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Original Article

Characterization of the biological and transcriptomic signatures of natural killer cells derived from cord blood and peripheral blood

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Abstract: Longitudinal studies have indicated the pivotal role of natural killer cells (NKs) in the elimination of certain infections and malignancies. Currently, perinatal blood (PB) and cord blood (CB) have been considered with promising prospective for autogenous and allogeneic NKs transplantation, yet the similarities and differences at the biological and molecular levels are largely obscure. We isolated mononuclear cells (MNCs) from PB and CB, and compared the biological phenotypes of resident NKs by flow cytometry and cell counting. Then, we turned to our well-established "3ILs" strategy and co-culture for NK cell activation and cytotoxicity analyses, respectively. Finally, with the aid of transcriptomic analyses, we further dissected the signatures of PB-NKs and CB-NKs. CB-NKs revealed superiority in cellular vitality over PB-NKs, together with variations in subpopulations. CB-NKs showed higher cytotoxicity over PB-NKs against K562 cells. Furthermore, we found both NKs revealed multifaceted conservations and differences in gene expression profiling and genetic variations, together with gene subsets and signaling pathway. Collectively, both NKs revealed multifaceted similarities and diverse variations at the cellular and transcriptomic levels. Our findings would benefit the further exploration of the biological and transcriptomic properties of CB-NKs and PB-NKs, together with the development of NK cell-based cytotherapy.

Keywords: Natural killer cells, peripheral blood, cord blood, cytotoxicity, transcriptomic properties

Introduction

Natural killer cells (NKs) are advantaged innate lymphoid cells (ILCs) with broad distribution, which play a crucial role in both innate and adaptive immune responses [1, 2]. Longitudinal studies have indicated the pivotal role of NKs with activating and inhibitory receptor expression in eliminating the tumor cells and pathogenic microorganism dispense with presensitization [3]. Meanwhile, Zalfa and Paust summarized the recent updates of NK cell interaction with the heterogeneous myeloid derived suppressor cells (MDSCs) with potent immuno-

suppressive activity in the tumor microenvironment (TME) for cancer immunotherapy, which would help improve the NK cell-based antitumor immunotherapy [4, 5].

To date, NKs have been recognized as the main effector cells against cancer and multiple adjacent cells in innate immunity with considerable heterogeneous in the circulatory system and the tumor microenvironment, which thus has been expected as next generation of immunooncology treatments in cancer immunotherapy [6-8]. For instance, NKs have revealed high cytotoxicity against diverse subtypes of cancers via direct killing effect, antibody-dependent cell mediated cytotoxicity (ADCC), the release of granuloenzyme and perforin [9, 10]. On the one hand, a certain number of literatures have verified the feasibility of NKs-based regimens for the administration and immunosurveillance of diverse hematological malignancies, including Chédiak-Higashi syndrome, X-linked lymphoproliferative syndrome, chronic lymphocytic leukemia (CLL), myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), multiple myeloma (MM), and aggressive non-Hodgkin lymphoma [11, 12]. On the other hand, NKs have been applied to the management of multiple solid tumors in both preclinical and clinical investigations, including gastric carcinoma, pancreatic cancer, non-small cell lung cancer (NSCLC), and recurrent or refractory neuroblastoma [11, 13, 14].

State-of-the-art renewal has also indicated the challenges in limiting NK cell therapeutics for a wide-ranging cancer treatment, and in particular, the source of stability and the concomitant variations in cellular vitality [15]. Currently, NKs have been amplified and activated from a variety of origins, including NK cell lines (e.g., YT, NK-92), peripheral blood, perinatal blood (e.g., cord blood, placental blood), memory-liker NK cells, and even differentiated from hematopoietic stem cells (HSCs) and pluripotent stem cells (PSCs) [11, 15, 16]. Of them, peripheral blood and cord blood have been considered with the widest applications and robust application ability, respectively [16, 17]. However, the systematic and detailed comparison of the biological phenotypes and transcriptomic properties of the indicated NKs from peripheral blood (PB-NKs) and cord blood (CB-NKs) are largely obscure.

Therewith, in this study, we isolated mononuclear cells (MNCs) from both peripheral blood and cord blood, and verified the diverse variations in the biological phenotypes including total and subsets of resident NKs. Meanwhile, we took advantage of our "3ILs"-based strategy for the further analyses of activated NKs, and found that CB-NKs revealed superiority in cellular vitality and cytotoxicity over PB-NKs. Furthermore, with the aid of transcriptomic analyses, we observed the conservation and multifaceted variations in gene expression profiling and genetic variations. Collectively, our data indicated the similarities and differences in biological and transcriptomic properties, which would benefit the further dissection of CB-NKs and PB-NKs and help facilitate the CB-NKs-based cytotherapy in future.

Materials and methods

Isolation of MNCs from CB and PB

MNCs were isolated from both cord blood (CB-MNCs) and peripheral blood (PB-MNCs) of heathy donors under the supervision of the Ethics Committee of Gansu Provincial Hospital according to the guideline of Helsinki (2022-088, 2023-120). In details, the aforementioned CB-MNCs and UC-MNCs were respectively isolated using the Ficoll (Sigma-Aldrich, USA) density gradient centrifugation as we reported before [17, 18].

Expansion and activation of NKs from CB-MNCs and UC-MNCs

The *in vitro* expansion and activation of NKs were accomplished using the "3ILs"-based strategy (3ILs refers to rhIL-2, rhIL-15, rhIL-18) as we recently reported [17]. Briefly, 2×10^6/ml CB-MNCs or PB-MNCs were cultured in NK MACS Medium (Miltenyi Biotech, Germany) with 1000 U/mL (100 ng/mL) rhIL-2, 10 ng/mL rhIL-15, 50 ng/mL rhIL-18 (PeproTech Inc., USA) addition for 14 days (the culture medium was changed every two days, and the cell concentration was adjusted to 2×10^6/ml) in 37°C, 5% CO_2 [17]. Cell counting was accomplished with Trypan Blue staining, and the proportion of the total or subpopulations of NKs were accomplished with flow cytometry (FCM) assay. The detailed information of the cytokines was available in Table S1.

Cell cycle and apoptosis detection

The cell cycle and apoptosis of the indicated NKs was analyzed as we reported before [16, 19]. In details, NKs were fixed with 70% (v/v) cooled ethanol at 4°C. Then, the cells were washed with 1×PBS, and turned to the Propidium iodide (PI) staining and the BD LSR II (BD Biosci, USA) for cell cycle detection according to the manufacturer's instructions. As to apoptosis analysis, 1×10^6 cells in the indicated groups (rCB-NK, rPB-NK, eCB-NK, ePB-NK) were washed with 1×PBS for twice at 4°C, and incubated with Annexin V Apoptosis Detection Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. After that, the cells were washed with 1×PBS for twice at 4°C in dark, and turned to apoptosis analysis by utilizing the FACS Canto II (BD Biosci, USA) and the FlowJo 10.0 software (Tree Star, USA).

Flow cytometry (FCM) assay

The proportion of MNCs and NKs were measured with the FCM assay as we recently reported [16, 19]. In details, the aforementioned cells in the indicated time points were collected and washed with 1×PBS for twice. Then, 1×10^6 cells were incubated with the indicated fluorescence-conjugated antibodies at 4°C in dark. After that, cells were washed with 1×PBS for twice and turned to FACS Canto II (BD Biosci, USA) for FCM detection, including total NKs ($CD3^-CD56^+$), subpopulations of NKs ($CD16^+$, $NKG2D^+$, $CD25^+$, $NKp44^+$, $NKp46^+$) and the relative subsets (Annexin V^+, 7-AAD^+, $CD4^+$, $CD8^+$, $CD107a^+$). The data were analyzed using the FlowJo 10.0 software (Tree Star, USA). The information of the indicated antibodies was available in Table S2.

Cytotoxicity assay of NKs

To compare the cytotoxicity of eCB-NKs and ePB-NKs, we took advantage of the co-culture model as reported before [16, 17]. In brief, the human myeloid leukemia cell line (K562) was collected and labelled with CellTrace Violet (Invitrogen, USA) as we reported before [17, 20]. Then, eCB-NKs or ePB-NKs were co-cultured with the aforementioned K562 cells at a series of effector-to-target ratios (E:T=3:1, 1:1, 1:3, 1:5) for 8 hrs. After that, the cells were labeled with the indicated antibodies (e.g., CD3, CD56, CD107a) and the Precision Count Beads (BioLegend, USA). After washing with 1×PBS for twice, the cells were turned to FACS Canto II (BD Biosci, USA) for detection. Cytotoxicity of NKs = $(1 - N_2/N_1) \times 100\%$. N_1 and N_2 represent the total living K562 cells in the control and experimental groups, respectively [17, 19].

RNA-SEQ analysis and bioinformatics analyses

To verify the potential similarities and variations in transcriptomic properties, eCB-NKs and ePB-NKs at day 14 of *in vitro* culture were lysed with the TRIZol reagent (ThermoFisher, USA) for total mRNA preparation according to the manufacturer's instructions [19, 21]. After that, the mRNAs were sent for RNA-sequencing (RNA-SEQ). Then, a variety of bioinformatics analyses were conducted for dissecting the transcriptomic features of eCB-NKs and ePB-NKs, including HeatMap diagrams, Principal Component Analysis (PCA), hierarchical cluster analysis, Volcano Plot, Gene Set Enrichment Analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology biological process (GOBP) as we reported before [22, 23]. The raw data of the gene expression in eCB-NKs and ePB-NKs at mRNA level was available in Table S3.

Statistical analyses

As we reported before, statistical analyses were conducted with the Graph Pad Prism 6.0 (San Diego, USA) software [24, 25]. For the analysis of the data between two unpaired groups and the data among multiple unpaired groups, we conducted the student's unpaired T test and one-way ANOVA test, respectively. All data were shown as mean ± SEM (N=3 independent experiments). Only the *P* value less than 0.05 ($P<0.05$) was considered as statistically significant. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; NS, not significant.

Results

Perinatal blood showed higher percentage of NKs whereas cord blood with more robust amplification

To dissect the multifaceted biological features of resident and activated NKs (rNKs, aNKs) in PB and CB, we primarily enriched mononuclear cells (MNCs) and conducted the "3ILs"-based

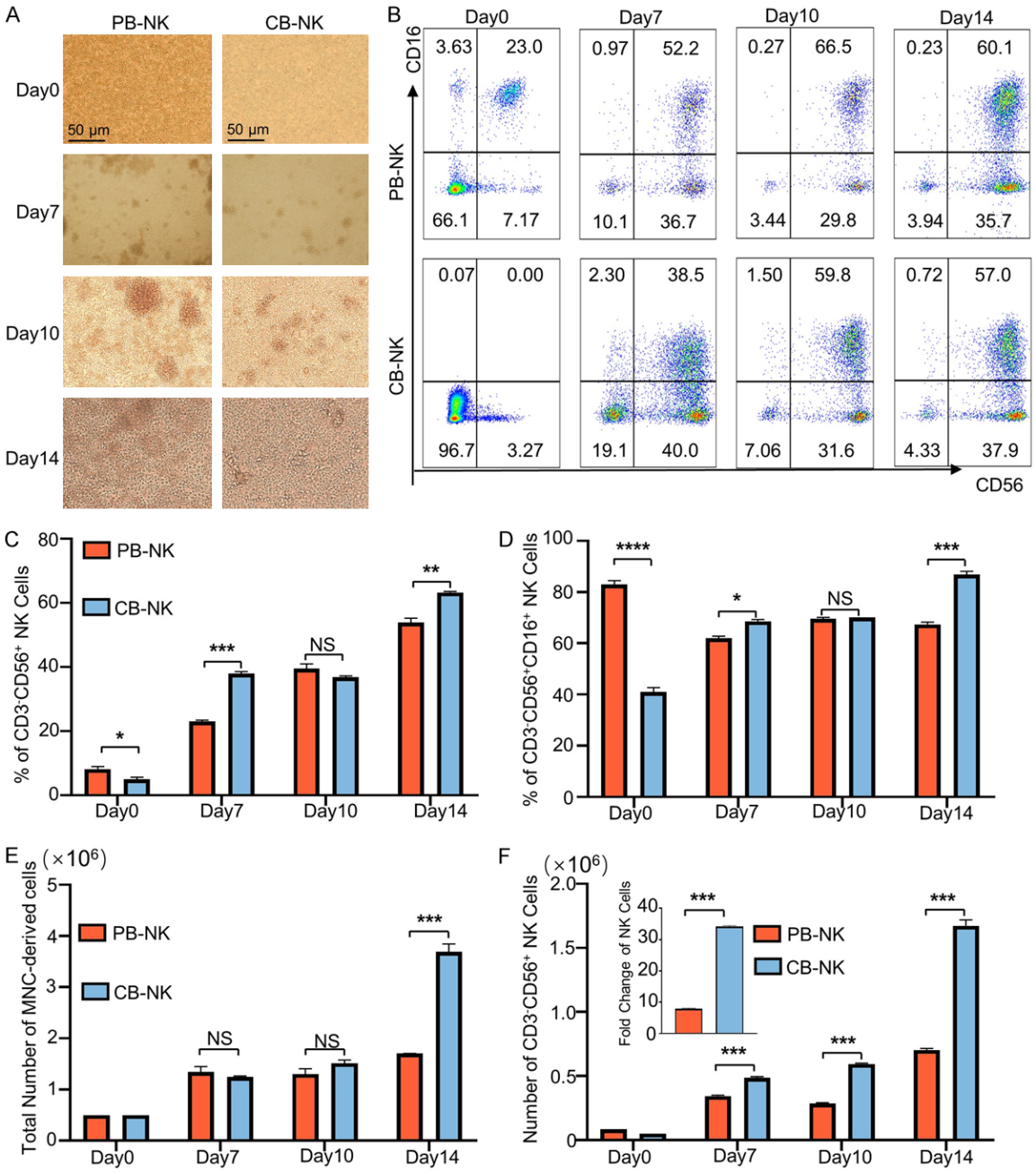


Figure 1. The comparison of resident NKs in MNCs enriched from PB and CB. (A) Representative phase contrast images of MNC-derived NKs at the indicated time points (day 0, 7, 10, 14) during the 14-days' *ex vivo* induction. Scale bar =50 μm. (B-D) Representative flow cytometry (FCM) diagrams (B) and statistical analysis of total $CD3^-CD56^+$ (C) and $CD3^-CD56^+CD16^+$ (D) resident NKs (rNK) in PB-derived MNCs (rPB-NK) and CB-derived MNCs (rCB-NK). (E, F) Statistical analyses of total number of MNC-derived cells (E) and $CD3^-CD56^+$ NKs (F) at the indicated time points (day 0, 7, 10, 14) during the 14-days' *ex vivo* induction. All data were shown as mean ± SEM (N=3). NS, not significant; ****, P<0.0001.

strategy for NK cell activation and amplification (**Figure 1A**). Intuitively, both the resident PB-NKs and CB-NKs revealed typical spheroidal morphology (Day 0), followed by clumping structure as *in vitro* differentiation (**Figure 1A**).

With the aid of FCM assay, we found the proportion of resident CB-NKs (Day 0) was higher than the concomitant resident PB-NKs (**Figure 1B**). Interestingly, after a 14-day's amplification and activation, the percentages of total NKs ($CD3^-$

$CD56^+$) and total activated NKs ($CD3^-CD56^+CD16^+$) in the CB-NK group were higher than those in the PB-NK group, respectively (**Figure 1B-D**). By conducting living cell counting, we found the numbers of MNCs and total NKs in the CB-NK group were higher compared with the CB-NK group, respectively (**Figure 1E**, **1F**). Taken together, CB-NKs revealed more robust *in vitro* expansion and activation activities over PB-NKs.

Expanded CB-NKs revealed moderate superiority in activation and cellular vitality over PB-NKs

Having preliminarily verified the phenotype of the indicated NKs, we next aim to dissect the similarities and variations in the subpopulations. As shown by the FCM diagrams and statistical analyses, resident CB-NKs showed higher percentage of $NKG2A^+$ NK cell subset but with lower $NKp46^+$ subset compared to resident PB-NKs (**Figure 2A**, **2B**). Instead, compared to the activated PB-NKs, higher proportions of $NKG2D^+$, $NKp46^+$ and $NKp44^+$ subsets were observed in activated CB-NKs (Day 14) (**Figure 2A**, **2B**).

With the aid of FCM assay, we found the proportion of $7\text{-}AAD^+$Annexin V^+ apoptotic NKs in PB-NK group was higher than that in the CB-NK group, whereas the proportions of $7\text{-}AAD^-$ Annexin V^+ and Annexin V^+ subsets showed the reverse tendency (**Figure 2C**, **2D**). As to the indicated subpopulations of NKs in the G_0/G_1, S and G_2/M stages, there were minimal differences between the indicated two groups (**Figure 2E**, **2F**). Taken together, both the activated PB-NKs and CB-NKs revealed diverse differences in the subpopulations and apoptotic cells of NKs.

Expanded CB-NKs exhibited higher cytotoxicity over PB-NKs

To dissect the cytotoxicity of the indicated NKs, we co-cultured PB-NKs or CB-NKs with the myeloid leukemia cell line K562. As shown in **Figure 3A**, **3B**, the percentage of NKs with CD107a expression in the CB-NK group was lower than that in the PB-NK group at diverse effector-to-target ratios (E:T=3:1, 1:1, 1:3, 1:5) for 8 hrs (**Figure 3A**, **3B**). Furthermore, we noticed that CB-NKs revealed higher cytotoxicity against K562 tumor cells over that of PB-NKs at a various E:T ratios (**Figure 3C**, **3D**). Collectively, our data indicated that CB-NKs showed moderate superiority over PB-NKs in cytotoxicity upon K562 tumor cells over PB-NKs.

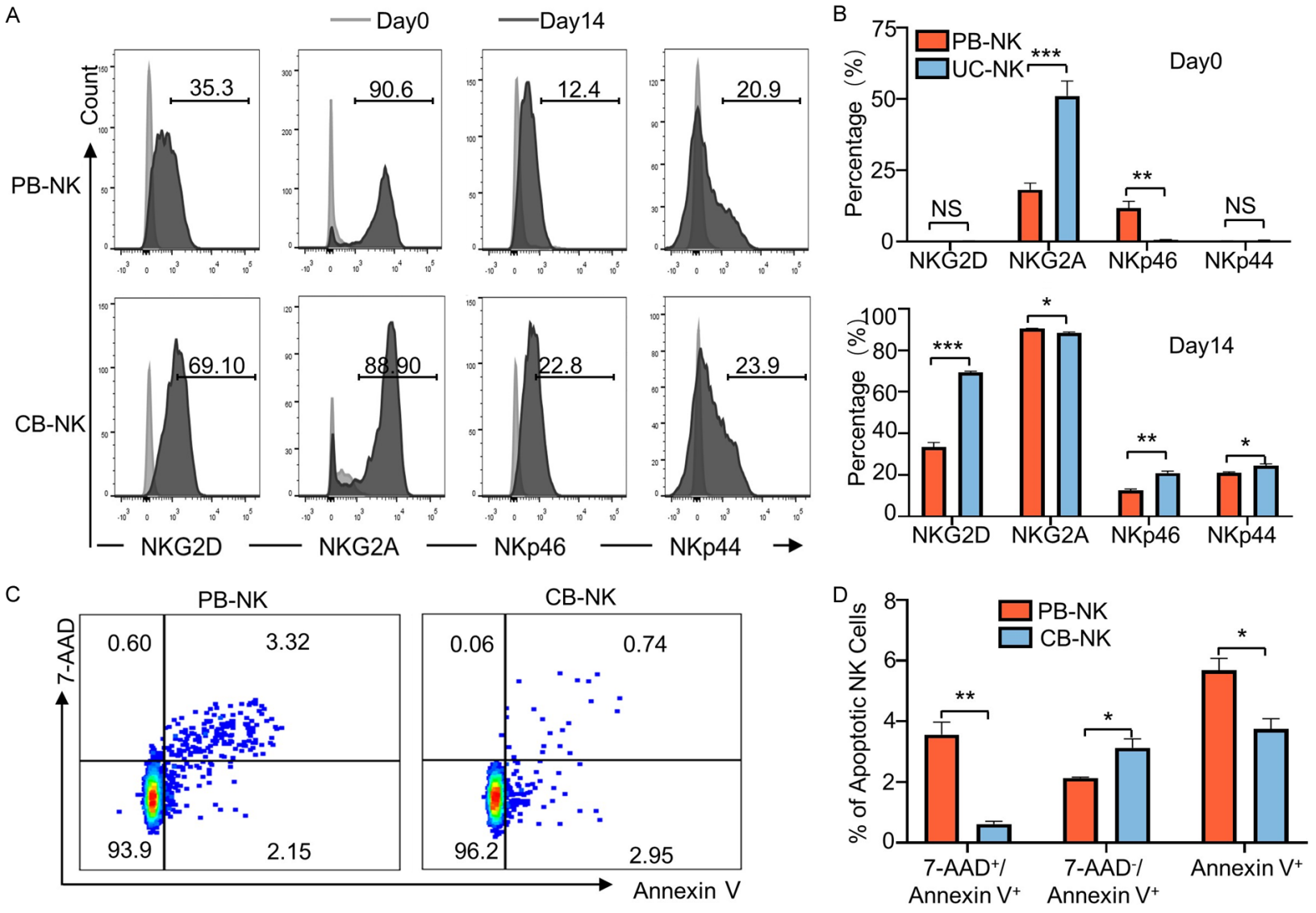
Expanded CB-NKs and PB-NKs revealed multifaceted similarities and diversity in gene expression profiling

Having compared the biological properties, we next turn to verify the similarities and variations of CB-NKs and PB-NKs at the transcriptomic level. Based on the boxplots and accumulation map of gene expression, we found both CB-NKs and PB-NKs revealed similarities in gene expression profiling (**Figure 4A**, **4B**). As shown by the principal component analysis (PCA), there's no tendency in the distribution of CB-NKs and PB-NKs (**Figure 4C**).

According to the Venn map diagram and volcano plot, 622 and 834 genes were upregulated and downregulated in CB-NKs when compared to PB-NKs, respectively (**Figure 4D**, **4E**). Interestingly, we observed the PB-NKs (PB-1, PB-2, PB-3) and CB-NKs (CB-1, CB-2, CB-3) revealed stronger clustering relationships within groups (**Figure 4F**). Simultaneously, we didn't find obvious differences in the distribution of genes with genetic variations or variable shear event (VSE) between PB-NKs and CB-NKs according to the Circos diagrams and VSE charts (**Figure 4G**, **4H**). Collectively, our data indicated the conservations and diverse differences in gene expression profiling and genetic variation.

The landscapes of gene subsets and gene ontology of expanded CB-NKs and PB-NKs

To further dissect the transcriptomic features, we took advantage of Gene Set Enrichment Analysis (GSEA), and found that CB-NKs and PB-NKs showed significant differences in diverse biological processes, including co-translational protein targeting to membrane, humoral immune response, antibiotic metabolic process, translational initiation, defense response to bacterium, and antibiotic metabolic process, which were mainly involved in cytotoxicity- and metabolism-associated processes (**Figure 5A**). Simultaneously, we also noticed the variations of specific gene subsets between CB-NKs and PB-NKs in signaling pathway, including TNF-α


A
Day0
Day14
Count
PB-NK
CB-NK
35.3
90.6
12.4
20.9
69.10
88.90
22.8
23.9
NKG2D
NKG2A
NKp46
NKp44
B
PB-NK
UC-NK
Day0
Percentage (%)
NS
***
**
NS
Day14
*
C
PB-NK
CB-NK
7-AAD
0.60
3.32
93.9
2.15
0.06
0.74
96.2
2.95
Annexin V
D
PB-NK
CB-NK
% of Apoptotic NK Cells
7-AAD+/ Annexin V+
7-AAD-/ Annexin V+
Annexin V+

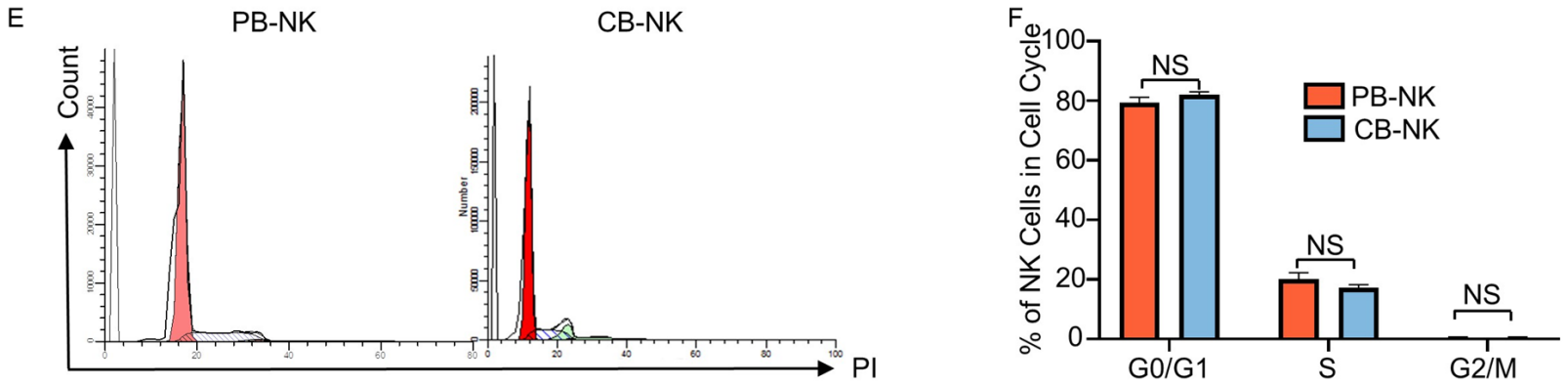


Figure 2. The comparison of the expanded NKs in content and cellular vitality. (A, B) Representative FCM diagrams (A) and statistical analysis (B) of the percentages of NK cell subsets (NKG2D$^+$, NKG2A$^+$, NKp46$^+$, NKp44$^+$) from the resident NKs (rNK) at day 0 to the corresponding expanded NKs (eNK) at day 14. (C, D) Representative FCM diagrams (C) and statistical analysis (D) of apoptotic NKs in ePB-NK and eCB-NK at day 14. (E, F) The representative distribution (E) and statistical analysis (F) of ePB-NK and eCB-NK in the indicated sub-stages of cell cycle. All data were shown as mean ± SEM (N=3). NS, not significant; **, P<0.01; ****, P<0.0001.

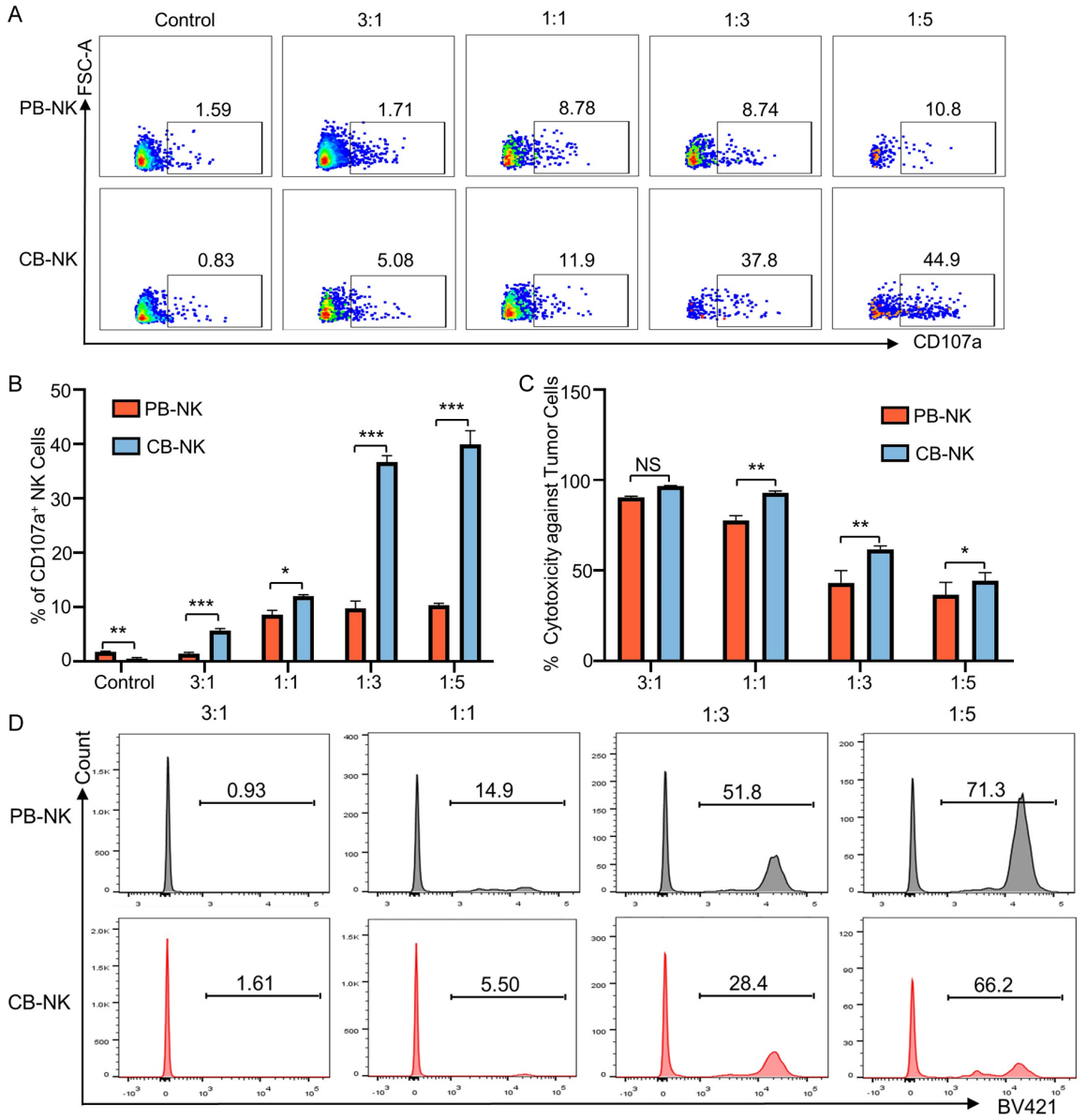
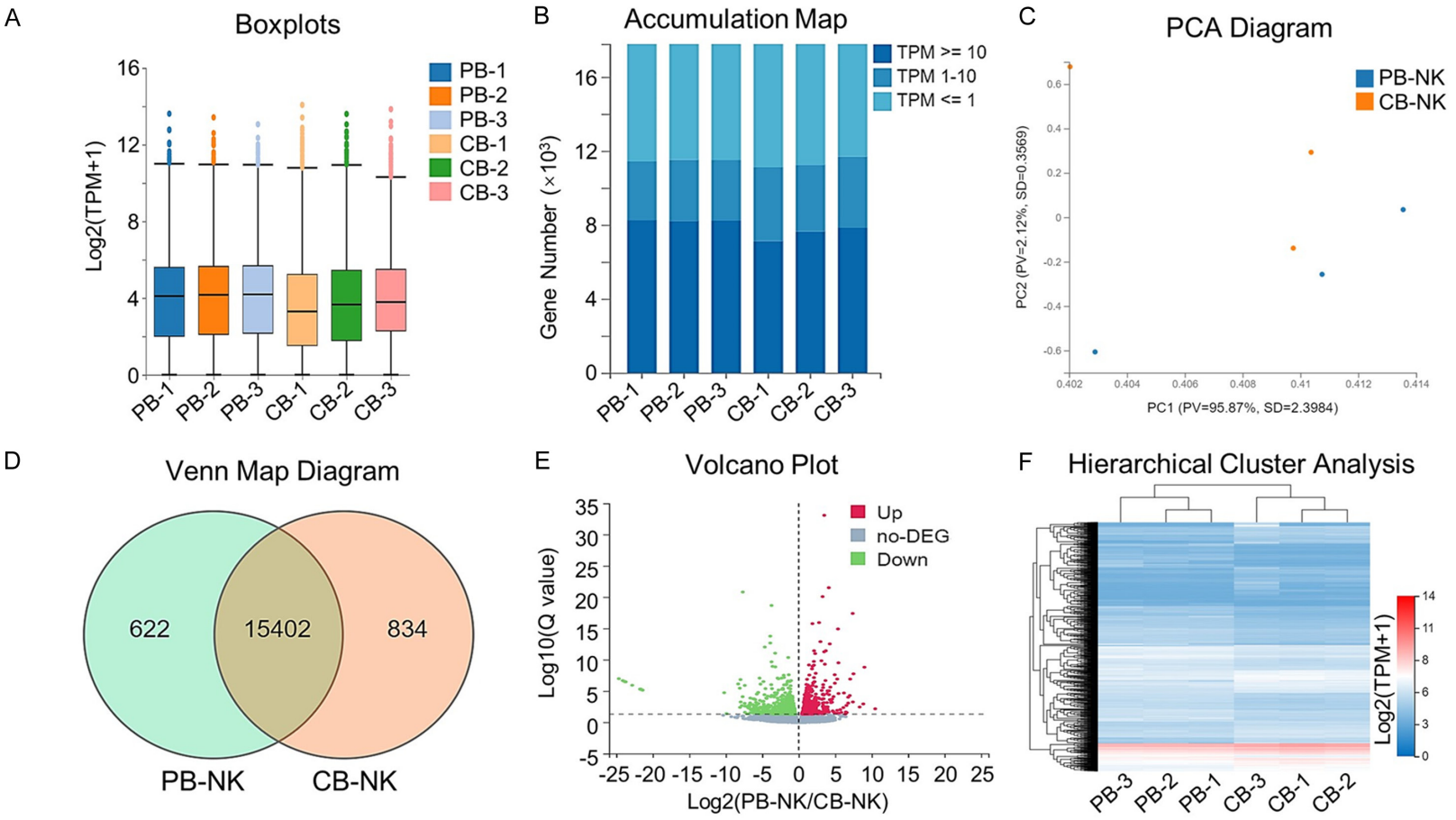


Figure 3. Comparison of *ex vivo* tumor killing cytothoxicity of the expanded PB-NKs and UC-NKs. (A, B) Representative FCM diagram (A) and statistical analysis (B) of CD107a$^+$ ePB-NK and eCB-NK in coculturing with K562 cell line at the effector-to-target ratios (E:T=3:1, 1:1, 1: 3, 1:5); (C, D) The comparison of the cytotoxicity of the expanded PB-NK and UC-NK against the aforementioned K562 cell line at the effector-to-target ratios based on the calculations of BV-421$^+$ cells (C) and proportion of tumor cells (D). All data were shown as mean ± SEM (N=3). NS, not significant; *, P<0.05; ***, P<0.001.

signaling via NF-κB, KRAS signaling, and relative biological processes (e.g., coagulation, EMT, complement, myogenesis) (**Figure 5B**).

Furthermore, we turned to gene ontology biological process (GOBP) analysis, and found that the differentially expressed genes (DEGs) between CB-NKs and PB-NKs were mainly involved in NK cell-associated bio-functions, such as immune response, inflammatory response, positive regulation of tumor necrosis factor (TNF) and immune system process (**Figure 5C**). Notably, as shown by the Kyoto Encylopaedia of Genes and Genomes (KEGG) analysis, the DEGs were enriched in cytotoxicity- and cellular vitality-associated signals, including TNF signaling pathway, cytokine-cytokine receptor interaction, NF-κB signaling path-


A
Boxplots
Log2(TPM+1)
PB-1
PB-2
PB-3
CB-1
CB-2
CB-3
B
Accumulation Map
Gene Number (×10³)
TPM >= 10
TPM 1-10
TPM <= 1
C
PCA Diagram
PB-NK
CB-NK
PC2 (PV=2.12%, SD=0.3569)
PC1 (PV=95.87%, SD=2.3984)
D
Venn Map Diagram
622
15402
834
PB-NK
CB-NK
E
Volcano Plot
Up
no-DEG
Down
Log10(Q value)
Log2(PB-NK/CB-NK)
F
Hierarchical Cluster Analysis
Log2(TPM+1)

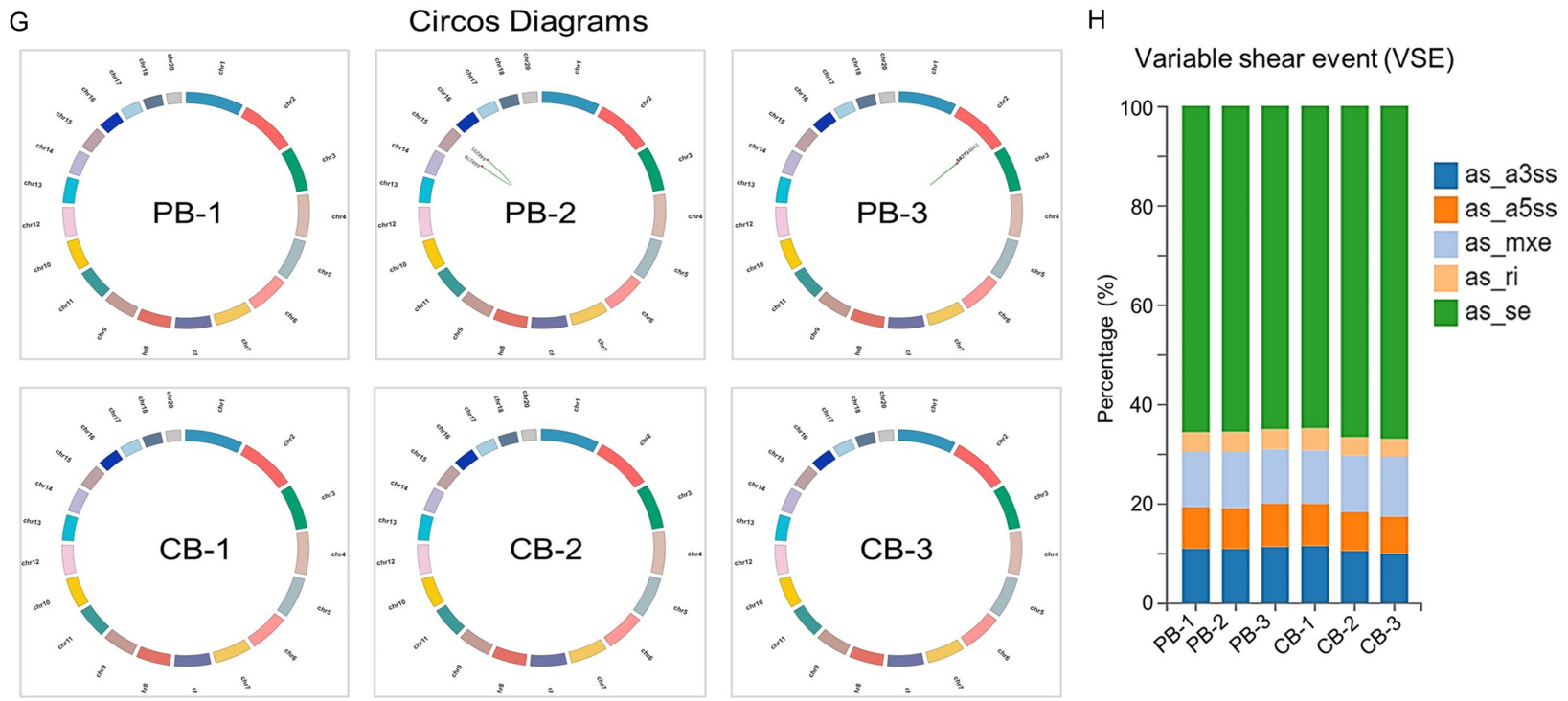


Figure 4. Comparison of the gene expression profiling of the expanded PB-NKs and UC-NKs. (A, B) The bar chart (A) and accumulation map (B) of gene numbers with the indicated expression levels based on TPM values in the expanded PB-NK (PB-1, PB-2, PB-3) and eCB-NK (CB-1, CB-2, CB-3). (C) The PCA diagram of the indicated the expanded PB-NK and CB-NK based on FPKM values. (D) The Venn Map Diagram revealed the number of genes in the expanded PB-NK and CB-NK. (E, F) Volcano Plot (E) and HeatMap diagram (F) of the differentially expressed genes (DEGs) in the expanded PB-NK and CB-NK. (G) Circos diagrams revealed the distribution of fusion genes with genetic variations between the expanded PB-NK and CB-NK. (H) The histogram showed the percentage of genes with variable shear event (VSE) between the expanded PB-NK and CB-NK.

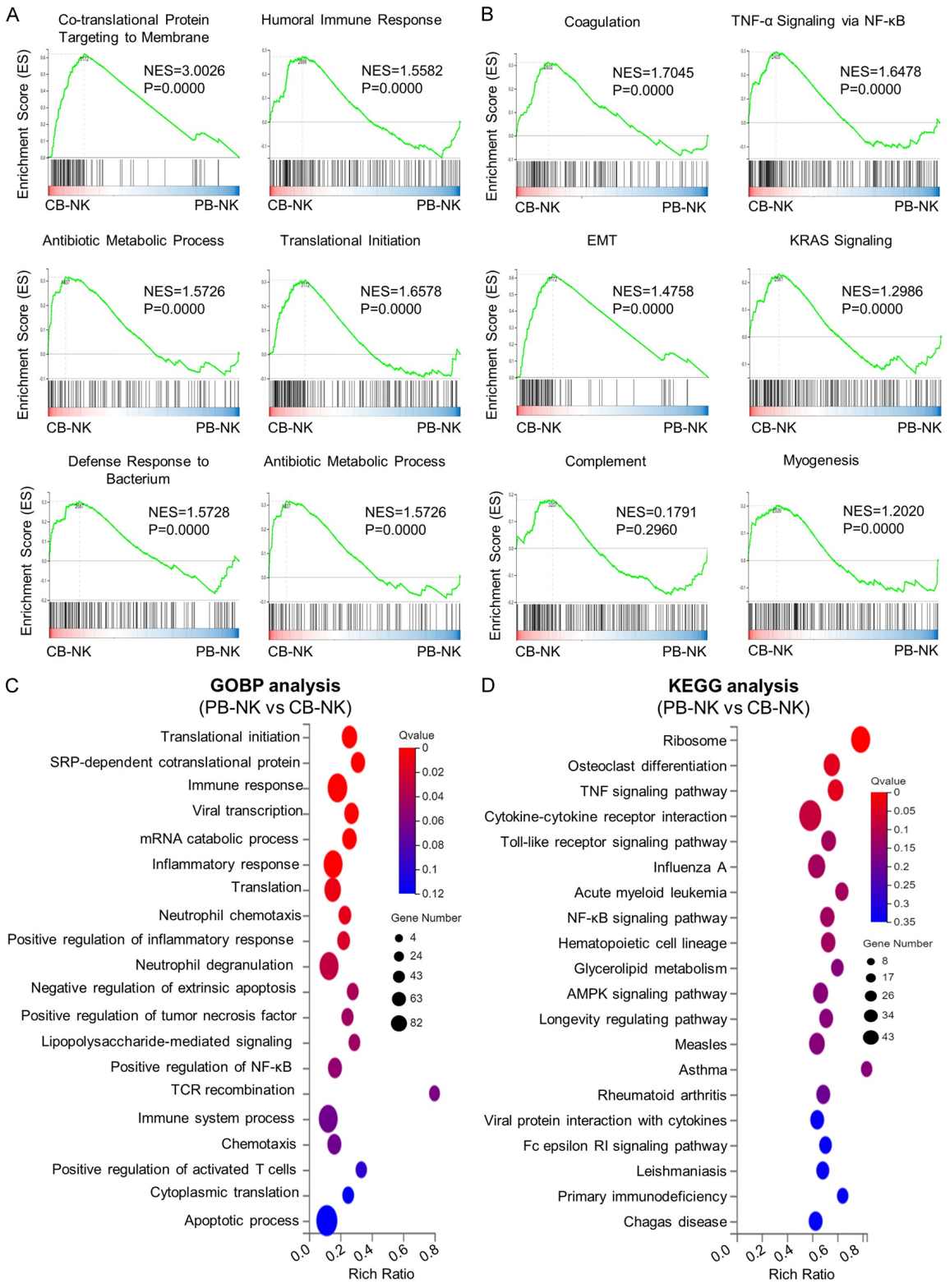


Figure 5. Signaling pathway analysis and variation spectrums between the expanded PB-NKs and UC-NKs. (A, B) GSEA diagrams of significantly different biofunction-associated gene sets (A) and signaling pathway-associated gene sets (B) between the expanded PB-NK and UC-NK. (C, D) GOBP analysis (C) and KEGG analysis (D) of the DEGs in the expanded PB-NKs and CB-NKs.

way, AMPK signaling pathway, and Toll-like receptor signaling pathway (**Figure 5D**). Overall, these data further indicated the variations in the landscape of gene subsets and signaling pathways between CB-NKs and PB-NKs.

Discussion

Longitudinal studies have indicated the promising prospect of NKs for the administration of infectious diseases and cancers including hematologic malignancies and metastatic solid tumors via orchestrating the diverse mode of action [7, 13, 26]. Recently, we and Zhang *et al* respectively demonstrated the multifaceted variations in the efficacy of cell transplantation on graft-versus-host disease (GvHD) mice and acute liver failure (ALF) mice, which suggested the prerequisites of cellular vitality for cytotherapy [27, 28]. Herein, we found CB-NKs and PB-NKs with multidimensional similarities and differences in biological and transcriptomic properties, and confirmed the superiority of CB-NKs in cellular vitality (e.g., more robust *ex vivo* proliferation, less apoptotic cells, higher cytotoxicity) and cytotoxicity-associated characteristics (e.g., inflammatory response, NF-κB signaling pathway), which would benefit the further dissection of the biofunction of CB-NKs and the concomitant cancer immunotherapy in future.

To date, cancers have become a leading cause of deaths and a major public health burden worldwide [29, 30]. For example, over 4,568,000 patients were newly diagnosed with diverse cancers in China, and 3,002,000 cases were died from cancers and the related fatigue in 2020 [31, 32]. Currently, a variety of implementation strategies are developed for tumor survivorship and treatment, including chemotherapy [33], radiotherapy [34], peptide-based neoantigen vaccine [35, 36], gene therapy [37-39], oncolytic virotherapy [40], RNA vaccine [41, 42], photothermal therapy (PTT) [43-45], and the nanomaterial-mediated nanotheranostics [11, 46]. However, the diverse inherent shortcomings of the therapeutic strategies have partially hindered the further improvement in cancer management, including off-target effects, severe toxicity, drug delivery barriers, graft-versus-host disease [11, 46]. Distinguish from the abovementioned treatment, cellular immunotherapy of diverse kinds has been considered as promising remedy for cancer administration, such as tumor infiltrating lymphocytes (TILs), NKs, macrophages (Mφ), cytokines-induced killer cells (CIKs), chimeric antigen receptor-transduced T cells (CAR-Ts) or CAR-transduced NKs (CAR-NKs) [13, 47-51]. Of them, allogeneic NK cell-based immunotherapy has been considered with advantaged characteristics in immune defense and cancer administration over the relative counterparts via simultaneously modulating the innate and adaptive immune response [1, 11].

For decades, NKs have been generated from various sources such as peripheral blood, cord blood, placental blood, bone marrow, hematopoietic stem cells (HSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and even NK cell lines (e.g., NK-92, YT) [11, 13, 52]. Differ from bone marrow with limitations for *ex vivo* NK cell amplification, stem cells (e.g., HSCs, ESCs, iPSCs) and NK cell lines are adequate for large-scale homogeneous NK cell generation but with diverse defects (e.g., high-cost, deficiency in cytotoxicity, potential safety hazards due to the differentiation efficiency) [13, 53]. Instead, cord blood and peripheral blood have been considered with diverse superiorities and preferable application prospects for allogenic NK cell preparation [11]. Generally, NKs occupy less than 5% of MNCs in cord blood cells, whereas with a proportion of 5% to 20% of MNCs in peripheral blood [11, 54]. Longitudinal studies has indicated the unique capacity of PB-NKs in killing a broad spectrum of tumor cells, which have also been considered as the dominant ingredient for NK cell generation in clinical practice but with limitations in stability and yield [55]. State-of-the-art literatures have turned to cord blood for large-scale NK cell production because the generated CB-NKs have been indicated with vigorous cytolytic activity [11]. In this study, we took advantage of our well-established “3ILs”-based strategy for *ex vivo* NK cell generation, and further verified the similarities and variations of resident NKs (rNKs) and activated NKs (aNKs) from the aspects of biological and transcriptomic properties, and in particular, CB-NKs showed preferable *ex vivo* amplification and cellular vitality, enhanced cytotoxicity over PB-NKs. In particular, by conducting RNA-SEQ analyses, we verified that ePB-NKs and eCB-NKs showed multifaceted similarities and variations

at the transcriptomic level including specific gene expression pattern and signaling cascades (e.g., TNF-α signaling, NF-κB signaling, AMPK signaling, KRAS signaling, complement) and genetic variations (e.g., VSE). Interestingly, we noticed the further activation of aNKs compared to rNKs, which further indicated the pivotal role of "NK cell education" for functional maturation of NKs.

Collectively, we verified the multidimensional properties of CB-NKs and PB-NKs from the aspects of biological phenotypes and transcriptomic features, and indicated the moderate superiority of CB-NKs in cellular vitality and cytotoxicity. Our data suggested cord blood as excellent candidates for the "off-the-shelf" antitumor immunotherapy and the concomitant next-generation of allogeneic CAR-NK cell preparation with both NK cell receptor-dependent and CAR-dependent mode of action. The indicated CB-NKs would also effectively avoid the diverse adverse effects of CAR-T regimens such as cytokine release syndrome (CRS), immune cell-associated neurotoxicity syndrome (ICANS), GVHD and neurotoxicity, which thus represented the novel therapeutic paradigm for facilitating the body immunity to reinforce anti-tumor responses and eventually obliterate malignancies [13, 53].

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Disclosure of conflict of interest

None.

Abbreviations

CB, Cord blood; NK, natural killer; DEGs, differentially expressed genes; HSCs, hematopoietic stem cells; PCA, principal component analysis; FPKM, fragments per kilobase million; VSE, variable shear event; DVSE, differentially variable shear event; PPI, protein-protein interaction; GSEA, gene set enrichment analysis; KEGG, Kyoto encyclopedia of genes and genomes; GOBP, gene ontology biological process; TPM, transcripts per kilobase of exon model per million mapped reads; MNCs, mononuclear cells; rNKs, resident natural killer cells; eNKs, expanded natural killer cells; CRS, cytokine release syndrome; ICANS, immune cell-associated neurotoxicity syndrome; NSCLC, non-small cell lung cancer.

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Table S1. The list of the cytokines

Reagent	Cat. No.	Conc.	Source
Recombinant Human IL-2 (rhIL-2)	200-02	100 ng/uL	PeproTech Inc., USA
Recombinant Human IL-15 (rhIL-15)	200-15	10 ng/uL	PeproTech Inc., USA
Recombinant Human IL-18 (rhIL-18)	119-BP-100	50 ng/uL	R&D Systems, USA

Table S2. The list of the antibodies

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
PE anti-human IgG	409304	BioLegend
Percision 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