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Original Article

Decoding the multidimensional signatures of resident and expanded natural killer cells generated from perinatal blood

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Abstract: Natural killer (NK) cells are lymphocytes and play a pivotal role in innate and adaptive immune responses against infections and malignancies. Longitudinal studies have indicated the feasibility of perinatal blood for large-scale NK cell generation, yet the systematic and detailed comparations of the signatures of resident and expanded NK cells (rNKs, eNKs) are largely obscure. Herein, we harvested rNKs from umbilical cord blood (rUC-NKs) and placental blood (rP-NKs) as well as the corresponding eNKs (eUC-NKs, eP-NKs). Furthermore, the biological properties and transcriptomic signatures including cellular subpopulations, cytotoxicity, gene expression profiling, genetic characteristics, signaling pathways and gene set-related biological process were investigated. The enriched rNKs and eNKs exhibited diversity in biomarker expression pattern, and eNKs with higher percentages of $NKG2D^+$, $NKG2A^+$, $NKp44^+$ and $NKp46^+$ subsets. rNKs or eNKs with different origins showed more similarities in transcriptomic signatures than those with the same origin. Our data revealed multifaceted similarities and differences of the indicated rNKs and pNKs both at the cellular and molecular levels. Our findings provide new references for further dissecting the efficacy and molecular mechanisms of rNKs and eNKs, which will collectively benefit the fundamental and translational studies of NK cell-based immunotherapy.

Keywords: Perinatal blood, resident NK cells (rNKs), expanded NK cells (eNKs), biological phenotype, transcriptomic signatures

Introduction

Natural killer (NK) cells, comprising the cytotoxic $CD3^-CD56^{dim}CD16^{high}$ and IFN-γ-producing $CD3^-CD56^{bright}CD16^+$ counterparts, are pivotal members of the innate lymphoid cells (ILCs) family with spontaneous anti-viral infection and tumor-killing capacity [1-3]. As the first line of host's defense, NK cells together with other ILCs including lymphoid tissue inducer (LTi) cells, ILC1s, ILC2s and ILC3s function to maintain spatio-temporal homeostasis and protect local microenvironments from tissue insults without prior sensitization [4, 5]. To date, adoptive NK cell-based cellular immunotherapy has emerged as a novel pillar with oncology man-

agement, which therefore occupies worldwide enthusiasm and concerns for refractory and relapsing cancers including hematologic malignancies and metastatic solid tumors [2, 5-7].

During the past four decades, extensive investigations have been conducted to disclose the conventional NK (cNK) cells-derived from mouse spleen and circulating blood. Recently, pioneering investigators have identified the newfound tissue-resident NK (trNK) cells, which are distinct from cNK cells or conventional memory NK cells and preferentially distribute in many non-lymphoid tissues (e.g., uterus, salivary gland, liver and adipose) [1, 3, 8]. For instance, the uterine NK (uNK) cells are mostly derived from circulating NK cells and occupy the most abundant lymphocytes, and thus play an extremely important role in placental vascular remodeling and pregnant uterus [1, 5]. Additionally, current updates have also enlightened the feasibility of chimeric antigen receptor modified NK (CAR-NK) cells in reinforcing tumor-specific targeting and cytotoxic efficacy without causing cytokine storms [9].

Meanwhile, we and other investigators are committed to explore alternative NK cell sources for satisfying the clinical needs of cancer immunotherapy [2, 3, 10]. Of the cell sources (e.g., NK-92 cell line, iPSCs), peripheral blood-derived mononuclear cells (PBMCs) are the dominant ingredient for autologous or allogeneic NK cell preparation, which can be conveniently and immediately collected after the aseptic apheresis and thus diminish the possibility for NK cell contamination [5, 11]. Distinguish from the PBMCs, mononuclear cells (MNCs)-derived from perinatal blood including umbilical cord blood and placental blood possess preferable characteristics, and in particular, the low immunogenicity and the capability in triggering forceful antitumor reactions [12-14]. State-of-the-art renewal has also suggested the unique biological properties of expanded perinatal blood-derived NK cells such as vigorous cellular vitality and cytotoxicity [13, 15]. However, to our knowledge, the systematic and detailed comparation of the similarities and diversities between the resident and expanded NK cells-derived from perinatal blood are largely unprocurable.

Herein, we enriched resident NK cells from umbilical cord blood (rUC-NKs) and placental blood (rP-NKs) as well as expanded NK cells from the corresponding perinatal blood (eUC-NKs, eP-NKs), respectively. On the one hand, we disclosed the biological phenotypes of the indicated NK cells such as subpopulations and cytotoxicity. On the other hand, we verified the multifaceted variations of transcriptomic signatures and the accompanied biological processes as well. Collectively, our findings supply overwhelming new references for further decoding the diversity of perinatal blood-derived NK cells and the resultant cellular immunotherapy.

Materials and methods

Perinatal blood and mononuclear cell (MNC) preparation

Human perinatal blood including umbilical cord blood and placental blood was acquired with the consents of healthy donors (HDs) and the approval of Ethics Committee of Shangrao ETD-Health & Biotech Hospital and the guideline of Helsinki Declaration (KLL-2020-04) according to the guidelines of Helsinki Declaration. The mononuclear cells (MNCs) were isolated and enriched by utilizing the standard Ficoll-based density gradient centrifugation method as we described recently [10, 16].

Enrichment of resident and expanded NK cells

As to resident umbilical cord blood and placental blood NK cell (rUC-NKs, rP-NKs) enrichment, we conducted magnetic cell sorting (MACS) method for total $CD3^-CD56^+$ NK cell isolation from the aforementioned MNCs. As to expanded umbilical cord blood and placental blood NK cell (eUC-NKs, eP-NKs) enrichment, the corresponding resident NK cells (rUC-NKs, rP-NKs) were seeded in NK MACS Medium (Miltenyi Biotec, Germany) supplemented with 1% NK MACS Supplement (Miltenyi Biotec, Germany), 1% penicillin/streptomycin (ThermoFisher, USA) and 5% human AB serum (Sigma-Aldrich, St Louis, MO, USA), 1000 U/ml rhIL-2 (PeproTech Inc, USA), 10 ng/ml rhIL-15 (PeproTech Inc, USA) and 50 ng/ml rhIL-18 (R&D Systems, USA) as we recently reported [10]. After 14-days' *ex vivo* culture, the total expanded $CD3^-CD56^+$ NK cells (eUC-NKs, eP-NKs) were isolated by MACS, respectively. The detailed information of the indicated cytokines was available in Supplemental Information: Table S1.

Flow cytometry assay

The resident (rUC-NKs, rP-NKs) and expanded (eUC-NKs, eP-NKs) NK cells were washed with 1× PBS for twice and incubated with the indicated fluorescence conjugated antibodies including CD3, CD56, CD16, NKp44, NKp46, NKG2A, NKG2D, CD107a, BV421 in dark for 30 min. After that, the aforementioned resident and expanded NK cells were washed with 1× PBS for twice and analyzed by Canto II and FlowJo V10.0 (BD, USA) as we reported before [10, 17, 18]. The detailed information of the indicated antibodies was available in Supplemental Information: Table S2.

Cytotoxicity assessment of NK cells

The cytotoxicity of the eUC-NKs and eP-NKs was evaluated by utilizing the coculture model with K562 cell line at a series of effector-to-target ratios (E:T ratios) as we recently reported with several modifications [10]. Briefly, the K562 cells labelled with CellTrace Violet (Invitrogen, Carlsbad, USA) were solely cultured (Control) or cocultured with eUC-NKs or eP-NKs for 4 hrs in 96-well plate well. After that, total cells were labelled with anti-CD3, anti-CD56 and anti-CD107a and turned to BD Canto II for cytotoxicity assessment according to the formula: Cytotoxicity = (1 - living K562 cell number in the experimental group/living K562 cell number in control group) ×100%.

RNA-SEQ and bioinformatic analysis

RNA samples were prepared from the resident (rUC-NKs, rP-NKs) and expanded (eUC-NKs, eP-NKs) NK cells by utilizing the TRIZol reagent (ThermoFisher, USA) as we described before according to the manufactures' instructions [19-21]. The RNAs were sent to quality test and BGI Genomics (Shenzhen, China) for transcriptome sequencing (RNA-SEQ). The bioinformatic analysis of the RNA-SEQ data was performed with the existing software and platforms such as Venn Map diagram, Gene Set Enrichment Analysis (GSEA), HeatMap, Volcano Plot, Gene Ontology Biological Process (GOBP), Principal Component Analysis (PCA), Kyoto Encyclopedia of Genes and Genomes (KEGG) as we recently described [16, 19, 22].

Statistical analysis

The statistical analysis was performed with the GraphPad Prism 6.0 (GraphPad Software, USA) software as we described recently [22-25]. Generally, the data was shown as means ± SEM and One-way ANOVA assay was used for comparisons of three groups. Statistically significant difference was confirmed only when $P<0.05$. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, Not Significant.

Results

Resident and expanded UC-NKs and P-NKs revealed diversity in subpopulations

To explore the potential similarities and differences among the indicated NK cells, we initially isolated and enriched resident UC-NKs (rUC-NKs) and P-NKs (rP-NKs) from umbilical cord blood and placental blood, respectively. Meanwhile, we took advantage of the "3ILs"-based strategy for expanded UC-NKs (eUC-NKs) and expanded P-NKs (eP-NKs) generation and assessment (**Figure 1A**). As shown by the FCM diagrams, high percentages of total $CD3^-CD56^+$ rUC-NKs and rP-NKs were enriched by utilizing the MACS method (**Figure 1B**, **1C**). As to the subsets of total resident NKs, both rUC-NKs and rP-NKs showed similarities in the high contents of $CD16^+$ or $NKG2A^+$ subpopulation whereas with minimal proportion of $NKG2D^+$, $NKp44^+$ or $NKp46^+$ subset (**Figure 1D**, **1E**). Interestingly, we found that the percentage of $NKG2D^+$ NKs was higher in rUC-NKs than that in the rP-NKs ($P=0.0036$) (**Figure 1E**).

Simultaneously, we enriched total $CD3^-CD56^+$ eUC-NKs and eP-NKs derived from rUC-NKs and eP-NKs, respectively (**Figure 2A**, **2B**). Distinguish from eUC-NKs, eP-NKs with higher proportions of $CD16^+$, $NKG2A^+$, $NKp44^+$ and $NKp46^+$ subsets whereas comparable content of $NKG2D^+$ cells was observed in eUC-NKs and eP-NKs instead (**Figure 2C**, **2D**). Longitudinal studies showed that both the eUC-NKs and eP-NKs with 14-days' *ex vivo* culture manifested higher level of activation over that in corresponding rUC-NKs and eP-NKs based on the statistical analysis of the indicated subpopulation of NKs (**Figure 2E**, **2F**).

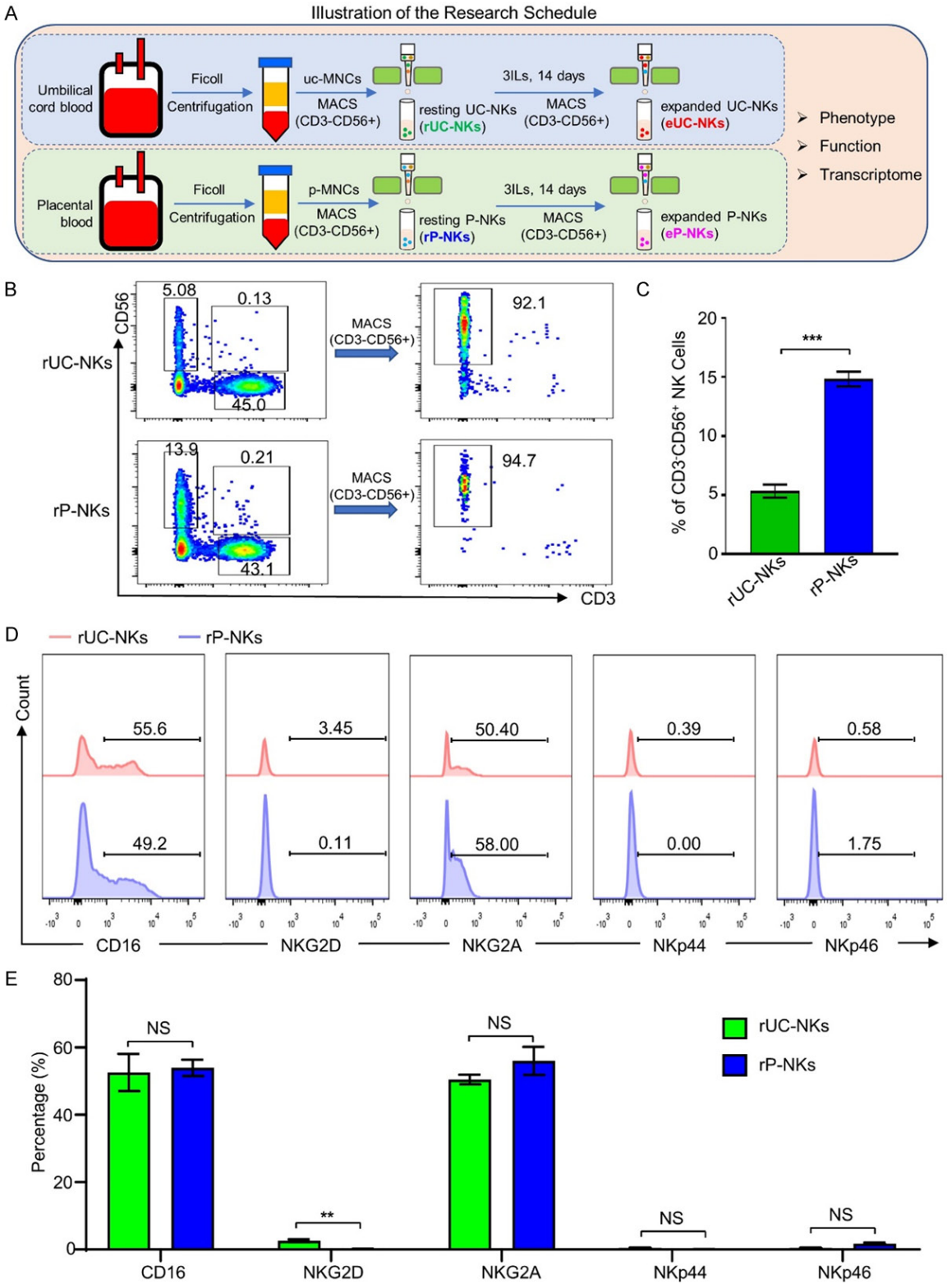

Figure 1. Comparation of the biological phenotype of resident UC-NKs and resident P-NKs. (A) Illustration of the research schedule. Resident UC-NKs (rUC-NKs) and resident P-NKs (rP-NKs) were isolated from umbilical cord blood- and placental blood-derived monocytes (UC-MNCs, P-MNCs), respectively. The ex vivo expanded UC-NKs (eUC-NKs) and expanded P-NKs (eP-NKs) were enriched after 14 days' culture; (B, C) Representative FCM diagrams (B) and statistical analyses (C) of total ($CD3^-CD56^+$) cells in UC-TNCs and P-TNCs and the enriched rUC-NKs and rP-NKs; (D, E) FCM diagrams (D) and statistical analyses (E) of the subpopulations of NK cells ($CD16^+$, $NKG2D^+$, $NKG2A^+$, $NKp44^+$, $NKp46^+$) in rUC-NKs and rP-NKs. Data was shown as mean ± SEM. ***, $P<0.001$; NS, not significant.

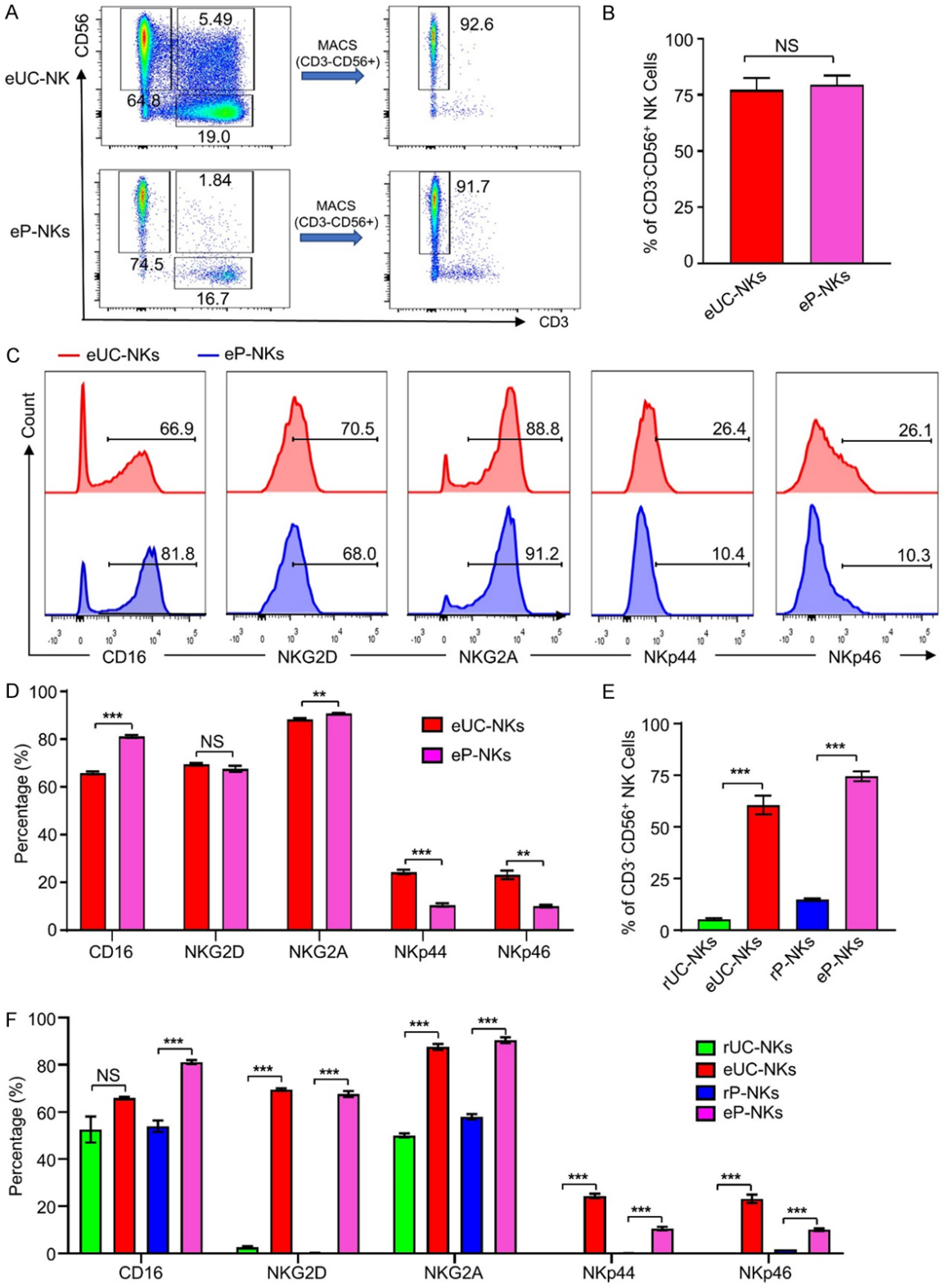

Figure 2. Comparation of the biological phenotype of expanded UC-NKs and expanded P-NKs. (A, B) Representative FCM diagrams (A) and statistical analyses (B) of total ($CD3^-CD56^+$) in the enriched eUC-NKs and eP-NKs; (C, D) FCM diagrams (C) and statistical analyses (D) of the subpopulations of NK cells ($CD16^+$, $NKG2D^+$, $NKG2A^+$, $NKp44^+$, $NKp46^+$) in eUC-NKs and eP-NKs; (E, F) Statistical analyses of total ($CD3^-CD56^+$) cells (E) and the subpopulations of NK cells ($CD16^+$, $NKG2D^+$, $NKG2A^+$, $NKp44^+$, $NKp46^+$) (F) in the indicated groups. Data was shown as mean ± SEM. **, P<0.01; ***, P<0.001; NS, not significant.

Expanded eUC-NKs exhibited superiority in cytotoxicity over eP-NKs in vitro

Due to the limitation of resident NKs in cell count and immaturity as reported before, we turned to the expanded and more mature eUC-NKs and eP-NKs for *ex vivo* cytotoxicity evaluation. Thus, we took advantage of the K562 cells and NKs coculture model at various effector-target (E:T) ratios and found that the eUC-NKs with higher percentages of $CD107^+$ subsets than those in the eP-NKs (**Figure 3A**, **3B**). Furthermore, the cytotoxicity of eUC-NKs against the target K562 cells was better than that of eP-NKs, which as further confirmed by BV421-based fluorescent quantitation (**Figure 3C**, **3D**). Collectively, our data showed that the eUC-NKs had moderate advantage in *ex vivo* cytotoxicity over eP-NKs.

Resident and expanded UC-NKs and P-NKs showed diversity in gene expression profiling

Having dissected the detailed information of cellular phenotype among the resident NKs (rUC-NKs and rP-NKs) and expanded NKs (eUC-NKs and eP-NKs), we are further curious about the potential similarities and differences in genetic characteristics. For the purpose, we conducted transcriptome sequencing and multifaceted bioinformatic analyses. Notably, the distribution of gene expression in resident NKs (rUC-NKs and rP-NKs) and expanded (eUC-NKs and eP-NKs) NKs revealed greater diversity to each other, which was confirmed by Pearson correlation analysis and collectively indicated the distinctions before and after *ex vivo* expansion and activation (**Figure 4A**, **4B**). From the view of PC1 (69.99%) and PC2 (20.30%) by principal component analysis (PCA), we congruously demonstrated the more closely related relationship between eUC-NKs and eP-NKs when compared with rUC-NKs or rP-NKs (**Figure 4C**). As shown by the volcano plot diagrams and Venn Map diagrams together with the statistical analysis, we could intuitively observe the expression pattern of total genes as well as the differentially expressed genes (DEGs) between each other (**Figure 4D-G**). Furthermore, hierarchical cluster analysis and KDA analysis revealed the detailed distribution of DEGs among the indicated resident and expanded NKs (**Figure 4H**, **4I**). Collectively, these transcriptomic data were consistent with the variations of aforementioned biological signatures among resident NKs and expanded NKs in the landscape of gene expression profiling.

Resident NKs or expanded NKs from different sources revealed variations in multifaceted transcriptomic signatures

To illuminate the potential variations in multifaceted biological processes, we initially carried out the KEGG analysis and found that the DEGs between rUC-NKs and rP-NKs or between eUC-NKs and eP-NKs revealed similarities in multiply signaling pathways such as chemokine signaling pathway, cytokine-cytokine receptor interaction, antigen processing and presentation (**Figure 5A**, **5B**). Meanwhile, we also noticed that the NK maturation and activation-associated signaling were consistently enriched in expanded NKs including TNF signaling pathways, IL-17 signaling pathway and natural killer cell mediated cytotoxicity (**Figure 5B**). Simultaneously, with the aid of GOBP analysis, we observed cell chemotaxis, immune response, cell adhesion and regulation of signaling receptor activity-associated biological processes were synchronously enriched in both resident and expanded NKs (**Figure 5C**, **5D**). Furthermore, as shown by gene set enrichment analysis (GSEA), IFN-γ and IFN-α response, coagulation and inflammatory response-associated datasets were uniquely enriched in rP-NKs compared to rUC-NKs, while the G2M checkpoint, NOTCH signaling, IL6/JAK-STAT3 signaling, IL2-STAT5-associated datasets were enriched in eP-NKs compared to eUC-NKs instead (**Figure 5E**, **5F**). Taken together, the transcriptomic characteristics between the indicated resident NKs (rUC-NKs vs rP-NKs) or expanded NKs (eUC-NKs vs eP-NKs) further indicated the variations and underlying mechanism in biological properties.

Resident NKs and expanded NKs from the same source revealed diversity in multifaceted transcriptomic signatures

For decades, we and other investigators have devoted to decode the characteristics of NK cells, yet the comparation of the transcriptomic signatures between the resident NKs and expanded NKs are largely obscure [2, 3, 10, 26]. Therefore, we conducted longitudinal study upon P-NKs (rP-NKs and eP-NKs) and UC-NKs (rUC-NKs and eUC-NKs), respectively. As to UC-NKs, the DEGs between rP-NKs and

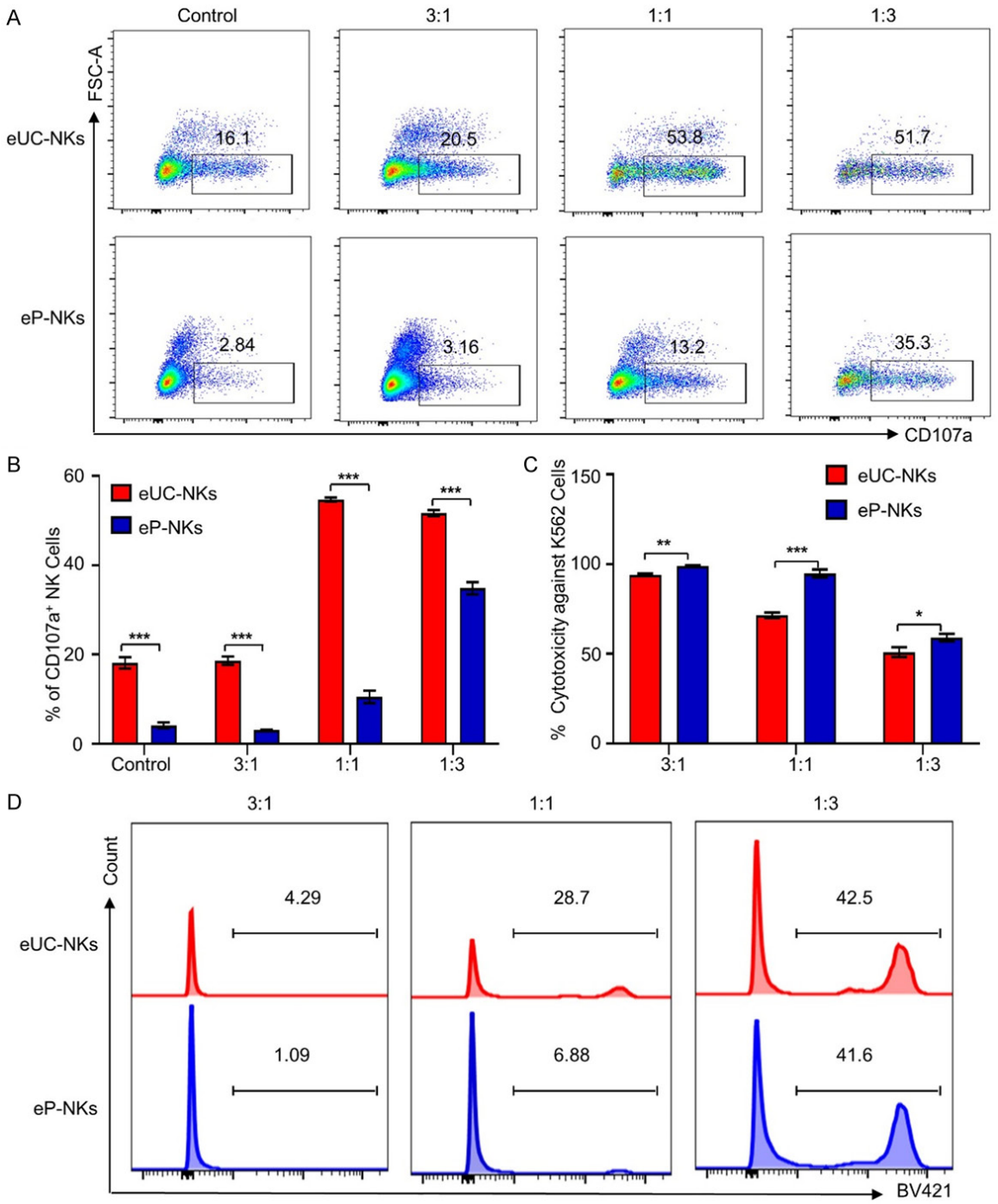

Figure 3. Comparation of the *in vitro* cytotoxicity of eUC-NKs and eP-NKs upon K562 cells. (A, B) FCM diagram (A) and statistical analysis (B) of CD107a$^+$ eUC-NKs and eP-NKs in coculturing with K562 cells at various effector-to-target ratios (E:T=3:1, 1:1; 1:3); (C, D) The comparation of the cytotoxicity of eUC-NKs and eP-NKs against K562 cells at the aforementioned E:T ratios. All data was shown as mean ± SEM. *, P<0.05; **, P<0.01; ***P<0.001; NS, not significant.

eP-NKs were mainly involved in biological processes such as cytokine-cytokine receptor interaction, cell cycle, metabolic pathways and Jak-STAT signaling pathway, while those between rUC-NKs and eUC-NKs were principally related to hematopoietic cell lineage, P53 signaling pathway, cell cycle and cytokine-cytokine receptor interaction (**Figure 6A**, **6B**).

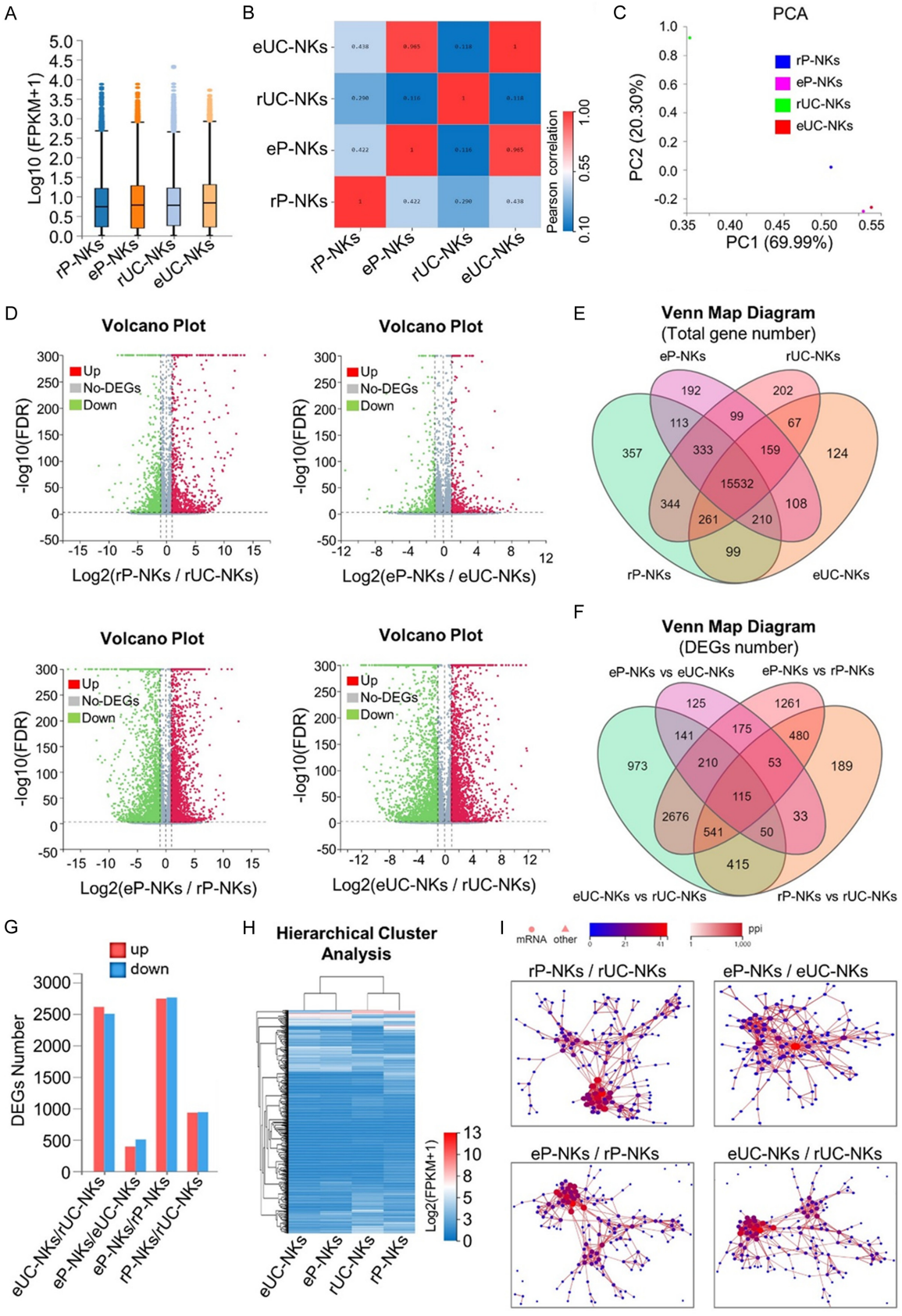
A
Log10 (FPKM+1)
rP-NKs
eP-NKs
rUC-NKs
eUC-NKs
B
Pearson correlation
C
PCA
PC2 (20.30%)
PC1 (69.99%)
D
Volcano Plot
Up
No-DEGs
Down
-log10(FDR)
Log2(rP-NKs / rUC-NKs)
Log2(eP-NKs / eUC-NKs)
Log2(eP-NKs / rP-NKs)
Log2(eUC-NKs / rUC-NKs)
E
Venn Map Diagram
(Total gene number)
F
Venn Map Diagram
(DEGs number)
eP-NKs vs eUC-NKs
eP-NKs vs rP-NKs
eUC-NKs vs rUC-NKs
rP-NKs vs rUC-NKs
G
up
down
DEGs Number
eUC-NKs/rUC-NKs
eP-NKs/eUC-NKs
eP-NKs/rP-NKs
rP-NKs/rUC-NKs
H
Hierarchical Cluster Analysis
Log2(FPKM+1)
I
mRNA
other
ppi
rP-NKs / rUC-NKs
eP-NKs / eUC-NKs
eP-NKs / rP-NKs
eUC-NKs / rUC-NKs

Figure 4. The landscape of gene expression profile of resident NKs and expanded NKs. (A) The distribution of gene expression in resident NKs (rUC-NKs, rP-NKs) and expanded NKs (eUC-NKs, eP-NKs); (B) The correlation of the aforementioned resident NKs and expanded NKs; (C) Principal component analysis (PCA) of the indicated resident NKs and expanded NKs; (D) The visualized distribution of the gene expression in the indicated resident NKs and expanded NKs; (E, F) Venn Map diagram reveals the number of the total overlapped genes (E) and differentially expressed genes (DEGs) (F) among the indicated resident NKs and expanded NKs; (G, H) The number (G) and hierarchical cluster analysis (H) of the upregulated (up) and downregulated (down) DEGs in the indicated resident NKs; (I) PPI analysis of the 300 DEGs (log2FC>2.0) between the indicated NKs.

Consistently, as shown by the GOBP analysis, the DEGs between P-NKs (rP-NKs and eP-NKs) or UC-NKs (rUC-NKs and eUC-NKs) were concerned with biological processes including immune system process and innate immune response, cell cycle and apoptotic process, DNA replication, phosphorylation and cell surface receptor signaling pathways (**Figure 6C**, **6D**). To further disclose the potentially genetic characteristics, we turned to the aforementioned GSEA diagrams and confirmed that cell cycle (e.g., G2M checkpoint, MYC targets, mTORC1 signaling) and immune (e.g., IL2-STAT5 signaling)-related datasets were specifically enriched in eP-NKs (FDR<0.001), which was largely consistent with those in eUC-NKs except IL2-STAT5 signaling (FDR>0.05) (**Figure 6E**, **6F**). Overall, by comparing rNKs with the corresponding eNKs, we noticed multidimensional diversities in the underlying biofunction and molecular mechanism, which collectively indicated the maturation and activation of rNKs after *in vitro* stimulation.

Discussion

Currently, millions of people endure and even die from hematologic malignancies or solid tumors with high morbidity and mortality worldwide, and thus result in tremendous pressure to tumor patients and their guardians both physically and mentally [27-32]. Despite the encouraging progress in oncotherapy by traditional remedies including surgery, radiation and chemotherapy [33, 34], yet the inherent shortcomings such as graft-versus-host disease, neurotoxicity, off-target effects and drug delivery barriers are collectively constitute the core dilemmas in cancer therapy [2, 7, 31]. To date, NK cell-based cellular immunotherapy has become an emerging therapeutic strategy for cancer immunosurveillance, intracellular pathogen clearance and hematopoietic reconstitution [35-37]. Considering the promising prospect of perinatal blood-derived NK cells in clinical therapeutics, it's of great importance to dissect the multidimensional characteristics before large-scale applications [15]. For the purpose, we verified the subpopulations and cytotoxicity of the enriched resident (rUC-NKs and rP-NKs) and expanded (eUC-NKs and eP-NKs) NK cells as well as the diversities in transcriptomic signatures, which collectively depicted the panorama of NK cells derived from perinatal blood and would ultimately benefit the understanding of NK cell-based immunotherapy in clinical applications.

Current studies have indicated numerous newly discovered inhibitory checkpoints and activating receptors of NK cells together with the corresponding engagement by cognate ligands on malignant cells modulate NK cell cytotoxicity [38-41]. Meanwhile, a certain number of immunotherapeutic approaches in the setting of recipient-donor KIR/HLA mismatch have been developed to potentiate NK cell activity in cancer management and allogeneic hematopoietic stem cell transplantation [38, 42]. In this study, we observed the relative conservation of the proportion of subpopulation (e.g., $CD16^+$, $NKG2A^+$, $NKG2D^+$, $NKp44^+$, $NKp46^+$) between rUC-NKs and rP-NKs or between eUC-NKs and eP-NKs whereas resident and expanded NK cells of the same origin (rUC-NKs vs eUC-NKs, rP-NKs vs eP-NKs) showed significant diversity, which collectively indicated the maturation of *ex vivo* expanded NK cells rather than resident NK cells. Additionally, the cytotoxicity of eP-NKs was superior to eUC-NKs upon K562 cells after 14-days' expansion and activation with "3ILs" (IL-2, IL-15, IL-18) stimulation, which was confirmed by the increased proportion of active NK cell subsets ($NKG2D^+$, $NKp44^+$ and $NKp46^+$) and the decreased inhibitory $NKG2A^+$ counterpart as well as the preferable properties in multifaceted transcriptomic signatures (e.g., INF-γ and other cytokine-associated signaling pathway, natural killer cell mediated cytotoxicity, and cytokine-cytokine receptor interaction). Furthermore, considering the large amount of placenta perfusate, robust *ex vivo*

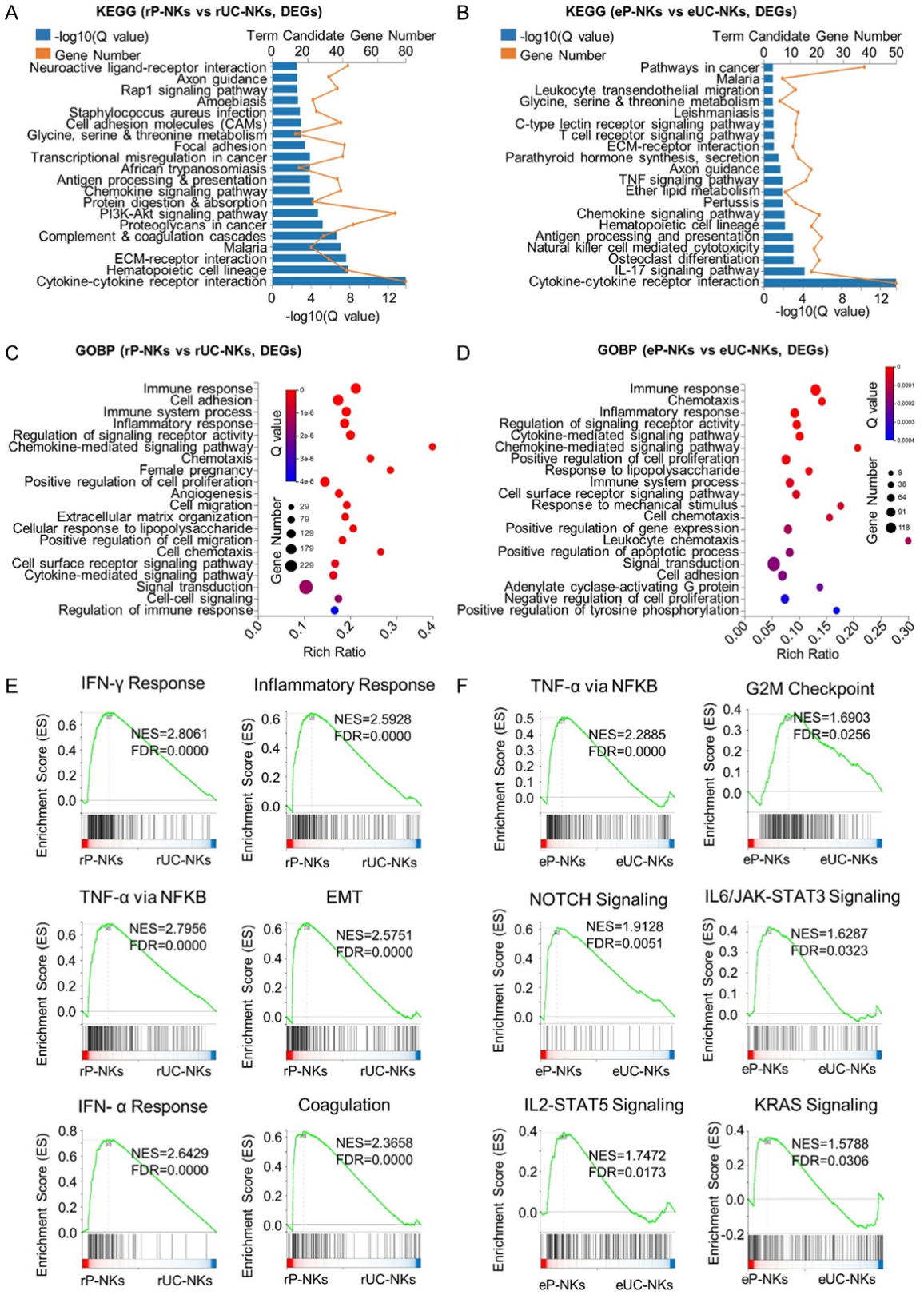

Figure 5. Potential variations between rUC-NKs and rP-NKs or between eUC-NKs and eP-NKs in multidimensional transcriptomic signatures. (A, B) KEGG analysis of the DEGs between rUC-NKs and rP-NKs (A) or between eUC-NKs and eP-NKs (B); (C, D) Potential variations between rUC-NKs and rP-NKs (C) or between eUC-NKs and eP-NKs (D) in representative gene ontology biological processes (GOBP); (E, F) Gene Set Enrichment Analysis (GSEA) of the DEGs between rUC-NKs and rP-NKs (E) or between eUC-NKs and eP-NKs (F).

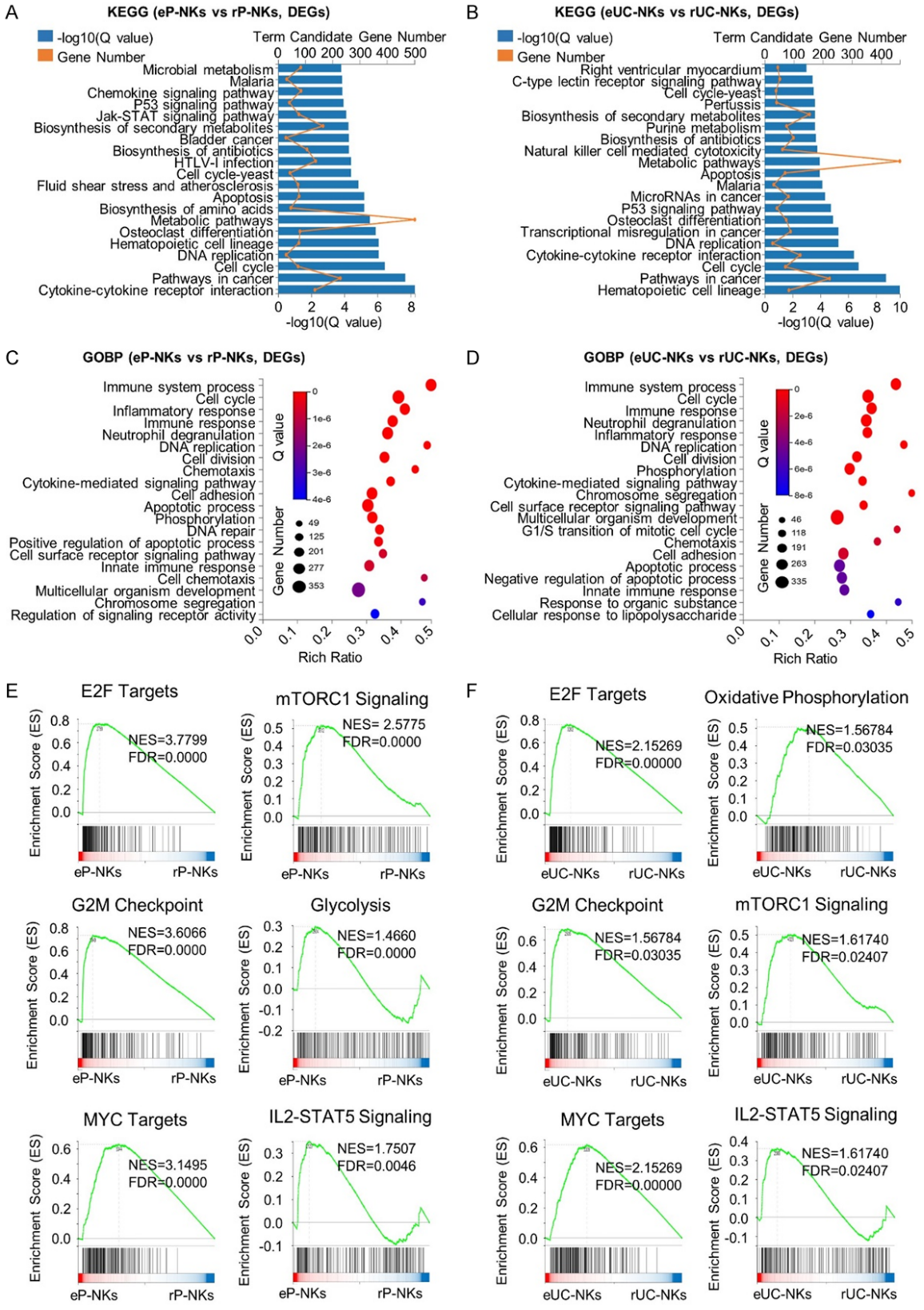

Figure 6. Potential variations of resident NKs and expanded NKs in multidimensional transcriptomic signatures. (A, B) KEGG analysis of the DEGs between rP-NKs and eP-NKs (A) or between eUC-NKs and rUC-NKs (B); (C, D) Potential variations between eP-NKs and rP-NKs (C) or between eUC-NKs and rUC-NKs (D) in representative gene ontology biological processes (GOBP); (E, F) Gene Set Enrichment Analysis (GSEA) of the DEGs between rP-NKs and eP-NKs (E) or between eUC-NKs and rUC-NKs (F).

expansion potential and elevated levels of multiple surface marker expression, placental blood hold better prospects for efficiently clinical-grade eP-NKs preparation [2, 15].

For decades, numerous cellular immunotherapies for cancer management have been raised and developed including the tumor infiltrating lymphocytes (TILs), cytokine-induced killer cells (CIKs), NK cells, dendritic cells (DCs), DC-CIKs, macrophages (Mø), CAR-transduced immune cells (e.g., CAR-T, CAR-NK, CAR-Mø) and T cell receptor (TCR) chimeric T cells [43-45]. Differ from the most of the abovementioned adoptive cellular immunotherapy, NKs are adequate for tumor immunosurveillance via rapidly removing tumor cells with oncogenic transformation-related surface marker expression and simultaneously enhancing antibody and T cell responses [46, 47]. Therefore, NK cells are promising "off-the-shelf" products and function by mode of direct cytolytic effect, antibody-dependent cell-mediated cytotoxicity (ADCC) and paracrine effects (IFN-γ, GM-CSF, G-CSF) [45, 48]. Moreover, numerous preclinical and clinical studies have indicated that the "tumor escape" phenomenon attributes to the resistance of residual tumor cells to endogenous resident NK cells can be overcome via *ex vivo* expansion and activation of NK cells as well as genetic modification [47]. Notably, our findings also confirmed the capacitation and conversion of resident NKs after *ex vivo* stimulation. Moreover, to our knowledge, our study for the first time systematically expounded the similarities and diversities of perinatal blood-derived resident and expanded UC-NKs and P-NKs from the overview of biological phenotype and transcriptomic signatures. For instance, eNKs (eUC-NKs, eP-NKs) manifested multifaceted transcriptomic signatures in cancer immunotherapy-associated processes (e.g., Jak-STAT, pathways in cancer, cytokine-cytokine receptor interaction, innate immune response) when compared to the corresponding rNKs, which was consistent with the aforementioned biological signatures such as NK cell activation, maturation and cytokine secretion (e.g., INF-γ, TNF-α). Therefore, these data will ultimately enlighten the exploration of NK cell-based cytotherapy and benefit the multipronged cancer immunotherapy for hematological malignancy and metastatic solid tumor management.

Overall, we have systematically dissected the similarities and diversities of both resident and expanded NK cells derived from perinatal blood including umbilical cord blood and placental blood. Our findings enlightened the feasibility of perinatal blood for large-scale generation of clinical grade NK cells and would supply new references to NK cell-based immunotherapy and investigation new drug (IND) development.

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Disclosure of conflict of interest

None.

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Table S1. The cytokines used in this study

Reagent	Cat. No.	Source
Recombinant Human IL-2 (rhIL-2)	200-02	PeproTech Inc, USA
Recombinant Human IL-15 (rhIL-15)	200-15	PeproTech Inc, USA
Recombinant Human IL-18 (rhIL-18)	119-BP-100	R&D Systems, USA

Table S2. Antibodies for flow cytometry assay in the study

Antibody	Cat. NO.	Source
Anti-CD3-PE	981004	BioLegend
Anti-CD3-APC-Cy7	300316	BioLegend
Anti-CD4-PE	357403	BioLegend
Anti-CD8-PE-Cy7	344711	BioLegend
Anti-CD16-FITC	302005	BioLegend
Anti-NKG2D-Percp-cy5.5	320817	BioLegend
Anti-CD56-APC	362503	BioLegend
Anti-CD56-Percp-cy5.5	362505	BioLegend
Anti-CD107a-PE-Cy7	328617	BioLegend
7-AAD-Percp-cy5.5	559925	BD Pharmigen
Precision Count Beads	424902	BioLegend
DAPI	MBD0015	Sigma-Aldrich
Cell Cycle and Apoptosis Detection Kit	C1052	Beyotime Biotehnology
CellTrace Voilet	C34557	Invitrogen™
Annexin V-FITC	AO2001-02G	Tianjin Sungene Biotech
Annexin V binding buffer (10X)	AB2000-G	Tianjin Sungene Biotech