samples) and the Ethics Committee of the School of Basic Medical Sciences, Central South University, China (Approval No. 2020-KT61). All individuals participating in the study provided written informed consent.

2.2. Human blood samples

French UCB samples were obtained from the Biological Resource Center Collection of the University Hospital of Montpellier. PB samples were sourced from the French national blood center "EFS" and from health donors recruited at Central South University, China. Human peripheral blood mononuclear cells (PBMCs) and umbilical cord blood mononuclear cells (UCBMCs) were isolated using density gradient centrifugation with Ficoll-Paque™ (Cytiva) followed by cryopreservation in FBS (Gibco) containing 10% DMSO (Miltenyi).

2.3. Stimulation of the CD16 signaling pathway using immobilized 3G8 abs

Prior to stimulating NK cells with PMA and Ionomycin (P/I) cocktail, 3G8, and target cells, we typically allowed thawed PBMCs or UCBMCs to recover in the incubator for 48 hours. The culture medium was RPMI1640 supplemented with 10% FBS, 5% human AB serum, 100 IU/ml IL-2. As expected, we observed that NK cells downregulated the expression of CD25 after IL-2 pre-stimulation. Stimulation of the CD16 signaling pathway with immobilized 3G8 Abs followed the method outlined by Liu et al.[16]

2.4. NK cell activation by mAbs-opsonized target cells

MDA-MB-468 cells ($EGFR^{high}$) were seeded at 30,000 cells per well in a 96-well plate. The next day, culture supernatant was replaced with 100 µl of either 2 µg/ml cetuximab (CET) or SDH-CET. Fc-enhanced CET was produced by RD-Biotech (Besançon, France) via adding the following four amino acid substitutions in the upper CH2 of cetuximab: S239D/H268F/S324T/I332E. This variant exhibits an enhanced affinity for IgGs and ADCC.[17] The tumor cells were opsonized with the mAbs for 1 hour at 37°C. Following this, NK cells were stimulated for 6 hours and subsequently collected for phenotypic and functional analysis as supplementary experimental procedures.

2.5. Cell staining and flow cytometry (FCM) analysis

FCM for phenotypic characterization and cytokine secretion assays of NK cells detailed in the supplementary experimental procedures.

2.6. Statistical analysis

Statistical analysis was conducted using Excel and GraphPad Prism 8, with significance levels denoted as follows: ns ($p > 0.05$), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$). Paired or unpaired Student's t-tests and two-way ANOVA test were applied to all measurements.

3. Results

3.1. Comparison of g-NK cells in PBMCs versus UCBMCs

g-NK cells were characterized by their low FcRγ expression in the $CD56^+$ $CD3^-$compartment (Figure 1a). T cells were used as an internal control for FcRγ-negative expression. The proportion of g-NK was very variable, with different donors displaying diverse patterns (Figure 1a, upper graphs). FcRγ expression in UCB-NK cells was more homogenous than in PB-NK cells (Figure 1a, lower graphs). This posed a challenge in identifying g-NK cells in certain donors. UCB tended to contain lower percentages of g-NK cells, although more donors expressed significant levels of g-NK cells (proportion >10%) (Figure 1b,c). In the classical scenario, FcRγ expression was positively correlated with high NKG2C expression and low PLZF expression in PB-NK cells (Supplementary Figure S1a and S1b). g-NK cells from PB, but not from UCB, co-expressed NKG2C in higher proportions than c-NK cells (Figure 2a).

3.2. Phenotypic analysis of c-NK and g-NK cells: contrasting markers and variations between PB and UCB

Phenotypic analysis encompassed both c-NK and g-NK cells from both PB and UCB sources, probing into 10 distinct NK cell markers. Notably, g-NK cells from both PB and UCB exhibited lower expression levels of CD16 and SYK compared to their c-NK counterparts (Figure 2a and Supplementary Figure S2a). PB-g-NK cells displayed reduced levels of NKp30 and Siglec-7, while showing an increase in CD57 expression. In contrast, UCB-g-NK cells expressed significantly lower levels of NKp44, CD2, NKp46, CD7, and NKG2A compared to UCB-c-NK cells (Figure 2a and Supplementary Figure S2a).

Killer cell immunoglobulin-like receptors (KIRs) are essential in dictating the education, maturation, and function of NK cells.[18] Further examination revealed that UCB-NK cells exhibited decreased expression levels of KIR2DL2/L3 but elevated expression of KIR3DL1 when compared to PB-NK cells (Figure 2b and Supplementary Figure S2b). Additionally, UCB-g-NK cells demonstrated higher expression levels of KIR2DL1 than PB-g-NK cells, while the reverse pattern was observed in the c-NK subsets (Figure 2c). Overall, g-NK cells displayed distinct phenotypes between PB and UCB samples.

Classically, the phenotypic profile of adaptive NK cells encompasses various subsets, including $NKG2A^-$ $NKG2C^+$, $NKG2C^+$ $CD57^+$, $NKG2A^-$ $CD57^+$, and $FcR\gamma^-$ $NKG2C^{\pm}$

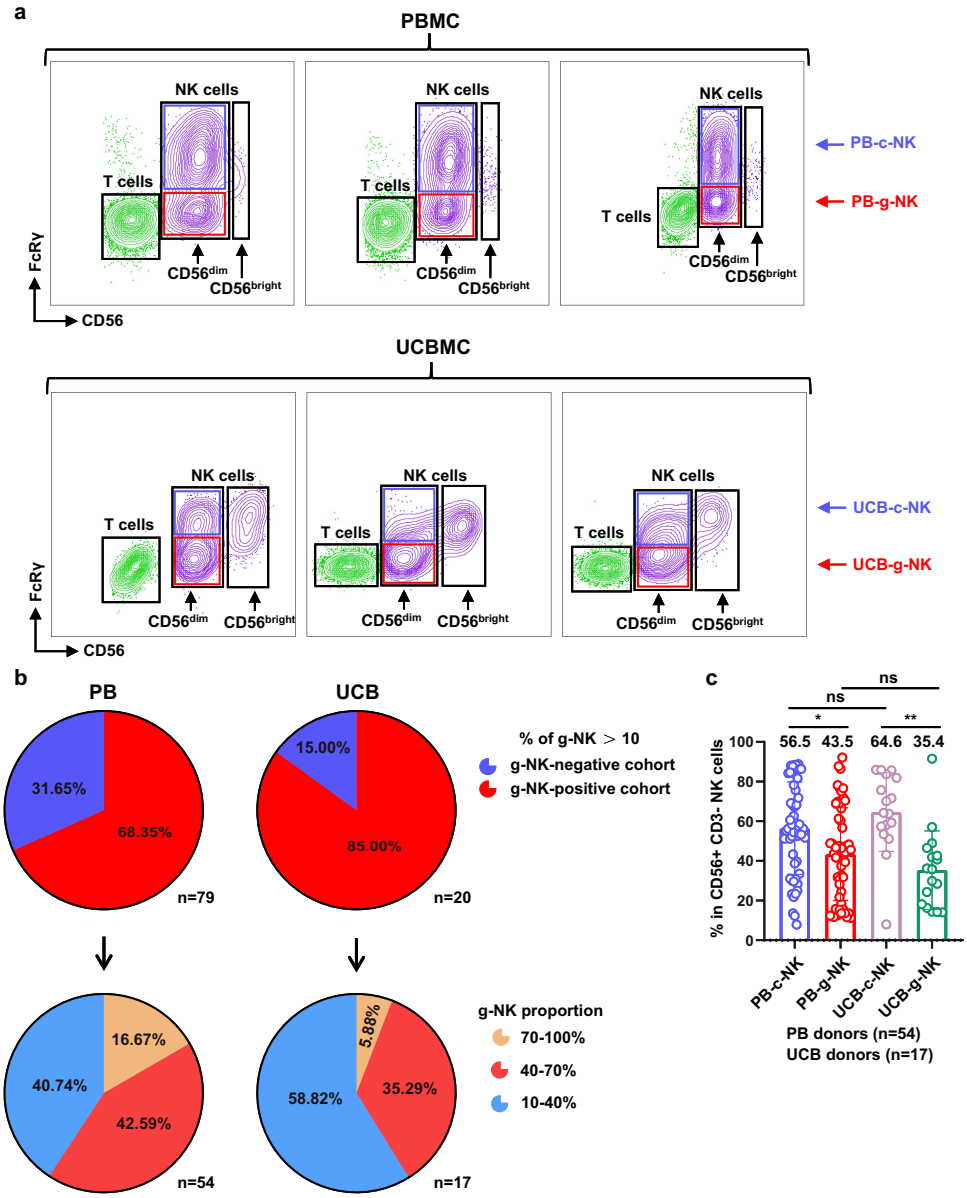

Figure 1. Comparative distribution analysis of g-NK cells in PB and UCB. (a) contour plots depicting gating strategies for c-NK and g-NK cells in PB and UCB samples from three donors each. T cells (green), lacking FcRγ expression, served as an internal control to delineate FcRγ$^+$ and FcRγ$^-$ subsets in NK cells (purple). (b) prevalence of g-NK cells in PB and UCB, categorized as positive or negative using a 10% arbitrary cut-off value. (c) mean proportions of PB-c-NK, PB-g-NK, UCB-c-NK, and UCB-g-NK cells compared. Error bar represented mean SD. Statistical analyses were done by unpaired student's t-test (c). ns ($p > 0.05$); *($p < 0.05$); **($p < 0.01$).

subsets.[19] To assess the heterogeneity in the expression of adaptive NK cell markers between PB- and UCB-NK cells, we employed tSNE dimensionality reduction analysis (Figure 3a). Notably, in mature PB-NK cells, the deep green cluster characterized by NKG2C$^+$ NKG2A$^-$ was closely aligned with the NKG2C$^+$ FcRγ$^-$ cluster, the CD57$^+$ NKG2A$^-$ cluster, and the NKG2C$^+$ CD57$^+$ cluster, representing the classical adaptive NK cell subsets. However, intriguingly, these four subsets did not completely align in UCB-NK cells, with CD57 expression notably absent in UCB-g-NK cells (Figure 2a and Figure 3b). Additionally, it was noted that UCB-g-NK and PB-g-NK cells did not exhibit clustering, suggesting significant distinctions between these two populations (Figure 3a; Supplementary Figure S3a and S3b).

3.3. PB-g-NK and UCB-g-NK differ in CD16 signaling response and cytotoxicity

To assess potential differences in functional responses between g-NK cells from PB and UCB, we stimulated PBMCs or UCBMCs with P/I cocktail. Compared to PB-c-NK cells, PB-g-NK cells exhibited an elevated capability in generating IFN-γ and TNF-α, as well as expressing CD107a (Figure 4a). However, P/I did not increase UCB-g-NK cell cytokine production compared to UCB-c-NK cells (Figure 4b), indicating distinct responses of g-NK cells from both sources to the same stimulation.

P/I stimulation bypasses membrane events; hence, we next stimulated cells with immobilized anti-CD16 3G8 Abs, which induces CD16 pathway-mediated NK cell activation (Figure 4c,d). PB-g-NK cells demonstrated higher production of IFN-γ and TNF-α than PB-c-NK cells, but not CD107a,

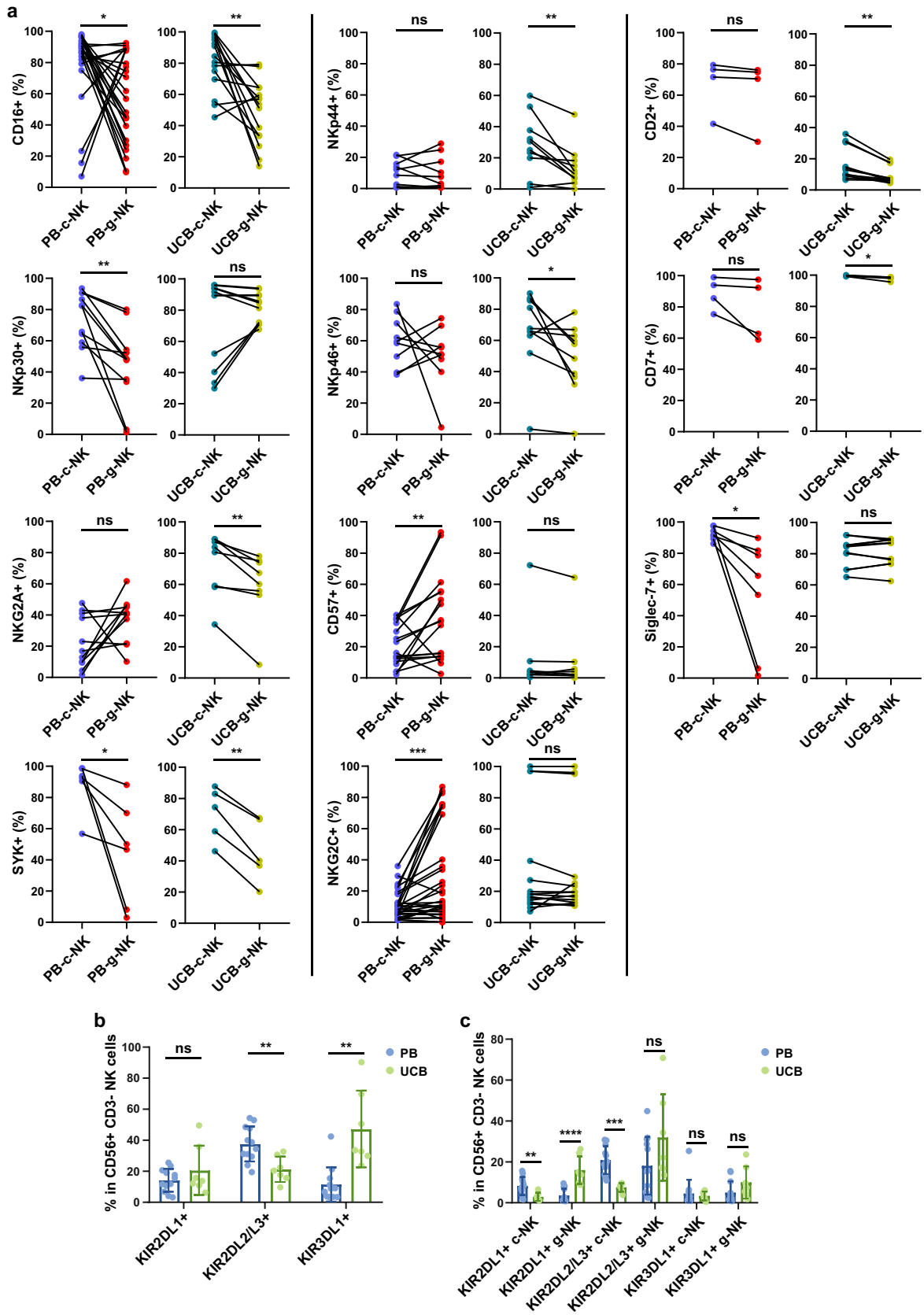

Figure 2. UCB-g-NK cells differ phenotypically from PB-g-NK cells. (a) comparison of NK-related molecule expression in PB-c-NK, PB-g-NK, UCB-c-NK, and UCB-g-NK cells. (b) distinct expression levels of KIRs (KIR2DL1, KIR2DL2/L3, and KIR3DL1) in PB-NK and UCB-NK cells (PBMCs, n=12; UCBMCs, n=7). (c) differential expression of KIR molecules in c-NK and g-NK cells from PB and UCB sources (PBMCs, n=12; UCBMCs, n=7). Error bar represented mean SD. Statistical analyses were done by paired (a) and unpaired (b and c) student's t-tests. ns ($p > 0.05$); *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); ****($p < 0.0001$).

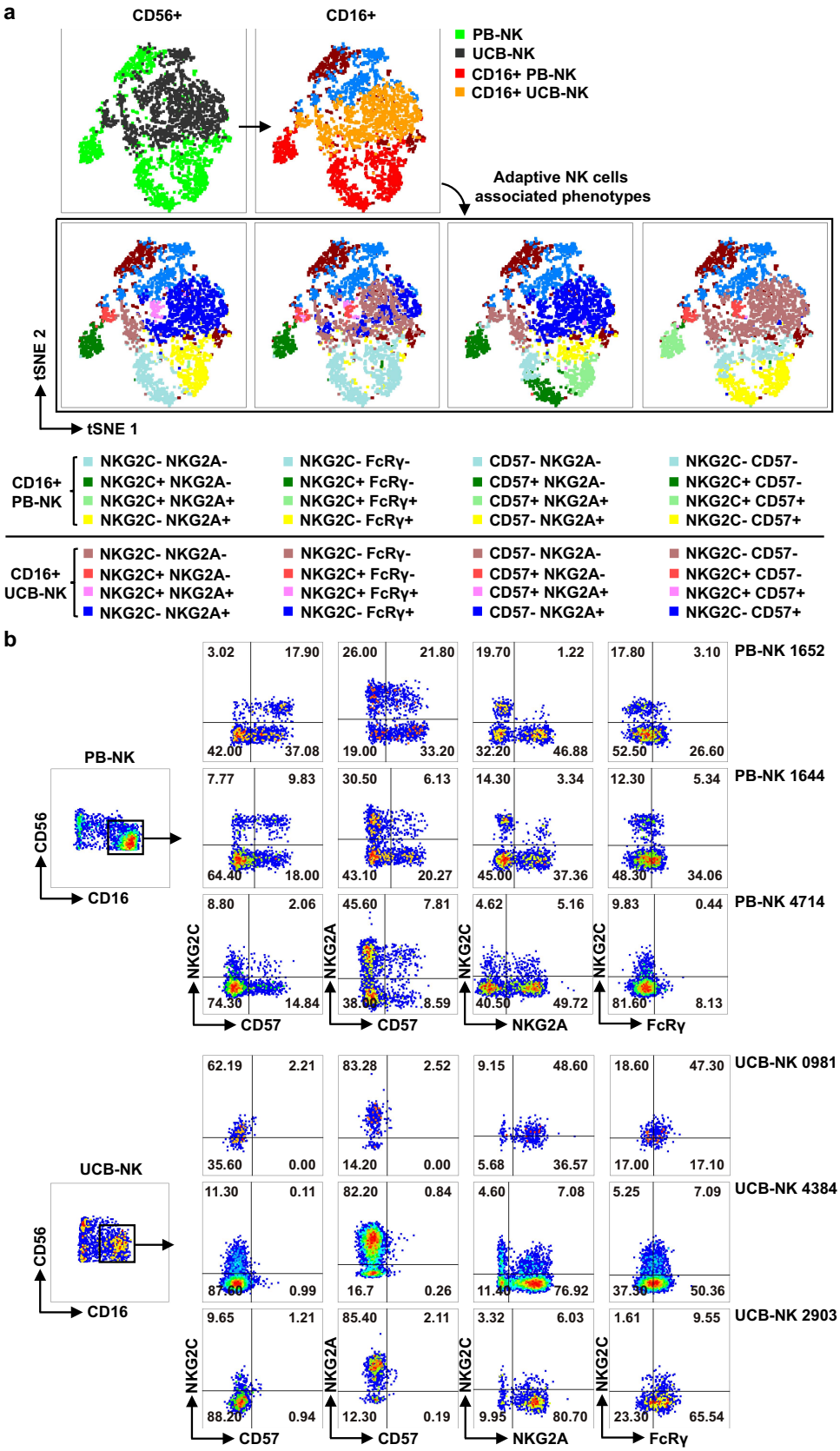

Figure 3. Unsupervised analysis distinguishes PB-g-NK and UCB-g-NK cells. (a) tSNE dimensionality reduction analysis of concatenated representative PB-NK and UCB-NK cells revealed distinct expression patterns of adaptive NK cell-associated phenotypes in mature NK cells ($CD3^-$ $CD56^{dim}$ $CD16^+$). (b) dot plots illustrating the expression of the adaptive NK cell-associated molecules NKG2C, NKG2A, and CD57 in 3 samples each from PBMCs and UCBMCs.

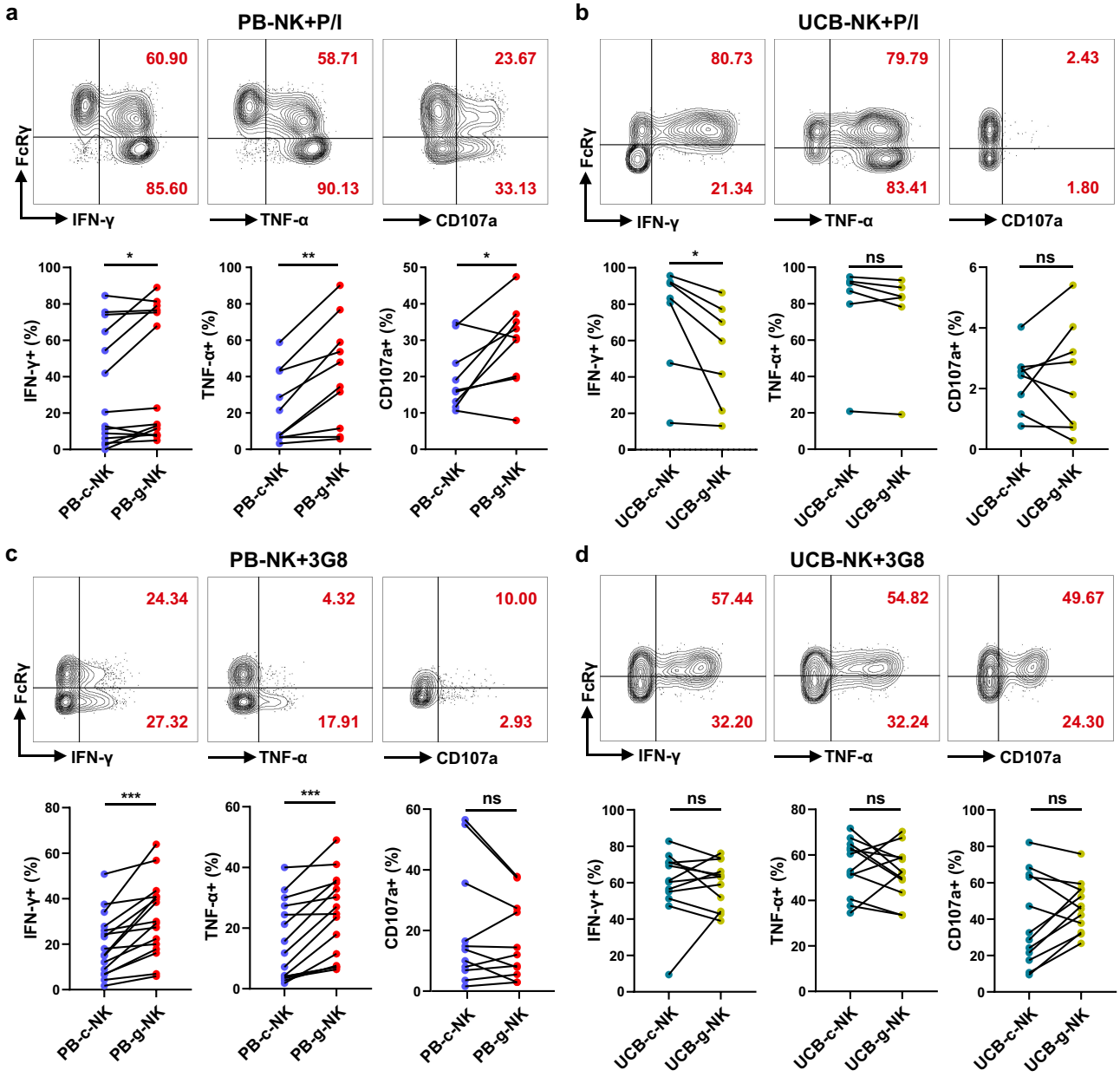

Figure 4. FcRγ deficiency enhances the responsiveness of the CD16 pathway in PB-g-NK cells, not in UCB-g-NK cells. FCM analysis assessed cytokine production and degranulation of NK cells after 6 hours of stimulation with a 1× activation mixture containing PMA and ionomycin (P/I). Panels: a (PB-NK cells) and b (UCB-NK cells). Sample sizes: a (IFN-γ n=14, TNF-α n=10, CD107a n=9); B (IFN-γ n=7, TNF-α n=6, CD107a n=8). Comparisons were drawn between c-NK and g-NK cells from the same source. Additionally, FCM analysis assessed cytokine production and CD107a expression on PB-NK (c) and UCB-NK (d) cells after 6-hour stimulation with immobilized 3G8. Sample sizes: C (IFN-γ n=16, TNF-α n=15, CD107a n=11); D (IFN-γ n=12, TNF-α n=12, CD107a n=12). Statistical analyses were done by paired student's t-test (A-D). ns ($p > 0.05$); *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$).

indicating stronger CD16 signaling leading to cytokine production in this g-NK subset (Figure 4c). Again, UCB-g-NK cells did not show variations compared to UCB-c-NK cells (Figure 4d).

Under a more physiological condition, PB-g-NK cells demonstrated comparable IFN-γ and TNF-α production to PB-c-NK cells when encountering MDA-MB-468 cells (Figure 5a). However, when the anti-EGFR mAb CET was present, PB-g-NK cells demonstrated greater cytokine production than PB-c-NK cells, resembling the impact of CD16 pathway activation through 3G8. Consistently, when we employed SDH-CET to opsonize target cells before adding PB-NK cells. We observed similar results with PB-g-NK cells exhibiting a more robust capacity to secrete cytokines (Figure 5a). In contrast, UCB-g-NK cells produced fewer cytokines after encountering target cells compared to UCB-c-NK cells, regardless of the presence or absence of CET. Notably, even the replacement of CET with SDH-CET failed to effectively reverse this skewed difference in cytokine production between UCB-c-NK and UCB-g-NK cells (Figure 5b).

Analysis of CD16 expression revealed that PB-g-NK cells consistently displayed lower levels of CD16 compared to PB-c-NK cells; whereas UCB-g-NK cells showed elevated CD16 levels than UCB-c-NK cells (Supplementary Figure S4a and S4b). Stimulation

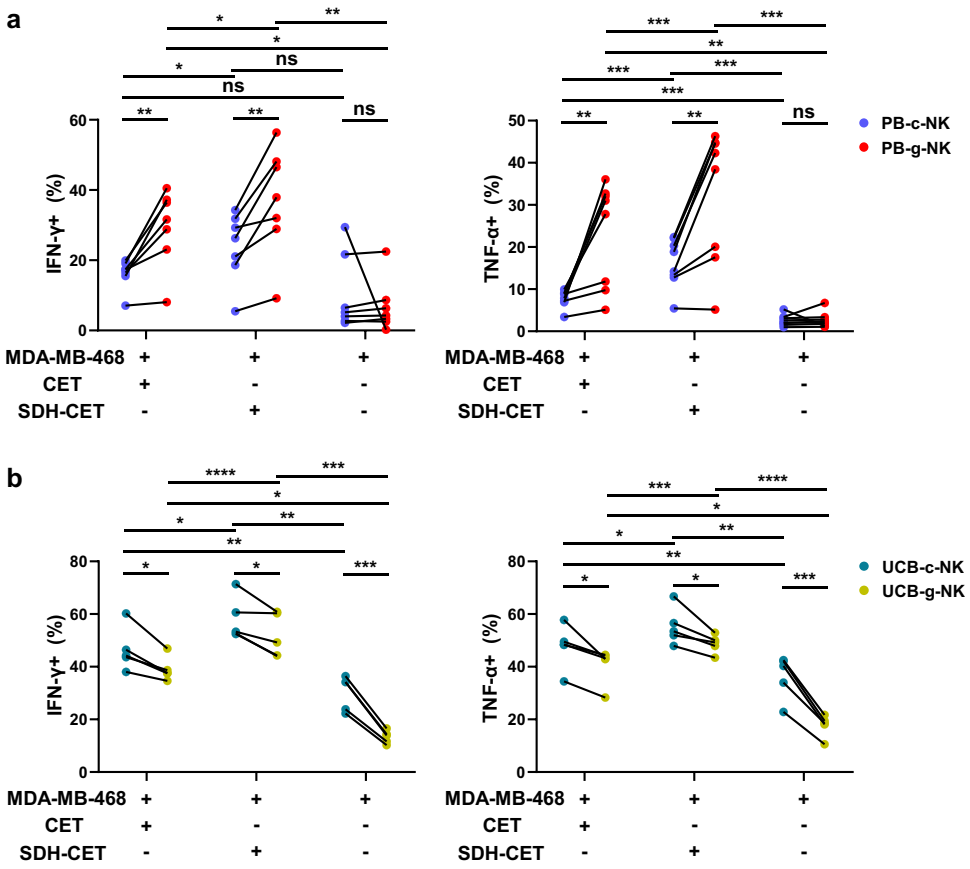

Figure 5. Contrasting cytokine production in PB-g-NK and UCB-g-NK cells upon mAb-opsonized target stimulation. MDA-MB-468 cells were opsonized with 2 µg/ml CET or SDH-CET and co-cultured with PBMCs or UCBMCs for 6 hours. Using FCM, we assessed IFN-γ and TNF-α production in PB-NK (a) and UCB-NK (b) cells, allowing for a comparison of cytokine production capacity between c-NK and g-NK cells from PB or UCB, activated through CD16 pathway or target cell stimulation. Sample sizes: a (IFN-γ n=7, TNF-α n=8); b (IFN-γ n=5, TNF-α n=5). Statistical analyses were done by paired student's t-test (a and b). ns ($p > 0.05$); *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$), ****($p < 0.0001$).

with P/I or 3G8 resulted in decreased CD16 expression in all NK cell types. Interestingly, encounters with target cells did not decrease CD16 expression in neither NK cell types. However, the addition of CET, particularly SDH-CET, led to reduced CD16 levels (Supplementary Figure. S4a and S4b). These findings underscore the intricate regulation of CD16 and its distinct expression profiles in response to diverse stimuli across g-NK and c-NK subsets originating from different sources.

Finally, we analyzed perforin and granzyme (Gzm) B expressions. PB-c-NK and PB-g-NK cells exhibited similar perforin expression (Supplementary Figure S5a). However, UCB-g-NK cells showed lower perforin expression than UCB-c-NK cells. In contrast, PB-g-NK cells displayed lower levels of Gzm B expression than PB-c-NK cells, while UCB-c-NK and UCB-g-NK cells expressed similar levels of Gzm B (Supplementary Figure S5b).

3.4. Serum from g-NK$^+$ donors and/or HCMV infection induce FcRγ downregulation and in vitro expansion of FcRγ$^{-/low}$ UCB NK subsets

Both PB-sourced NK and g-NK cells demonstrate a strong *in vitro* response to HCMV when exposed to plasma containing anti-HCMV Abs.[11] However, it is still uncertain whether NK and g-NK cells from UCB share similar characteristics with PB-sourced cells. We incubated UCB-NK or PB-NK cells with HCMV for 2 days. 6 hours prior to analysis, we introduced plasma from g-NK$^+$ donors into the co-culture system. HCMV combined with this plasma increased IFN-γ and TNF-α production in UCB-NK and also in the UCB-g-NK subset (Figure 6a), whereas PB-g-NK, which already showed higher basal levels, did not respond as much as UCB-g-NK subset. These findings underscore the potent cytokine-secreting potential of UCB-sourced NK and g-NK subpopulations under appropriate stimuli and also suggest that UCB-NK cells possess distinct regulatory mechanisms or necessitate specific factors for achieving optimal cytokine releasing.

The production of g-NK cells is positively associated with a history of HCMV infection and this can promote the expansion of g-NK cells from PB sources.[5,8,11] We also sought to validate if this phenomenon occurs in NK cells from UCB. To investigate the development of FcRγ$^{-/low}$ NK (g-NK-like) subpopulations in PB and UCB, we developed an *in vitro* co-stimulation system utilizing HCMV and the aforementioned plasma (Figure 6b). After 12–15 days of co-incubation, UCB-NK cells showed increased proportions of g-NK-like cells in the plasma alone, HCMV plus plasma, and doubled HCMV (+ +) plus plasma groups, as evidenced by changes in FcRγ mean fluorescence intensity (MFI) (Figure 6c). Plasma alone increased NK and g-NK-like cell numbers, but the presence

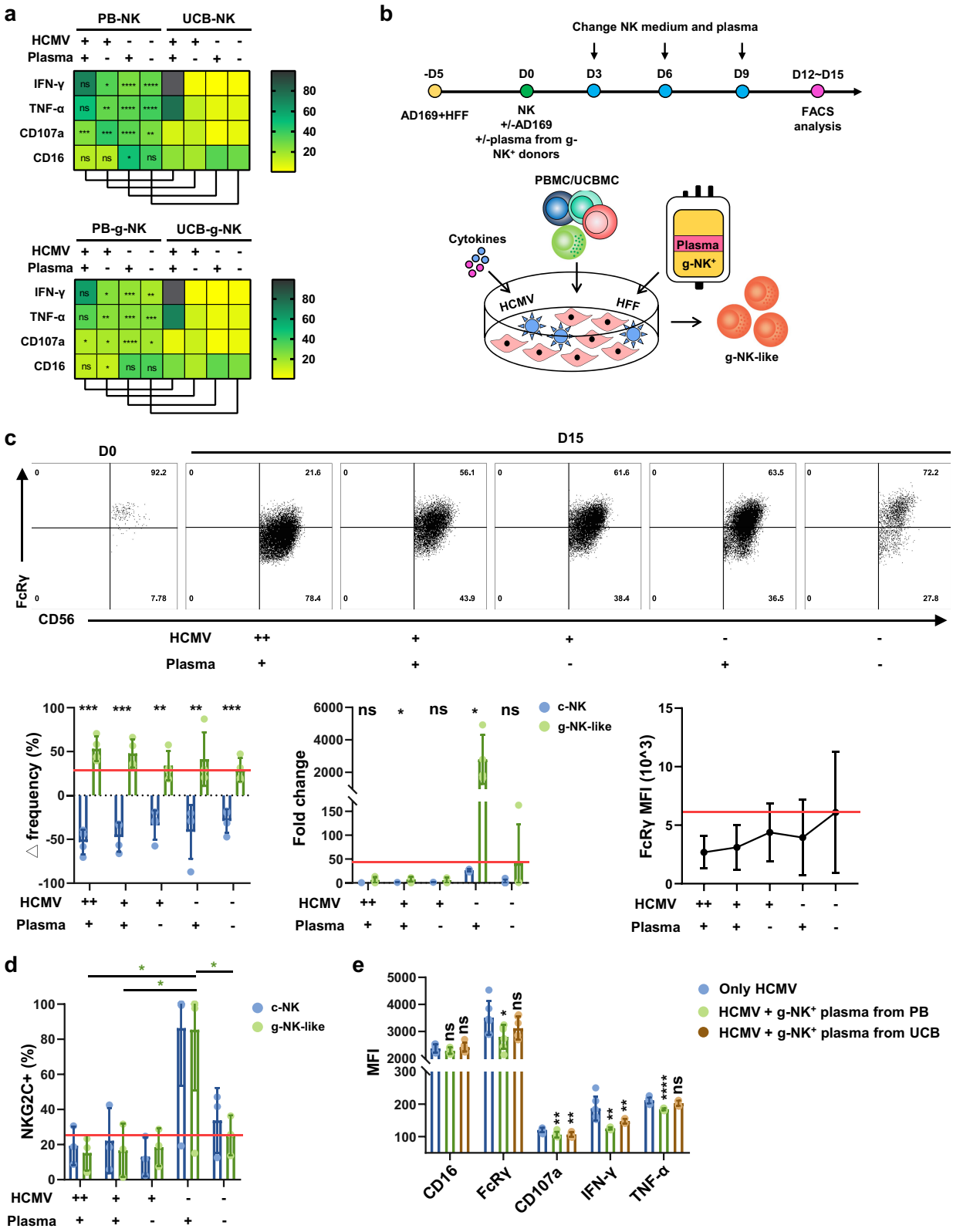

Figure 6. UCB-g-NK cells respond to HCMV and plasma from g-NK$^+$ donors. (a) PBMCs and UCBMCs were co-cultured with HCMV-infected HFF cells, supplemented with g-NK$^+$ plasma and anti-CD107a mAb. IFN-γ and TNF-α levels were assessed after 6 hours (PBMC samples=5; UCBMC samples=4). The heatmap depicted the average percentage of positive cells for each marker. Upper panel: comparison of PB-NK and UCB-NK cells. Lower panel: comparison of PB-g-NK and UCB-g-NK cells. (b) *in vitro* system demonstrating the influence of HCMV and/or g-NK$^+$ plasma on the transformation of PB-NK and UCB-NK cells into g-NK-like subpopulations. (c) expansion of g-NK-like subpopulations in UCB-NK cells after 15 days of *in vitro* stimulation with HCMV and/or g-NK$^+$ plasma. Δ% represents the percentage calculated by subtracting the initial percentage of FcRγ$^-$ NK cells from the percentage at day 15 after expansion. The expansion fold change of c-NK and g-NK-like subsets, as well as FcRγ mean fluorescence intensity (MFI) at 15 days, were also quantified. (d) changes in the proportion of NKG2C$^+$ subsets in c-NK and g-NK-like cells after 15 days of *in vitro* stimulation. (e) expression of CD16, FcRγ, IFN-γ, TNF-α, and CD107a in PLH-expanded UCB eNK cells (n=6) stimulated with HCMV alone or in combination with g-NK$^+$ plasma from PB or UCB sources. Error bar represented mean SD. Statistical analyses were done by unpaired Student's t-test (c and e) and two-way ANOVA test (d). ns ($p > 0.05$); *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); ****($p < 0.0001$).

of HCMV was required to increase the frequency of g-NK-like subset (Supplementary Figure S6a; Figure 6c). Stimulation with g-NK$^+$ plasma resulted in elevated expression of NKG2C in both the c-NK and g-NK-like subgroups, as well as in the FcRγ$^-$ NKG2C$^+$ and NKG2C$^+$ CD57$^-$ subgroups (Figure 6d; Supplementary Figure S6b and S6c). These findings indicate that the presence of g-NK$^+$ plasma contributes to the expansion and activation of NK cells, specifically the g-NK-like subpopulation characterized by higher NKG2C expression. For PB-NK cells, both HCMV and g-NK$^+$ plasma derived from PB led to a reduction in FcRγ expression (Supplementary Figure S7a). However, HCMV infection, whether it was a primary (×1) or secondary infection (×2), proved detrimental to the survival of PB-NK cells. The inclusion of g-NK$^+$ plasma from PB-NK cells co-cultured with HCMV was able to rescue this cell death. It is noteworthy that in the group where only g-NK$^+$ plasma was introduced, PB-NK cells exhibited efficient expansion, including c-NK and g-NK-like cells (Supplementary Figure S7b).

We next produced expanded and activated UCB eNK cells[20] and stimulated them with plasma from g-NK$^+$ UCB or PB donors and HCMV. The plasma emanated from the PB group downregulated FcRγ expression and decreased all cytokines production (Figure 6e). However, plasma from UCB only reduced the expression of CD107a and IFN-γ. The altered cytokine production pattern of the overall NK cell population may be attributed to the generation of g-NK-like subset. Notably, despite the CD16 pathway was activated by 3G8, the expanded UCB-g-NK (UCB eg-NK) cells did not exhibit higher CD107a expression compared to the expanded UCB-c-NK (UCB ec-NK) cells (Supplementary Figure S8a). When UCB eNK cells responded to HCMV and/or g-NK$^+$ plasma, their degranulation levels were elevated compared to stimulation with 3G8 alone. Interestingly, plasma obtained from the PB group nullified the differential CD107a expression between c-NK and g-NK-like cells, whereas this phenomenon was not observed with plasma from UCB donors (Supplementary Figure S8b). This indicates that g-NK$^+$ plasma could potentially transmit distinct stimulatory signals to NK cells, leading to variations in phenotype and functional diversity of NK cells.

4. Discussion

The origin and development of g-NK cells remain unclear. NKG2C$^+$ NK cells, crucial during HSCT, require active or subclinical HCMV Ag expression in recipients for clonal expansion.[21] Physiologically, HCMV infection or reactivation directly triggers the generation and expansion of NKG2C$^+$ NK or g-NK cells,[22–26] or it may be the outcome of co-evolution. The causal link between FcRγ downregulation and the rise in the NKG2C$^+$ NK fraction is still uncertain. Additionally, UCB is susceptible to HCMV infection.[27,28] This suggests that NKG2C$^+$ NK or g-NK cells induced by HCMV could potentially be generated in UCB. The discovery of NKG2C$^-$ g-NK cells warrants further research of g-NK heterogeneity.[29,30] There was no discernible difference between UCB-c-NK and UCB-g-NK cells in terms of CD57 and NKG2C expression. Since g-NK cells also occur in NKG2C$^{-/-}$ donors, this observation may be expected.[30] However, we lack HCMV serology data for correlation analysis with UCB-g-NK cell presence.

Phenotypic analysis revealed differences between PB-g-NK and UCB-g-NK cells, likely due to inherent disparities between UCB-NK and PB-NK cells. The expansion of NKG2C$^+$ NK cells is often driven by the dominance of single KIR clones.[10,31] The interplay between HLA-C1 and KIR2DL2/L3 facilitates the expansion of KIR2DL2/L3 single-positive /NKG2C$^+$ NK cells and is speculated to contribute to their education process.[32] Conversely, the CD57$^+$ NKG2C^{hi} NK subset lacks expression of KIR3DL1.[33] Our findings aligned with these previous studies, as PB-NK cells displayed higher expression of KIR2DL2/L3, while UCB-NK cells exhibited higher expression of KIR3DL1. PB-g-NK cells showed varying expression of KIR3DL1 or KIR2DL2/L3 or even no expression of KIR molecules, whereas UCB-g-NK cells were predominantly KIR2DL1$^+$.

To investigate changes in cytokine expression, we have quantified the percentage of cells positive for each analyzed cytokine in NK cells. However, this method has the limitation that we are unable to quantify the amount of cytokine produced by each subset of NK cells. We have presented some individual examples showing the relationship between the percentage of cell expressing the cytokine and the MFI levels (see Figure 4 and supporting material Fig S2), which showed that the percentage of positive cells just indicated this percentage and did not reflect whether the cells express higher levels of the given cytokines. Therefore, this is a technical limitation of our approach. While quantifying MFI can provide qualitative results, it is not designed for precise quantification. Although the enzyme-linked immunosorbent assay (ELISA) would be a more accurate method for quantifying cytokine secretion, it is not suitable for our study as we need to differentiate between the different subsets; *i.e.* g-NK cells from the whole NK cell population, which is characterized by the absence of an intracellular adaptor, FcRγ. In summary, using the percentage of positive cells gives a relatively good feed-back of cytokine secretion by one NK cell subset. However, it does not allow for the precise quantification of production levels.

Functional analysis revealed that UCB-g-NK cells did not respond as strongly as PB-g-NK cells to CD16 pathway activation or P/I stimulation. PB-g-NK and UCB-g-NK cells possibly serve different effector functions due to differential expression patterns of key molecules. Previous research implicates signaling proteins like FcRγ, CD3ζ, SYK, SHP-1, ZAP-70, and PLZF in the g-NK phenotype and function.[34] Knockout studies reveal nuanced effects: FcRγ deletion modestly increases TNF-α production, SYK elimination significantly boosts cytotoxicity and cytokine output, ZAP-70 deficiency weakens functionality, SHP-1 knockout heightens cytotoxicity but reduces cytokine output, and PLZF knockout doesn't enhance ADCC or cytokine production. SYK may indeed play a central role in augmenting their ADCC function in comparison to FcRγ. If this shortcut exists, it may shorten the time it takes for an active CD16 signaling to reach downstream ZAP70. Understanding these molecular nuances will shed light on the functional diversity of NK cells, necessitating further investigation.

Lee et al. demonstrated that PB-g-NK cell expansion is achieved through CD16 pathway activation with specific virus-binding

antiviral sera or Abs.[11] Furthermore, a robust correlation exists between the levels of Abs to HCMV-infected HFF cells and envelope gB whole-cell lysates and the proportion of g-NK cells. Moreover, there is a significant association between elevated levels of CXCL10 in the serum and g-NK cell proportion.[35] In our *in vitro* expansion system, g-NK-like subpopulations from PB and UCB demonstrated superior expansion and downregulated FcRγ expression in response to HCMV and/or g-NK$^+$ plasma stimulation. The impact of g-NK$^+$ plasma on the viability of g-NK-like subpopulations highlights its crucial role in long-term survival. These observations emphasize the critical role of g-NK and HCMV-specific plasma, acquired subsequently to primary infection, in the maintenance and control of HCMV reactivation. Nevertheless, the precise components of g-NK$^+$ plasma that ultimately are responsible for the described effects remain elusive.

In summary, our study reveals the heterogeneity of g-NK cells from different sources, *i.e.* PB- and UCB-sourced. FcRγ downregulation's impact on CD16 pathway responses varies by different NK cell source. However, our study has several limitations. Firstly, the uniform FcRγ expression in UCB-NK cells poses challenges in distinguishing UCB-g-NK from UCB-NK cells, affecting the precision of our assessments. Secondly, we have yet to fully understand the detailed regulatory mechanisms of HCMV and g-NK$^+$ plasma on UCB-NK cells, including the factors responsible for g-NK-like expansion and survival. Lastly, our study mainly focused on comparing the phenotypic and functional characteristics of PB- and UCB-g-NK cells, and further investigations are needed to elucidate their specific roles and regulatory mechanisms in *in vivo* immune responses.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

Corresponding authors can grant access to all study data upon reasonable request.

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