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In Vitro Expansion and Transduction of Primary NK Cells Using Feeder Cells Expressing Costimulatory Molecules and IL-21

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ABSTRACT

Natural Killer (NK) cells are an important population of the immune system, and NK cell-based therapy has shown great potential in the treatment of cancers. However, to apply NK cells clinically, producing a large number of cells with high cytotoxicity remains a challenge. Current strategies focus on employing different irradiated feeder cells to stimulate NK expansion, maturation, and cytotoxicity. While co-stimulatory signals play critical roles in promoting NK cell proliferation and activating their functions, the exploitation of these signals for expanding NK cells has not been fully explored. To identify the optimal engineered feeder cells for expanding umbilical cord blood-derived NK cells, we generated different feeder cells expressing the co-stimulatory molecules CD80, 4-1BBL, or membrane-bound IL-21 (mbIL21). We then evaluated the transduction efficacy of a chimeric antigen receptor (CAR) construct into expanded NK cells using various lentiviral vectors. Our results showed that CD80, in combination with 4-1BBL and mbIL21, induced the highest expansion of NK cells from cord blood. The expanded NK cells displayed higher cytotoxicity toward target cells compared to T cells following CAR transduction using BaEV lentivirus.

1 | Introduction

NK cells are key components of the innate immune system, known for their ability to detect and eliminate infected or abnormal cells without prior sensitization. They play a vital role in immune surveillance by balancing their activating and inhibitory receptors to target virus-infected cells, tumors, and other abnormalities [1, 2]. NK cells have emerged as a safe and effective alternative to T cells in adoptive transfer therapies. Unlike T cells, which are associated with severe adverse effects such as graft-versus-host disease, cytokine release syndrome, and immune effector cell-associated neurotoxicity syndrome, NK cells can recognize and eliminate

Abbreviations: CAR, chimeric antigen receptor; mbIL21, membrane-bound IL-21; MHC, major histocompatibility complex; NK, natural killer.

Thi Bao Tram Tran and Thi Van Anh Bui are contributed equally to this work.

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cancer cells independently of major histocompatibility complex (MHC) compatibility [3]. They benefit from reduced or absent MHC expression in certain tumors to enhance their killing activity [3]. CAR-engineered NK cells further amplify this cytolytic potential, effectively eliminating cancer cells while avoiding the limitations of CAR-T cells. Currently, over 40 clinical trials are evaluating the safety and efficacy of adoptive NK cell therapy for solid tumors, using either engineered or non-engineered NK cells [4, 5]. Notably, positive outcomes have been reported in neuroblastoma patients receiving allogeneic NK cells combined with GD2-targeting antibodies [6, 7]. Evidence from trials in hematologic malignancies and ovarian cancer also suggests promising outcomes of NK cell therapy [8–11].

A significant challenge in NK cell-based 'off-the-shelf' therapy is producing large quantities of NK cells with enhanced cytotoxicity for clinical use. NK cells typically comprise 10%–15% of lymphocytes in adult peripheral blood but can make up to 30% in umbilical cord blood [12]. Cord-blood NK cells often exhibit an immature phenotype with lower CD16 expression compared to peripheral blood NK cells [13, 14]. NK cells sourced from both sources can undergo in vitro expansion using feeder-free or feeder-dependent methods. Feeder-free approaches, reliant on cytokines like IL-2, IL-15, IL-21, along with IL-12 or IL-18 yield limited expansion, despite resulting in NK cells with enhanced cytotoxicity [15, 16]. Conversely, expansion methods involving irradiated feeder cells have demonstrated more robust yields, achieving over 40,000-fold expansions. Feeder cells are often engineered to express common ligands for co-stimulatory receptors such as 4-1BBL (41BBL), OX40L, or membrane-bound cytokines like IL-15 and IL-21 [17, 18]. In these studies, the expansion capacity of each stimulatory receptor or cytokine was assessed solely in comparison to the wild-type phenotype of the feeder cell line. While the search for novel NK cell expansion stimulators continues, existing feeder systems should be integrated into a standardized experimental design. Such an approach would provide clearer insights into the expansion levels achieved by each stimulatory factor and highlight the variability in their efficiency within *in vitro* NK cell cultures.

Often recognized as a costimulatory molecule, CD80 exhibits transient expression on B cells, macrophages, and dendritic cells upon activation [19, 20]. Its upregulation provides a crucial co-stimulatory signal to T cells, enhancing the activation initiated by the T cell's recognition of presented antigens via the T cell receptor. This sustains T cell responses with a lowered activation threshold, enhanced survival, and increased cytokine production, notably IL-2 [21–24]. The role of CD80 holds immense significance in tumor immune surveillance. The loss of CD80 alone enables tumors to evade immune cell attacks, leading to anergy and apoptosis among tumor-infiltrating T cells [25]. Intriguingly, the presence of CD80 on tumor cells can enhance recognition by NK cells, resulting in tumor lysis [26]. Several studies have highlighted the potential triggering effect of the CD80 molecule on NK cells. Initial findings in mice demonstrated that CD80 expression could prompt NK cell-mediated cell lysis. Interestingly, this effect was observed to surpass the protective role mediated by MHC class I molecules, as reported in one study [27]. Subsequent research by Wilson et al. provided further validation, showing that the introduction of human CD80, CD86, or CD80/86 molecules into murine B16.F1 melanoma cells increased their susceptibility to lysis by human NK cells [28]. Intriguingly, CD80 has not yet been explored as a target for engineering feeder cells used in NK cell expansion.

In addition to the challenge of expanding NK cells, their innate defense mechanisms, guided by pattern recognition receptors, make them resistant to viral transduction. This resistance poses a significant challenge for the development of CAR NK-based cell therapies [29, 30]. Recent exploration into different lentiviral systems as alternatives to the broadly tropic VSV-G systems has led to the identification of the Baboon envelope pseudotyped lentivirus, proving notably more effective in transducing primary human NK cells [31, 32].

In this study, we assessed the potential of CD80 as a stimulator for expanding and boosting cord-blood NK cells from different donors by expressing the gene, both alone and in combination with 41BBL and membrane-bound IL-21 (mbIL21), within the K562 cell line. Additionally, we explored the transduction of a CAR construct targeting carcinoembryonic antigen (CEA) into expanded NK cells using various lentiviral vectors. We further demonstrated the functionality of these cells post-transduction, comparing them with CAR-T cells in both 2D and 3D models.

2 | Materials and Methods

2.1 | Cell Lines

K562 cells were from ATCC. Engineered K562 cells expressing CD80, 41BBL, or mbIL21—individually or in combination—were generated via lentiviral transduction and sorted using the WOLFG2 Cell Sorter. These cells were cultured in RPMI 1640 (Gibco, USA) with 10% heat-inactivated FBS and 1% penicillin–streptomycin at 37°C with 5% CO_2 and validated by flow cytometry using anti-CD80 (#375409), 41BBL (#311505), and IL21 (#513003) antibodies (BioLegend). Lenti-X293T cells (Takara Bio, USA) were maintained in DMEM (Gibco, USA) with 10% FBS and 1% penicillin–streptomycin. MKN-45 cells were provided by Dr. Phu-Hung Nguyen (Thai Nguyen University of Science, Vietnam) and cultured in RPMI 1640 with 10% FBS and 1% penicillin–streptomycin. MCF-7 cells, courtesy of Dr. Thuy-Vy Nguyen (Ho-Chi-Minh City University of Science, Vietnam), were maintained in DMEM with 10% FBS and 1% penicillin–streptomycin. All cell lines were tested and confirmed mycoplasma-free.

2.2 | NK Cell Isolation and Expansion

Umbilical cord blood, collected with CPDA-1 anticoagulant, was diluted 1:2 with PBS and layered onto Lymphoprep (Stemcell Technologies, Canada) at a 2:1 ratio. Density-gradient centrifugation was performed for 30 min at 1000 g without the brake. Mononuclear cells were isolated from the white ring at the interface between Lymphoprep and plasma and washed with PBS. NK cells were isolated with the MojoSort Human NK Cell Isolation Kit (Biolegend, USA) and were cultured in AIM-V medium (Gibco, USA) with 10% heat-inactivated FBS (Cytiva, Sweden) and 200 IU/mL IL-2 (PeproTech, USA), with feeder cells. NK

cells in the feeder-free group were cultured with 500 IU/mL IL-2 and 20 ng/mL IL-15. The medium was refreshed every 2 days. For feeder-cell-dependent expansion, NK cells were stimulated at a 1:5 ratio with K562 or engineered K562 cells, irradiated with 100 Gy, and replenished every 7 days. Phenotype analysis was performed by staining for CD3, CD56, NKG2D, NKp46, NKp30, CD16, NKG2A, and TIGIT (Biolegend) markers, followed by flow cytometry analysis.

2.3 | RNA Sequencing

RNA from NK cells cultured with wild-type, 41BBL-mbIL21, and CD80-41BBL-mbIL21 K562 cells at D14 was isolated using Quick DNA/RNA Miniprep (Zymo). Total RNA was subjected to the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) to isolate intact poly(A) + RNA. RNA libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) and sequenced paired-end at 50× on the MGI DNBseq-G400 platform. Clean reads were obtained with Cutadapt (v3.4) and aligned to the GRCh38 reference genome using STAR (v2.7.9a). Reads mapped were counted with featureCounts (Subread v2.0.1) using GENCODE 43 gene annotation (Ensembl 109). Differentially expressed genes (DEG) were identified with the edgeR package (v4.1.2) [33]. Low-expression genes were excluded, and data were normalized using TMM. DEGs between groups were identified with glmQLFit in edgeR, applying an FDR-adjusted p-value < 0.05 and $|\log_2 FC| \geq 1$. Genes related to CD28 activity, signaling, proliferation, cytokine production, and cytotoxicity were visualized using volcano plots and heatmaps in R.

2.4 | Lentivirus Production and NK Cell Transduction

Lentiviruses were generated by transfecting Lenti-X293T cells (Takara Bio, US) with pMDLg/pRRE, pMD2.G, pRSV-Rev plasmids (NovoPro Bioscience, China), and pCDH-EF1a-DNAM-1 CAR-IRES-EGFP constructs (Epoch Life Science, USA) to express a CAR targeting CEA, consisting of VH and VL of αCEA, a CD8α hinge, DNAM-1 transmembrane domain, and 2B4/CD3ζ intracellular domain. For BaEV-pseudotyped lentiviral vectors, transfection included BaEVRLess plasmid along with pMDLg/pRRE, pRSV-Rev, pCDH-EF1a-DNAM-1 CAR-IRES-EGFP, and optionally pMD2.G. The BaEVRLess plasmid was provided by Prof. Verhoeyen Els and Dr. François-Loïc Cosset (CIRI, Inserm—U1111, Ecole Normal Supérieure, Lyon, France).

NK cell expansion for transduction was performed using CD80-41BBL-mbIL21 K562 cells at a 1:2 ratio for 5 days in AIM-V medium with 10% FBS and 200 IU/mL IL-2. Restimulation was done with CD80-41BBL-mbIL21 K562 cells at a 1:1 ratio for 2 days before transduction with CAR-lentivirus. Transduction occurred in 24-well plates precoated overnight with Retronectin (Takara Bio, USA) at 5 μg/cm^2. Lentiviral supernatant was added to the wells and centrifuged at 2000 g for 1.5 h at 4°C before adding NK cells, which were then centrifuged at 1000 × g for 1 h at 32°C. The medium was refreshed the next day and every 3 days thereafter, with CAR expression assessed 5 days post-transduction.

2.5 | Cytotoxicity Assay

NK, CAR-NK, and CAR-T cells were co-cultured with MKN-45 or MCF-7 cells labeled with CellTrace Far Red or CFSE (Invitrogen, USA) at various Effector:Target (E:T) ratios. After 5 h, cells were harvested, stained with Zombie Red (Biolegend, USA) for viability, and analyzed by flow cytometry. The cytotoxicity index was calculated by comparing target cell death in co-culture to the death rate in target cell monoculture, plus 100%.

For cytotoxicity assessment using the Cell Counting Kit-8 (CCK-8) assay, MKN-45 cells were co-cultured with NK cells at various E:T ratios for 4–5 h. After medium replacement with CCK-8, plates were incubated for 1 h. Absorbance (Abs) at 450 nm was measured using a GloMax Discover Reader. Cytolysis (%) was calculated as: % Cytolysis = 100 − [(Abs of test group − Abs of background) / (Abs of control group − Abs of background)] × 100.

2.6 | ELISpot Assay

NK cells cultured with WT, 41BBL-mbIL21, and CD80-41BBL-mbIL21 K562 at D14 were transferred to an ELISpot plate (Mabtech, Sweden) and incubated for 5 h at 37°C. The ELISpot assay was performed using the ELISpot Pro: Human IFN-γ (ALP) kit (Mabtech, Sweden) following the manufacturer's protocol. Reactivity was determined by comparing the number of spots in the different NK cell groups.

2.7 | Spheroid Formation

Spheroid formation was conducted as described [33]. MKN-45 cells were cultured in RPMI 1640 with 10% FBS and 1% penicillin–streptomycin. Upon confluency, cells were trypsinized and suspended in serum-free RPMI 1640 with MaxGel (Sigma-Aldrich, USA) at a 1:100 ratio, 100 ng/mL EGF, and 20 ng/mL FGF. Parental cells were seeded at 1000 cells per well in an ultra-low attachment plate (Corning, USA). Labeled NK/T effector cells were incubated with spheroids for 48 h. After washing and trypsinization, suspended cells were analyzed by flow cytometry, with effector cell percentage indicating infiltration.

2.8 | Spheroid Infiltration and Cell Death Induced by Immune Cells

T cells and NK cells were washed with PBS and resuspended in AIM-V medium with 10% FBS. Each spheroid was co-cultured with 10^4 immune cells for 48 h. After incubation, cells were collected, trypsinized, and stained with anti-CD3 or anti-CD56 antibodies to quantify T cell and NK cell infiltration. Cell death was assessed using Zombie Red fixable viability dye (BioLegend, USA). Infiltration was measured as the percentage of $CD3^+$ $Zombie^-$ or $CD56^+$ $Zombie^-$ cells, and cell death as the percentage of $CD3^-$ $Zombie^+$ or $CD56^-$ $Zombie^+$ cells, both analyzed by flow cytometry.

2.9 | Statistical Analysis

Statistical analyses were performed using Prism-GraphPad version 10.0.0 (GraphPad Software, USA). Data are presented as mean ± SEM. Two-tailed Student's t-tests were used for comparisons between two groups, and one-way ANOVA was used for three or more groups. Results were considered significant if the p-value was < 0.05.

3 | Results

3.1 | K562 Cells Co-Expressing CD80, 41BBL, and mbIL21 Enhance the Expansion of NK Cells Derived From Umbilical Cord Blood Collected From Multiple Donors

To develop a novel feeder cell for NK cell expansion, we established a platform of K562-based feeder cells. Co-stimulatory receptors, including CD80, 41BBL, and mbIL21, were selected for NK cell expansion and were engineered to express in K562 cells as single genes or in combination through consecutive transductions (Figure 1A). These transductions generated seven feeder cell pools with strong expressions of CD80, 41BBL, and mbIL21, either as a single molecule (41BBL, mbIL21, or CD80) or in combinations (41BBL-mbIL21, CD80-mbIL21, CD80-41BBL, or CD80-41BBL-mbIL21) for culturing NK cells (Figure S1). To evaluate the ability of these feeder cells to expand NK cells, we next used them to culture umbilical cord blood NK cells from six donors. NK cells from each donor were expanded for 14 days, with irradiated feeder cells replenished on Day 7 (Figure 1A).

Although there was high variability in NK cell expansion rates among the six donors, even when stimulated with the same feeder cells, NK cells cultured with K562 feeder cells showed higher levels of expansion compared to the feeder-free approach. Among the seven tested feeder cell types, CD80-41BBL-mbIL21 K562 consistently demonstrated the highest expansion rates across all six donors (Figure 1B). When comparing fold expansions normalized to wild-type K562 cells, CD80-41BBL-mbIL21 exhibited the highest fold change among the seven tested feeder cells (Figure 1B). NK cells exhibited significantly higher expansion rates when cultured with CD80-41BBL-mbIL21 compared to the conventional 41BBL-mbIL21 K562 cells commonly used by many research groups (34.1 ± 9.0 versus 5.1 ± 1.8, $p = 0.0003$, Figure 1B). These data suggest that the co-expression of the co-stimulatory molecules CD80 in the conventional 41BBL-mbIL21 feeder cells significantly enhances NK cell expansion across different donors.

3.2 | Expression Profiles of Activating and Inhibitory Receptors Were Similar Among Feeder Cell-Expanded NK Cells

We next examined whether culturing NK cells with the seven tested feeder cells altered the expression profiles of activating and inhibitory receptors. After 14 days of *in vitro* culture, we performed flow cytometry staining to assess the expression of NK activating receptors (CD16, NKG2D, NKp46, NKp30, DNAM1, 2B4, Figure S2) and NK inhibitory receptors (NKG2A, PD-1, and TIGIT, Figure S3). After gating on CD56+ cells, we did not observe any significant differences in the proportion of NK cells expressing either activating or inhibitory receptors when cultured with these feeder cells (Figure 1C).

3.3 | CD80-41BBL-mbIL21 K562 Cells Enhanced the Expression of Genes Regulating NK Cell Proliferation and Effector Function

To further investigate the impact of feeder cells on NK cell characteristics beyond changes in cell surface receptors, we performed RNA sequencing to comprehensively analyze the transcriptome of NK cells cultured with wild-type, 41BBL-mbIL21, or CD80-41BBL-mbIL21 K562 feeder cells. Transcriptomic profiling revealed distinct clusters for NK cells cultured with each feeder cell type, indicative of unique transcriptional programs (Figure S4). As expected, NK cells expanded with CD80-41BBL-mbIL21 K562 cells exhibited the highest upregulation of the CD80 ligand, *CD28*, compared to other conditions (Figure 2A,B). Consistent with our RNAseq data, we observed a higher proportion of CD28-expressing NK cells in the group expanded with CD80-41BBL-mbIL21 K562 feeders compared to 41BBL-mbIL21 K562 feeders ($75.75\% \pm 1.64\%$ vs. $55.85\% \pm 4.48\%$) and a trend toward increased CD28 expression (Figure S5). These findings suggest CD80 enhances the expansion of CD28-expressing NK cells. Furthermore, NK cells expanded with CD80-41BBL-mbIL21 feeders showed increased expression of CD28 downstream signaling molecules, including *FLNA*, *GRAP2*, and *VAV1*. These genes are critical for NK cell development and cytotoxic function [34, 35]. Consistent with this observation, CD80-41BBL-mbIL21 K562-stimulated NK cells demonstrated significant induction of 33 proliferation-associated genes, including *CCDC88A*, *ARHGEF11*, and *FLNB*—known regulators of mitotic spindle assembly in NK cells [36] (Figure 2C). Furthermore, genes implicated in cytokine production (*PIK3R3* [37], *KRAS* [38], *PIK3CD* [37], *CD86* [39], *CD44* [40], and *IRF4* [41]) and cytotoxic activity (*SH2D1A* [42], *ZAP70* [43], and *ITGB2* [43]) were also significantly upregulated in CD80-41BBL-mbIL21 K562-stimulated NK cells (Figure 2C). Expression levels of CD107a (*LAMP-1*), perforin (*PRF1*), and granzyme B (*GZMB*) were comparable among the three feeder cell groups (Figure S6), aligning with previous findings that K562 cells without co-stimulatory molecules induce these markers in NK cells [4]. Interestingly, granzyme H (*GZMH*) expression was significantly upregulated in NK cells stimulated with CD80-41BBL-mbIL21 K562 cells ($p < 0.05$; Figure S6), indicating enhanced NK cell cytotoxicity via *GZMH* induction. Additionally, CD80-41BBL-mbIL21 feeders significantly downregulated KIR expression in NK cells compared to wild-type or 41BBL-mbIL21 K562 cells, suppressing inhibitory KIR genes, including *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR2DP1*, *KIR2DS4*, *KIR3DL1*, *KIR3DL2*, and *KIR3DL3*. These findings suggest that CD80-41BBL-mbIL21 feeder cells enhance NK cell activation by downregulating inhibitory KIR expression and promoting CD28-mediated gene expression related to proliferation, cytokine production, and cytotoxic activity.

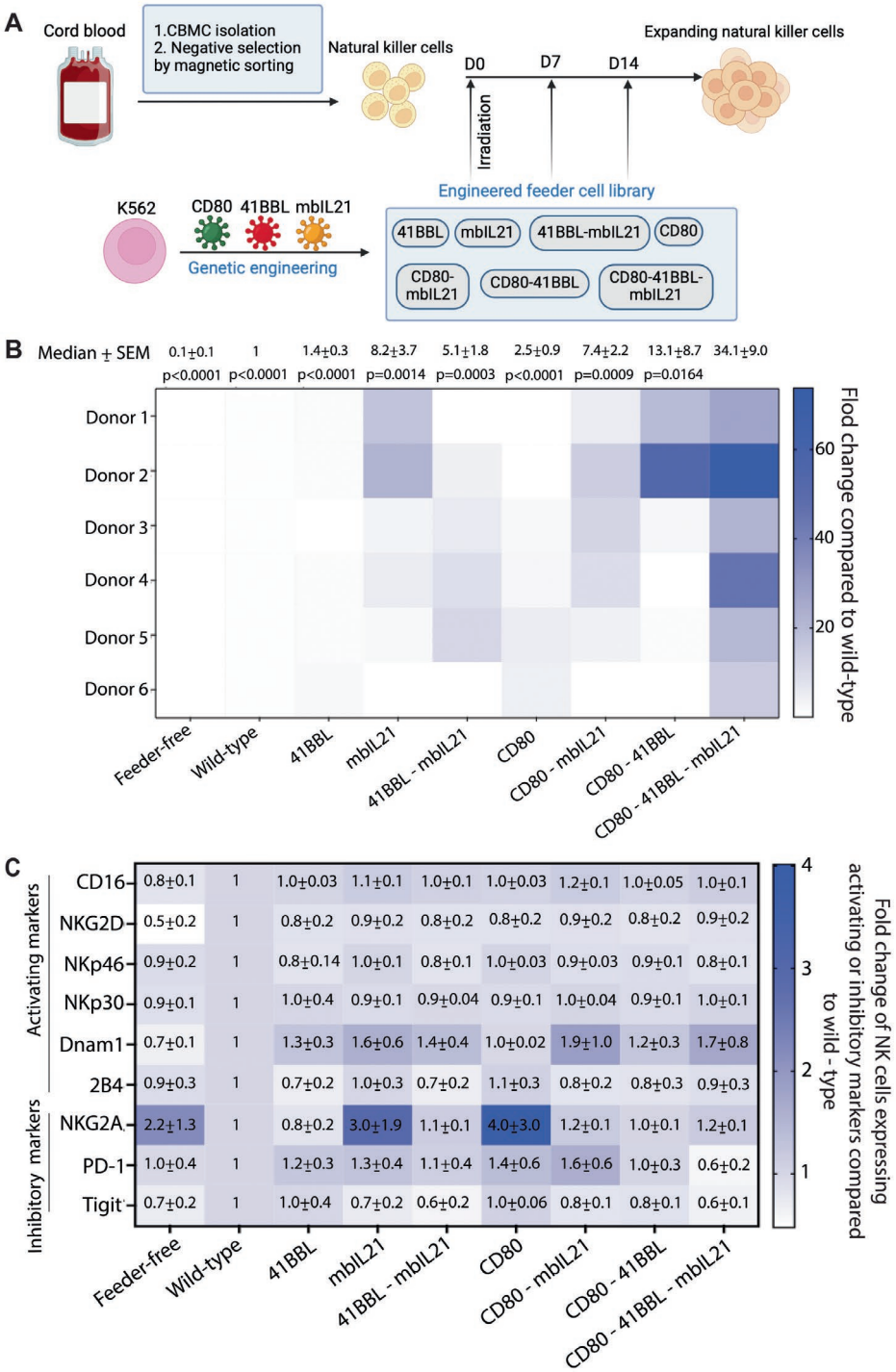

FIGURE 1 | Expansion and phenotype of expanded umbilical cord blood natural killer cells (NK) with different treatments. (A) Methods of NK cell expansion with different irradiated feeder cells. (B) Heatmap showing fold change in NK cell expansion from six donors cultured with or without engineered feeder cells, normalized to wild-type K562 feeders. (C) Heatmap showing the fold change in the percentage of NK cells expressing activating or inhibitory receptors when cultured with different feeder cells, normalized to wild-type feeder cells. CD56+ NK cells were gated, and double-positive percentages normalized to the wild-type group. Panels B and C present mean ± SEM from six donors. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test. NK cells co-cultured without feeders (Feeder-free) or with irradiated K562 derivatives: Wild-type; 41BBL; mbIL21; 41BBL-mbIL21; CD80; CD80-mbIL21; CD80-41BBL; and CD80-41BBL-mbIL21.

To confirm the RNA sequencing results, we examined the production of IFN-γ, an important effector cytokine, and the cytotoxicity of NK cells after culturing with the target feeder cells. NK cells cultured with CD80-41BBL-mbIL21 K562 cells released the highest levels of IFN-γ compared to the other two feeder cell types, including wild-type and 41BBL-mbIL21 K562 (Figure 3A,B). To evaluate NK cell cytolytic capabilities, expanded NK cells were incubated with wild-type K562 cells on day 14 of the expansion and processed for 5 h. The cytotoxicity index was calculated as the percentage of dead K562 cells to

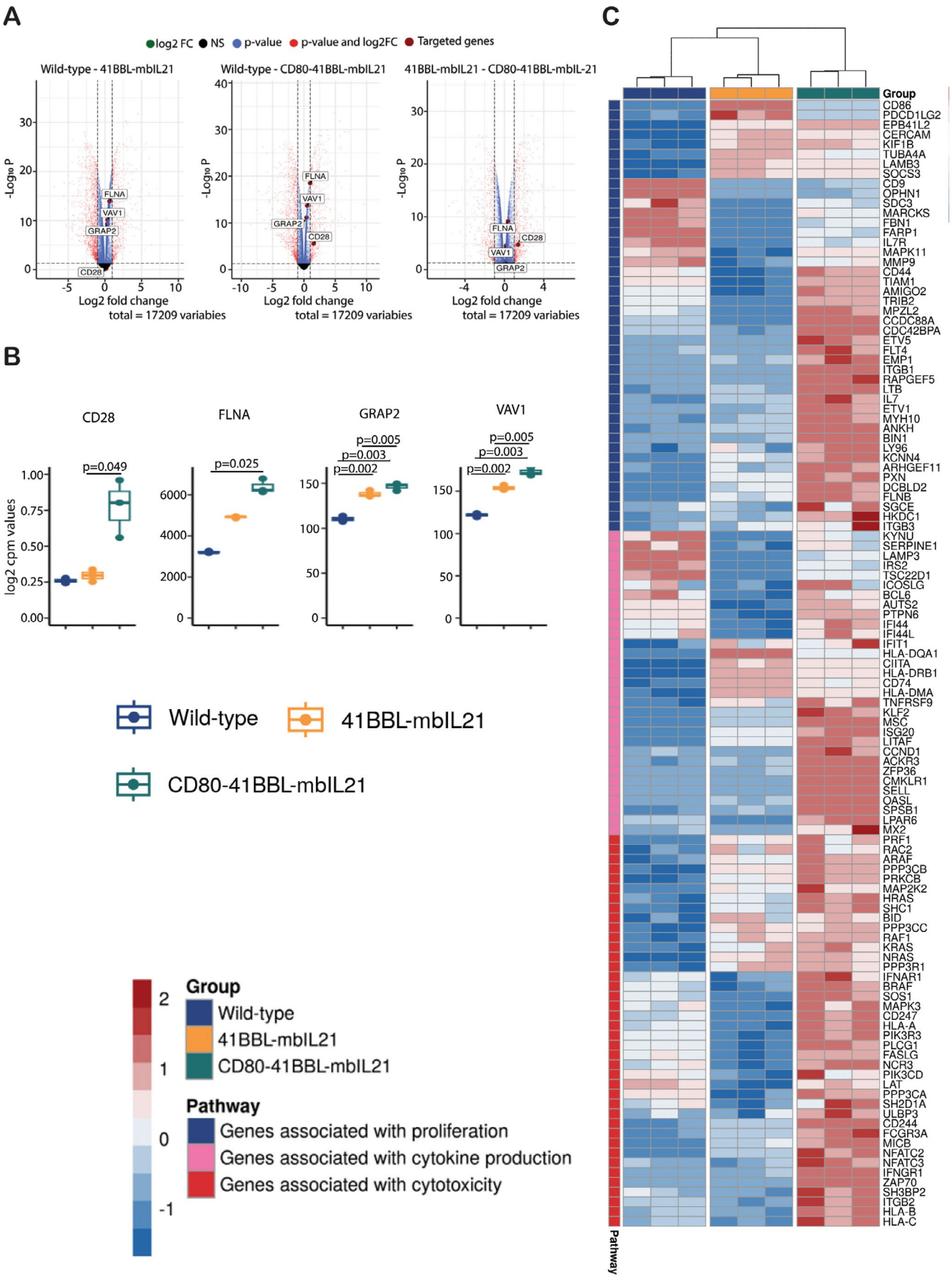

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FIGURE 2 | CD80-41BBL-mbIL21 K562 feeder cells induced the expression of genes associated with NK cell proliferation, cytokine production, and cytotoxicity. (A) Volcano plots show three pairwise comparisons: Wild-type vs. 41BBL-mbIL21, wild-type vs. CD80-41BBL-mbIL21, and 41BBL-mbIL21 vs. CD80-41BBL-mbIL21, highlighting genes linked to CD80 signaling. (B) Expression of CD80 signaling genes (*CD28*, *FLNA*, *GRAP2*, and *VAV1*) was analyzed using one-way ANOVA with Tukey's test. (C) Heatmap showing gene expression across three groups: Proliferation, cytokine production, and cytotoxicity pathways. Genes were considered differentially expressed if the FDR-adjusted p-value was <0.05 and $|\log_2 FC| \geq 1$. Panels B and C present mean ± SEM from three donors across three experiments. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons. NK cells were co-cultured with irradiated K562 derivatives: Wild-type, 41BBL-mbIL21, and CD80-41BBL-mbIL21.

live K562 cells during the incubation by flow cytometry method (Figure 3C,D). NK cell cytotoxicity was also tested with the cancer cell line, MCF-7 cells, which do not downregulate the expression of HLA class I (Figure S7). As expected, NK cells stimulated with CD80-41BBL-mbIL21 K562 cells showed significantly higher cytotoxicity against both K562 and MCF-7 cells compared to NK cells stimulated with 41BBL-mbIL21 or wild-type feeder cells. The data presented here support the conclusion that CD80 is crucial for achieving high levels of NK cell proliferation and activity.

3.4 | BaEV-Pseudotyped Lentivirus Enhanced Transduction Efficiency in Expanded Cord Blood-Derived NK Cells When Compared to the VSV-Pseudotyped Lentivirus

To expand the therapeutic potential of NK cells expanded with CD80-41BBL-mbIL21 feeder cells, we genetically engineered them to express a CAR targeting carcinoembryonic antigen (CEA) as a proof of concept (Figure 4A). Genetic engineering has posed significant challenges in NK therapy [44]. Conventional lentiviruses using vesicular stomatitis virus glycoprotein (VSV-G) as the envelope glycoprotein have shown unexpected inefficiency in transducing NK cells due to the absence of the low-density lipoprotein (LDL) receptor in these cells [45]. Recent studies have highlighted the BaEV-Baboon retroviral envelope glycoprotein-pseudotyped lentivirus as a promising vector for NK cells, as the BaEV receptor ASCT-2 is reported to be present in activated NK cells [31]. Jo et al. proposed combining VSV-G and BaEV within a single lentiviral particle to counter the low titer resulting from syncytia formation in BaEV receptor-less viruses [46]. To determine the most efficient virus, we transduced CD80-41BBL-mbIL21-K562 cell-expanded NK cells with a CAR construct targeting CEA (Figure 4A) packaged in lentiviruses pseudotyped with BaEV, VSV-G, or a combination of both (Figure 4B). Evaluation of CAR construct expression through the GFP reporter gene at 5 days post-transduction revealed BaEV lentivirus to be more effective in NK cell transduction, with efficiencies ranging from 24% to 60% across three different donors (Figure 4C,D). However, the combination of BaEV and VSV-G did not improve transduction efficiency in cord blood-derived NK cells compared to the BaEV-based approach, often yielding a transduction rate lower than 10% (Figure 4C,D).

We next assessed the cytolytic activity of NK cells following transduction with BaEV-pseudotyped lentivirus by co-culturing them with two target cell lines: The gastric cancer cell line MKN-45, which exhibits high CEA expression, and the breast cancer cell line MCF-7, which lacks CEA expression. Microscopic observation revealed reduced density and distinct apoptotic morphology of MKN-45 cells co-cultured with untransduced-NK cells (UTD-NK), which were more pronounced in those transduced with the CAR construct (Figure 4E). CCK-8 assay analysis showed a dose-dependent reduction in MKN-45 viability with CAR-NK, but not UTD-NK or mock-NK cells (Figure 4F). Results were consistent with Zombie Red-based flow cytometry. Furthermore, this effect was not observed in co-cultures of CAR-NK cells with MCF-7 cells (Figure S8), which do not express CEA. These results suggest that the specific cytolysis is driven by the expression of the CEA-targeting CAR construct.

3.5 | CAR-NK Cells Displayed Higher Cytolytic Activity and Superior Infiltration Capacity Compared to CAR-T Cells

Although NK and T cells utilize different cytotoxic mechanisms, we investigated whether NK cells expanded using CD80-41BBL-mbIL21 K562 feeder cells retain superior killing activity compared to T cells when both are engineered to express CARs, as shown in previous studies [47, 48]. To assess whether transduced-NK cells could outperform T cells engineered with the same CAR construct, we conducted a cytotoxicity assay against MKN-45 cells, which exhibit high levels of CEA. NK cells and T cells were transduced with lentivirus packaged with CAR constructs. Subsequently, CAR-expressing NK or T cells were isolated via cell sorting. NK and T cells, both untransduced (UTD) and CAR-expressing (CAR), were co-cultured with labeled MKN-45 cells at an identical E:T ratio for 5 h and then analyzed for viability. MKN-45 cells displayed sporadic signs of cell shrinkage upon co-culturing with untransduced T or NK cells and exhibited more noticeable signs upon co-culturing with CAR-T or CAR-NK cells (Figure 5A). Interestingly, CAR-NK cells significantly induced higher death rates in MKN-45 cells compared to CAR-T cells, resulting in a higher cytotoxicity index (8.6 ± 1.4 versus 3.5 ± 0.4, $p = 0.009$, Figure 5B).

To examine whether CAR-NK cells might excel over CAR-T cells in solid tumor contexts, we developed MKN-45 spheroid models (Figure 5C). MKN-45 spheroids were incubated with effector cells for 48 h, then collected and stained for CD56 and CD3 to quantify infiltrating NK and T cells. Generally, NK effector cells exhibited superior infiltration compared to T cells, with the highest infiltration rate observed in co-culture with CAR-NK cells (7.2% ± 1.1% versus 3.3% ± 0.3%, $p = 0.0024$, Figure 5D,E). To assess effector cell cytotoxicity, we stained trypsinized spheroids with a viability dye and gated on the CD56- and CD3- population. Corresponding with the infiltration trend, co-culture of MKN-45 spheroids with NK cells resulted in a significantly

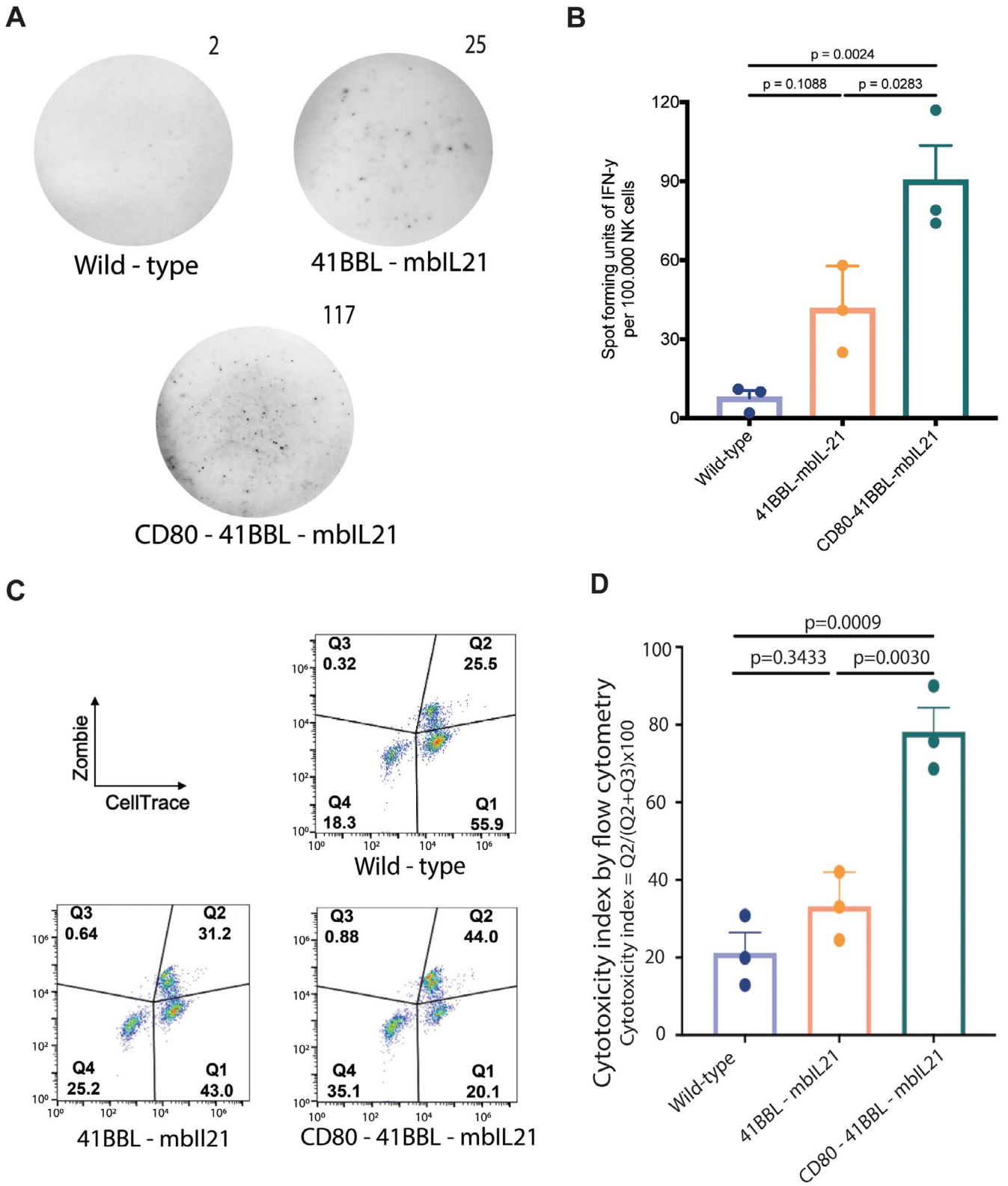

FIGURE 3 | CD80-41BBL-mbIL21 K562 feeder cells enhanced the effector functions of NK cells. (A) Representative ELISpot images showing IFNγ production by NK cells expanded with the indicated K562 feeder cells. (B) Box plot showing quantification of IFNγ spots by NK cells expanded from the indicated K562 cells. (C) FACS plots measuring the cytolytic activity of NK cells expanded with the indicated K562 cells and co-cultured with MK-542 cells. (D) Box plot showing the cytotoxic index of NK cells from the three tested groups. Data in B and D are from three experiments with three donors. Statistical significance was assessed by two-way ANOVA with Tukey's multiple comparisons. NK cells were co-cultured with irradiated K562 derivatives: Wild-type, 41BBL-mbIL21, and CD80-41BBL-mbIL21.

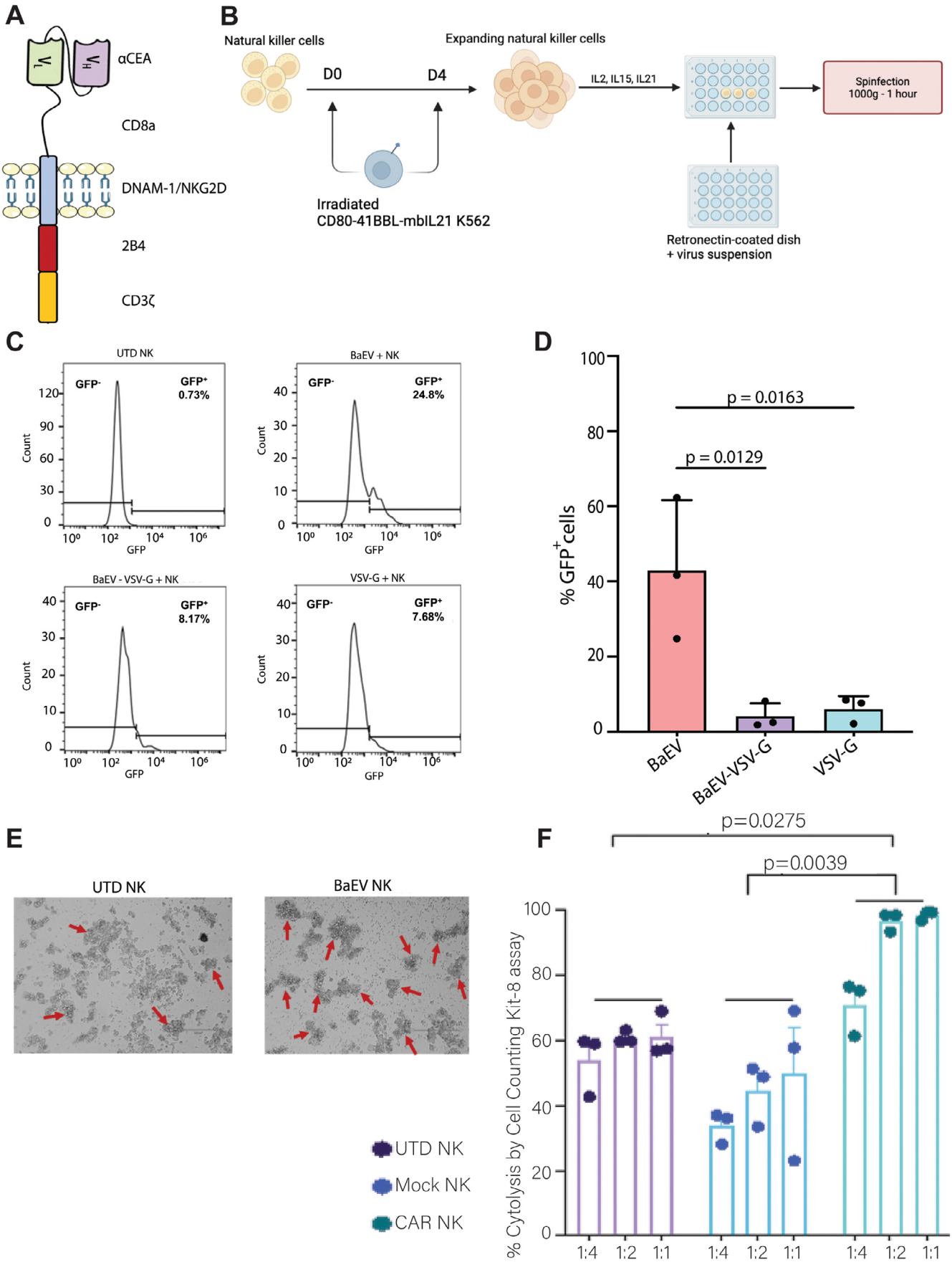

FIGURE 4 | Legend on next page.

FIGURE 4 | Transduction rate and cytotoxicity of CAR-NK cells. (A) Illustration of the CAR construct expressing CEA. (B) Transduction scheme of CAR construct into NK cells using BaEV, BaEV-VSV-G, and VSV-G lentiviral systems. (C) Representative FACS histograms of NK cells transduced with the indicated lentiviruses. (D) Percentage of GFP+ NK cells assessed in three different donors 5 days after transduction. (E) Microscopic observation of NK cell cytotoxic effects on MKN-45 cells. Red arrows indicate MKN-45 cells with damaged morphology. UTD: Untransduced NK cells; BaEV: CAR-NK cells generated with BaEV lentivirus vector. Scale bar = 200 μm. (F) Cytotoxicity of NK cells (CCK-8 assay) after a 4-h co-culture with MKN-45 cells at different effector-to-target cell ratios. UTD NK: Untransduced NK cells. Mock NK: NK cells with empty vector. CAR NK: NK cells expressing CAR. Statistical significance in D and F was determined using one-way and two-way ANOVA, respectively, with Tukey's post hoc test.

higher cell death rate compared to T cell effectors, with the highest rate achieved with CAR-NK cells (16% ± 1.15% versus 10% ± 0.58%, $p = 0.0222$, Figure 5F). There appeared to be a correlation between effector cell infiltration and observed cancer cell death in our model. These findings confirmed the integral functionality of transduced NK cells and highlighted the potential of CAR-NK cells in targeting cancer cells due to their higher cytolytic effect and improved infiltration compared to T cells and CAR-T cells.

4 | Discussion

Interest in NK-based cancer therapies is growing, driven by clinical success in hematologic malignancies [49]. NK cells offer a scalable, off-the-shelf alternative to CAR-T therapy, avoiding severe toxicities like cytokine release syndrome and graft-versus-host disease. However, their functionality depends on the immunophenotype from expansion methods and their low gene transfer efficiency with current genetic engineering techniques [50].

In our study, we tackled these obstacles by creating a library of genetically engineered feeder cells (Figure 1A). Our research demonstrated that K562 cells, expressing both the co-stimulatory molecule CD80, 41BBL, and mbIL21, offered optimal expansion rates across various donor samples (Figure 1B). Previous studies have shown varying expansion rates using engineered K562 cells. Zhao et al. achieved a 2833-fold expansion with mbIL21 and 41BBL after 3 weeks [51], Shman et al. reported a 171-fold expansion in 18 days [52], and Denman et al. observed a 31,747-fold expansion by day 21 with mbIL21 alone [53]. These differences arise from variations in culture methods and NK cell sources. Uniquely, our study incorporated multiple stimulatory genes, including the underexplored CD80. Remarkably, we achieved a 60,421-fold expansion in just 14 days, highlighting CD80's potential, especially alongside 41BBL and mbIL21, to enhance the expansion of cord-blood-derived NK cells.

Despite the enhanced expansion of NK cells observed with CD80-41BBL-mbIL21 feeder cells, we did not detect significant changes in the expression of activating or inhibitory surface receptors (Figure 1C). However, RNA-seq analysis revealed a distinct transcriptional profile in NK cells expanded with CD80-41BBL-mbIL21 K562 cells, characterized by the upregulation of genes involved in three key signaling pathways: proliferation, cytokine production, and cytotoxicity (Figure 2 and Figure S5). This observation aligns with our findings of increased NK cell expansion, IFN-γ production, and cytotoxicity in response to CD80-41BBL-mbIL21 K562 stimulation (Figure 3). The upregulation of critical genes like *CD28* and *VAV1*, known to be downstream of CD80-CD28 signaling [54, 55], further supports the role of CD80 in activating these pathways. Notably, prior research has demonstrated that CD28 is essential for NK cell proliferation [56]. Our findings show that treatment with CD80-41BBL-mbIL21 enhances CD28 expression compared to stimulation with 41BBL-mbIL21 K562 cells. Moreover, the induction of the PI3K signaling pathway, particularly *PIK3CD* [57], is consistent with previous reports on its importance in NK cell maturation and lytic function [37]. Additionally, the upregulation of *TNFRSF9* and *IRF4*, which interact to regulate cytokine production [41], suggests that CD80 stimulation promotes a pro-inflammatory response in NK cells. While CD107a, perforin, and granzyme B expression remained similar across groups (Figure S6), this is likely due to K562 cells' known upregulation of these markers, which may have masked group differences [58]. However, granzyme H expression significantly increased in CD80-41BBL-mbIL21 K562-stimulated NK cells. Granzyme H functions in an alternative cytotoxic pathway [59, 60], and its upregulation, along with KIR gene reduction, likely explains the enhanced cytotoxicity in this group. These findings suggest that CD80-mediated CD28 activation in NK cells promotes genes related to proliferation, cytokine production, and cytotoxicity, while reducing inhibitory markers. Overall, CD80 engagement on feeder cells is crucial for NK cell activation, highlighting the potential of CD80-based strategies in enhancing NK cell-mediated immunotherapy.

Genetic engineering has long been a significant challenge in NK cell-based therapy. To deliver diverse transgenes, including cytokines supporting cell persistence in vivo and genes countering the suppressive tumor microenvironment, lentiviruses are often preferred due to their higher efficiency compared to nonviral methods. In our study, we investigated three different lentiviruses: The conventional VSV-G pseudotype lentivirus, the BaEV pseudotype, and a combination of both. Our findings indicated that BaEV-pseudotyped lentivirus achieved superior transduction efficiency, stabilizing at 20%–30% (Figure 4C). This aligns with previous reports consistently highlighting the superior efficacy of BaEV pseudotype over VSV-G [31, 46, 61]. While VSV-G binds to the low-density lipoprotein receptor LDL-R, which is poorly expressed on activated NK cells, the Baboon-derived BaEV envelope binds to both ASCT-1 and ASCT2 [31, 62, 63]. These receptors are found at significantly higher levels in NK cells, potentially explaining the enhanced efficiency of transduction in NK cells. Unexpectedly, lentivirus pseudotyped with both BaEV and VSV-G resulted in a significant reduction in transduction (Figure 4C,D). This decrease is likely due to aberrant virus particle formation arising from the co-expression of both viral envelope proteins in the same producer cells.

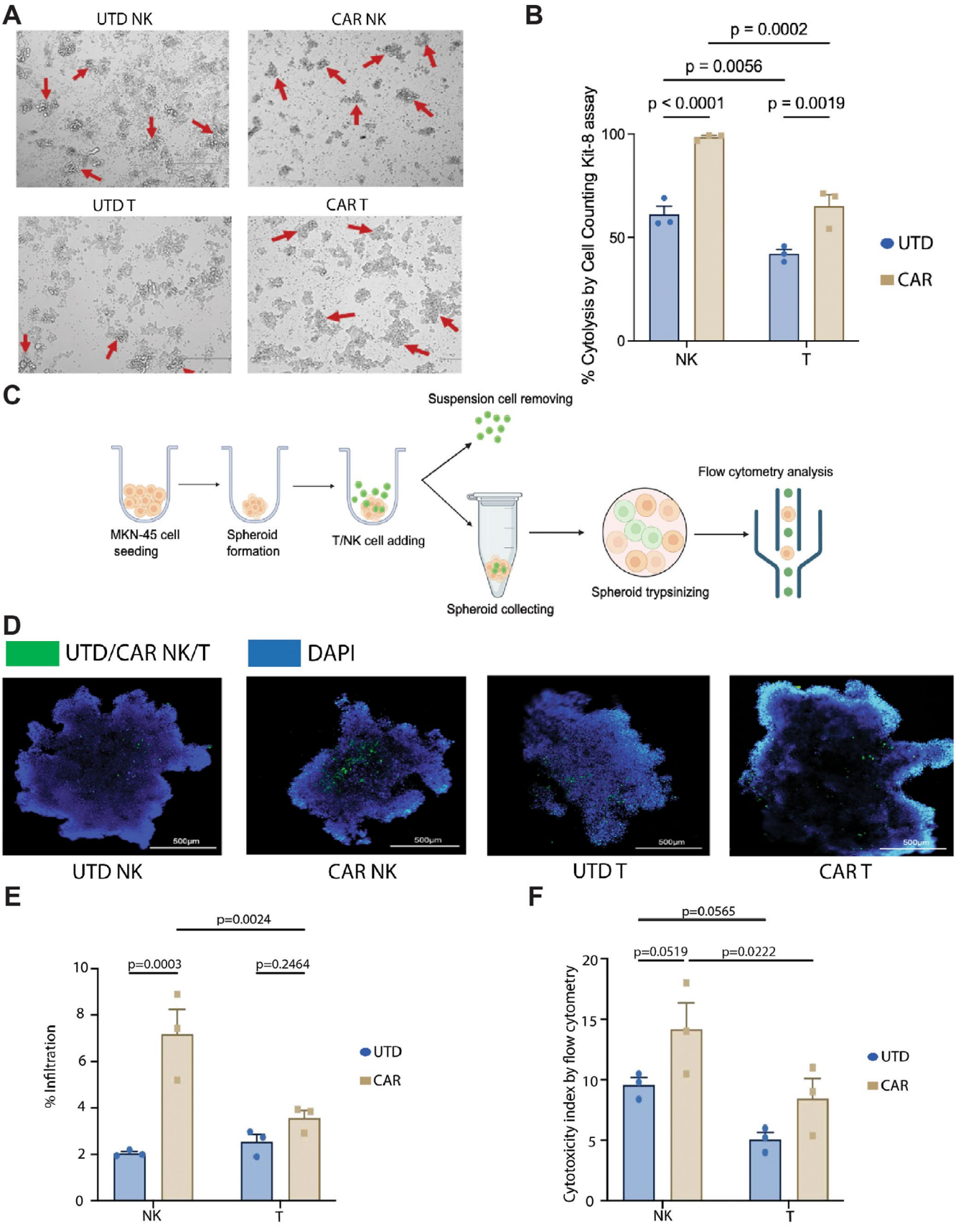

FIGURE 5 | Legend on next page.

FIGURE 5 | Cytotoxicity of CAR-NK and CAR-T cells in 2D culture and spheroid models. (A) Microscopic observation of NK or T cell cytotoxic effects on MKN-45 cells after 48-h co-culture in 2D culture. Scale bar = 200 μm. (B) Cytotoxicity index of CAR-NK/T cells measured after 48-h co-culture with MKN-45 cells. (C) Experimental procedure to assess the cytotoxicity of CAR-NK and CAR-T cells in cancer spheroids. (D) Confocal images show CAR-NK/T cell infiltration (green) into 3D cancer spheroids stained with DAPI (blue) after 48 h of co-culture. Images represent three experiments with three donors. Scale bar: 500 μm. (E) Quantification of CAR-NK and CAR-T cell infiltration in 3D cancer spheroids. (F) Cell death percentages in 3D cancer spheroids treated with CAR-NK/T cells. UTD: Untransduced; CAR: Chimeric antigen receptor. Data in B, E, and F are Mean ± SEM. Significance was analyzed using two-way ANOVA.

In addition to transduction of a CAR construct, we demonstrated the cytotoxic effect specific to the CAR target by CAR-transduced NK cells. Even with a modest CAR expression rate of 20%–30%, CAR-NK cells exhibited improved killing activity against target cells (Figure 4E,F). At comparable levels of CAR expression, NK cells proved more effective than T cells in eliminating target cells (Figure 5A,B). This difference is likely attributed to the distinct response rates between $CD8^+$ T cells and NK cells, as innate immunity highlights the rapid response of NK cells, positioning them as the first responders to infections [64]. Our results are consistent with previous findings showing that NK cells lyse their target cells more rapidly than $CD8^+$ T cell subsets [47]. Specifically, at a ratio of 1:1, $CD8^+$ T cells lysed approximately 20% of target cells, while NK cells eliminated over 60%. We observed a similar trend in our study. Furthermore, research has shown that CAR-NK therapy demonstrates superior therapeutic efficacy compared to CAR-T in in vivo models [48]. In a spheroid model, CAR-NK cells consistently exhibited greater efficiency, demonstrating higher infiltration capacity and increased spheroid cell death (Figure 5E,F).

Our study had some limitations. A larger sample size was difficult to obtain, and donor variability was substantial. Despite this, CD80 expression in K562 feeder cells consistently achieved high NK cell expansion rates across six donors. In addition to CD80, other stimulatory molecules, including CD86, CD40, and OX40L, may also complement 41BBL and mbIL21 in promoting NK cell expansion. However, evaluating their contributions would likely require a large library of genetically engineered feeder cells and high-throughput screening platforms, which we will explore in future studies. Transduction efficacy was often below 40%, aligning with recent studies, but could potentially improve with a second-generation retrovirus system using fewer helper plasmids. We also did not evaluate CAR expression stability in transduced NK cells, requiring further study to optimize transduction. Lastly, validating differences in infiltration will need in vivo models to better mimic the tumor microenvironment.

Author Contributions

Thi Bao Tram Tran: conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft. **Thi Van Anh Bui:** conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft. **Thi Minh Thu Tran:** methodology. **Nguyen Minh Nguyen:** methodology. **Hoang Thien Phuc Nguyen:** formal analysis, methodology. **Thi Phuong Diem Tran:** methodology. **Duc Minh Quan Nguyen:** investigation. **Thai Minh Quan Ngo:** formal analysis, methodology, visualization. **Thanh Binh Nguyen:** resources. **Els Verhoeyen:** resources, writing – review and editing. **Nhat Thang Tran:** resources. **Hoai-Nghia Nguyen:** conceptualization. **Le Son Tran:** conceptualization, data curation, formal analysis, investigation, supervision, writing – original draft, writing – review and editing.

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Ethics Statement

Approval of the research protocol by an Institutional Review Board: Ethics Committee-approved protocols (02/2024/CT-VDTYH).

Consent

The authors have nothing to report.

Conflicts of Interest

Hoai-Nghia Nguyen and Le Son Tran are affiliated with Gene Solutions JSC. Other authors declare no conflicts of interest.

References

1. A. M. Abel, C. Yang, M. S. Thakar, and S. Malarkannan, "Natural Killer Cells: Development, Maturation, and Clinical Utilization," *Frontiers in Immunology* 9 (2018): 1869.

2. N. K. Wolf, D. U. Kissiov, and D. H. Raulet, "Roles of Natural Killer Cells in Immunity to Cancer, and Applications to Immunotherapy," *Nature Reviews. Immunology* 23, no. 2 (2023): 90–105.

3. M. M. Berrien-Elliott, M. T. Jacobs, and T. A. Fehniger, "Allogeneic Natural Killer Cell Therapy," *Blood* 141, no. 8 (2023): 856–868.

4. N. Lamers-Kok, D. Panella, A. M. Georgoudaki, et al., "Natural Killer Cells in Clinical Development as Non-Engineered, Engineered, and Combination Therapies," *Journal of Hematology & Oncology* 15, no. 1 (2022): 164.

5. L. Tong, C. Jiménez-Cortegana, A. H. M. Tay, S. Wickström, L. Galluzzi, and A. Lundqvist, "NK Cells and Solid Tumors: Therapeutic Potential and Persisting Obstacles," *Molecular Cancer* 21, no. 1 (2022): 206.

6. S. M. Federico, M. B. McCarville, B. L. Shulkin, et al., "A Pilot Trial of Humanized Anti-GD2 Monoclonal Antibody (hu14.18K322A) With Chemotherapy and Natural Killer Cells in Children With Recurrent/Refractory Neuroblastoma," *Clinical Cancer Research* 23, no. 21 (2017): 6441–6449.

7. S. Modak, J. B. le Luduec, I. Y. Cheung, et al., "Adoptive Immunotherapy With Haploidentical Natural Killer Cells and Anti-GD2 Monoclonal Antibody m3F8 for Resistant Neuroblastoma: Results of a Phase I Study," *Oncoimmunology* 7, no. 8 (2018): e1461305.

8. S. Xie, J. Chen, M. Zhang, and Z. Wu, "Allogenic Natural Killer Cell Immunotherapy of Sizeable Ovarian Cancer: A Case Report," *Molecular and Clinical Oncology* 6, no. 6 (2017): 903–906.

9. J. S. Miller, Y. Soignier, A. Panoskaltsis-Mortari, et al., "Successful Adoptive Transfer and In Vivo Expansion of Human Haploidentical NK Cells in Patients With Cancer," *Blood* 105, no. 8 (2005): 3051–3057.

10. D. A. Knorr, V. Bachanova, M. R. Verneris, and J. S. Miller, "Clinical Utility of Natural Killer Cells in Cancer Therapy and Transplantation," *Seminars in Immunology* 26, no. 2 (2014): 161–172.

11. L. Kean, K. Hamby, T. Pearson, and C. Larsen, "NK Cells Efficiently Prevent Engraftment of Donor Stem Cells After Tolerigenic Bone Marrow Transplantation," *Blood* 106, no. 11 (2005): 3025.

12. M. Luevano, M. Daryouzeh, R. Alnabhan, et al., "The Unique Profile of Cord Blood Natural Killer Cells Balances Incomplete Maturation and Effective Killing Function Upon Activation," *Human Immunology* 73, no. 3 (2012): 248–257.

13. J. Gaddy, G. Risdon, and H. E. Broxmeyer, "Cord Blood Natural Killer Cells Are Functionally and Phenotypically Immature but Readily Respond to Interleukin-2 and Interleukin-12," *Journal of Interferon & Cytokine Research* 15, no. 6 (1995): 527–536.

14. Y. Wang, H. Xu, X. Zheng, H. Wei, R. Sun, and Z. Tian, "High Expression of NKG2A/CD94 and Low Expression of Granzyme B Are Associated With Reduced Cord Blood NK Cell Activity," *Cellular & Molecular Immunology* 4, no. 5 (2007): 377–382.

15. M. Granzin, J. Wagner, U. Köhl, A. Cerwenka, V. Huppert, and E. Ullrich, "Shaping of Natural Killer Cell Antitumor Activity by Ex Vivo Cultivation," *Frontiers in Immunology* 8 (2017): 458.

16. J. Wagner, V. Pfannenstiel, A. Waldmann, et al., "A Two-Phase Expansion Protocol Combining Interleukin (IL)-15 and IL-21 Improves Natural Killer Cell Proliferation and Cytotoxicity Against Rhabdomyosarcoma," *Frontiers in Immunology* 8 (2017): 676.

17. E. O. Ojo, A. A. Sharma, R. Liu, et al., "Membrane Bound IL-21 Based NK Cell Feeder Cells Drive Robust Expansion and Metabolic Activation of NK Cells," *Scientific Reports* 9, no. 1 (2019): 14916.

18. S. Kweon, M. T. T. Phan, S. Chun, et al., "Expansion of Human NK Cells Using K562 Cells Expressing OX40 Ligand and Short Exposure to IL-21," *Frontiers in Immunology* 10 (2019): 879.

19. B. Soskic, L. E. Jeffery, A. Kennedy, et al., "CD80 on Human T Cells Is Associated With FoxP3 Expression and Supports Treg Homeostasis," *Frontiers in Immunology* 11 (2020): 577655, https://doi.org/10.3389/fimmu.2020.577655.

20. M. A. Mir, "Chapter 2 - Concept of Reverse Costimulation and Its Role in Diseases," in *Developing Costimulatory Molecules for Immunotherapy of Diseases* (Academic Press, 2015), 45–81.

21. A. Viola and A. Lanzavecchia, "T Cell Activation Determined by T Cell Receptor Number and Tunable Thresholds," *Science* 273, no. 5271 (1996): 104–106.

22. L. H. Boise, A. J. Minn, P. J. Noel, et al., "CD28 Costimulation Can Promote T Cell Survival by Enhancing the Expression of Bcl-xL," *Immunity* 185, no. 7 (1995): 3788–3799.

23. C. B. Thompson, T. Lindsten, J. A. Ledbetter, et al., "CD28 Activation Pathway Regulates the Production of Multiple T-Cell-Derived Lymphokines/Cytokines," *Proceedings of the National Academy of Sciences of the United States of America* 86, no. 4 (1989): 1333–1337.

24. M. K. Jenkins, P. S. Taylor, S. D. Norton, and K. B. Urdahl, "CD28 Delivers a Costimulatory Signal Involved in Antigen-Specific IL-2 Production by Human T Cells," *Journal of Immunology* 147, no. 8 (1991): 2461–2466.

25. P. Zheng, S. Sarma, Y. Guo, and Y. Liu, "Two Mechanisms for Tumor Evasion of Preexisting Cytotoxic T-Cell Responses: Lessons From Recurrent Tumors," *Cancer Research* 59, no. 14 (1999): 3461–3467.

26. J. M. Kelly, P. K. Darcy, J. L. Markby, et al., "Induction of Tumor-Specific T Cell Memory by NK Cell-Mediated Tumor Rejection," *Nature Immunology* 3, no. 1 (2002): 83–90.

27. B. J. Chambers, M. Salcedo, and H. G. Ljunggren, "Triggering of Natural Killer Cells by the Costimulatory Molecule CD80 (B7-1)," *Immunity* 5, no. 4 (1996): 311–317.

28. J. L. Wilson, J. Charo, A. Martín-Fontecha, et al., "NK Cell Triggering by the Human Costimulatory Molecules CD80 and CD86," *Journal of Immunology* 163, no. 8 (1999): 4207–4212.

29. H. Kumar, T. Kawai, and S. Akira, "Pathogen Recognition by the Innate Immune System," *International Reviews of Immunology* 30, no. 1 (2011): 16–34.

30. E. Littwitz, S. Francois, U. Dittmer, and K. Gibbert, "Distinct Roles of NK Cells in Viral Immunity During Different Phases of Acute Friend Retrovirus Infection," *Retrovirology* 10 (2013): 127.

31. A. B. L. Colamartino, W. Lemieux, P. Bifsha, et al., "Efficient and Robust NK-Cell Transduction With Baboon Envelope Pseudotyped Lentivector," *Frontiers in Immunology* 10 (2019): 2873.

32. A. Girard-Gagnepain, F. Amirache, C. Costa, et al., "Baboon Envelope Pseudotyped LVs Outperform VSV-G-LVs for Gene Transfer Into Early-Cytokine-Stimulated and Resting HSCs," *Blood* 124, no. 8 (2014): 1221–1231.

33. Y. Chen, L. Chen, A. T. L. Lun, P. L. Baldoni, and G. K. Smyth, "edgeR v4: powerful differential analysis of sequencing data with expanded functionality and improved support for small counts and larger datasets," *Nucleic Acids Research* 53, no. 2 (2025): https://doi.org/10.1093/nar/gkaf018.

34. G. Chan, T. Hanke, and K. D. Fischer, "Vav-1 Regulates NK T Cell Development and NK Cell Cytotoxicity," *European Journal of Immunology* 31, no. 8 (2001): 2403–2410.

35. N. Kim, E. Yi, S. J. Kwon, H. J. Park, H. J. Kwon, and H. S. Kim, "Filamin A Is Required for NK Cell Cytotoxicity at the Expense of Cytokine Production via Synaptic Filamentous Actin Modulation," *Frontiers in Immunology* 12 (2021): 792334, https://doi.org/10.3389/fimmu.2021.792334.

36. A. Liberzon, C. Birger, H. Thorvaldsdóttir, M. Ghandi, J. P. Mesirov, and P. Tamayo, "The Molecular Signatures Database (MSigDB) Hallmark Gene Set Collection," *Cell Systems* 1, no. 6 (2015): 417–425.

37. E. M. Mace, "Phosphoinositide-3-Kinase Signaling in Human Natural Killer Cells: New Insights From Primary Immunodeficiency," *Frontiers in Immunology* 9 (2018): 445.

38. K. Kaur, H. H. Chang, P. Topchyan, et al., "Deficiencies in Natural Killer Cell Numbers, Expansion, and Function at the Pre-Neoplastic Stage of Pancreatic Cancer by KRAS Mutation in the Pancreas of Obese Mice," *Frontiers in Immunology* 9 (2018): 1229.

39. Y. Peng, G. Luo, J. Zhou, et al., "CD86 Is an Activation Receptor for NK Cell Cytotoxicity Against Tumor Cells," *PLoS One* 8, no. 12 (2013): e83913.

40. S. L. Sague, C. Tato, E. Puré, and C. A. Hunter, "The Regulation and Activation of CD44 by Natural Killer (NK) Cells and Its Role in the Production of IFN-Gamma," *Journal of Interferon & Cytokine Research* 24, no. 5 (2004): 301–309, https://doi.org/10.1089/107999004323065093.

41. J. Lu, T. Liang, P. Li, and Q. Yin, "Regulatory Effects of IRF4 on Immune Cells in the Tumor Microenvironment," *Frontiers in Immunology* 14 (2023): 1086803.

42. A. Liu, G. Klein, K. Bandobashi, E. Klein, and N. Nagy, "SH2D1A Expression Reflects Activation of T and NK Cells in Cord Blood Lymphocytes Infected With EBV and Treated With the Immunomodulator PSK," *Immunology Letters* 80, no. 3 (2002): 181–188.

43. A. A. van Vliet, M. G. C. N. den Hout, D. Steenmans, et al., "Bulk and Single-Cell Transcriptomics Identify Gene Signatures of Stem Cell-Derived NK Cell Donors With Superior Cytolytic Activity," *Molecular Therapy: Oncology* 32, no. 4 (2024): 200870, https://doi.org/10.1016/j.omton.2024.200870.

44. D. S. J. Allan, M. Chakraborty, G. C. Waller, et al., "Systematic Improvements in Lentiviral Transduction of Primary Human Natural Killer Cells Undergoing Ex Vivo Expansion," *Molecular Therapy–Methods & Clinical Development* 20 (2021): 559–571.

45. Y. Gong, R. G. J. Klein Wolterink, I. Janssen, A. J. Groot, G. M. J. Bos, and W. T. V. Germeraad, "Rosuvastatin Enhances VSV-G Lentiviral Transduction of NK Cells via Upregulation of the Low-Density Lipoprotein Receptor," *Molecular Therapy - Methods & Clinical Development* 17 (2020): 634–646.

46. D. H. Jo, S. Kaczmarek, O. Shin, et al., "Simultaneous Engineering of Natural Killer Cells for CAR Transgenesis and CRISPR-Cas9 Knockout Using Retroviral Particles," *Molecular Therapy - Methods & Clinical Development* 29 (2023): 173–184.

47. S. C. Chiang, J. Theorell, M. Entesarian, et al., "Comparison of Primary Human Cytotoxic T-Cell and Natural Killer Cell Responses Reveal Similar Molecular Requirements for Lytic Granule Exocytosis but Differences in Cytokine Production," *Blood* 121, no. 8 (2013): 1345–1356.

48. C. Quintarelli, S. Sivori, S. Caruso, et al., "CD19 Redirected CAR NK Cells Are Equally Effective but Less Toxic Than CAR T Cells," *Blood* 132 (2018): 3491.

49. L. Ruggeri, M. Capanni, E. Urbani, et al., "Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants," *Science* 295, no. 5562 (2002): 2097–2100.

50. S. Matosevic, "Viral and Nonviral Engineering of Natural Killer Cells as Emerging Adoptive Cancer Immunotherapies," *Journal of Immunology Research* 2018 (2018): 4054815.

51. X. Y. Zhao, Q. Jiang, H. Jiang, et al., "Expanded Clinical-Grade Membrane-Bound IL-21/4-1BBL NK Cell Products Exhibit Activity Against Acute Myeloid Leukemia In Vivo," *European Journal of Immunology* 50, no. 9 (2020): 1374–1385.

52. T. V. Shman, K. P. Vashkevich, A. A. Migas, et al., "Phenotypic and Functional Characterisation of Locally Produced Natural Killer Cells Ex Vivo Expanded With the K562-41BBL-mbIL21 Cell Line," *Clinical and Experimental Medicine* 23, no. 6 (2023): 2551–2560.

53. C. J. Denman, V. V. Senyukov, S. S. Somanchi, et al., "Membrane-Bound IL-21 Promotes Sustained Ex Vivo Proliferation of Human Natural Killer Cells," *PLoS One* 7, no. 1 (2012): e30264.

54. J. Xing, Y. Hu, W. Liu, et al., "The Interaction Between the Costimulatory Molecules CD80/86 and CD28 Contributed to CD4(+) T Lymphocyte Activation in Flounder (*Paralichthys olivaceus*)," *Fish & Shellfish Immunology* 148 (2024): 109482.

55. C. Charvet, A. J. Canonigo, S. Bécart, et al., "Vav1 Promotes T Cell Cycle Progression by Linking TCR/CD28 Costimulation to FOXO1 and p27kip1 Expression," *Journal of Immunology* 177, no. 8 (2006): 5024–5031, https://doi.org/10.4049/jimmunol.177.8.5024.

56. D. Nandi, J. A. Gross, and J. P. Allison, "CD28-Mediated Costimulation Is Necessary for Optimal Proliferation of Murine NK Cells," *Journal of Immunology* 152, no. 7 (1994): 3361–3369.

57. R. Ruiz-García, A. Vargas-Hernández, I. K. Chinn, et al., "Mutations in PI3K110δ Cause Impaired Natural Killer Cell Function Partially Rescued by Rapamycin Treatment," *Journal of Allergy and Clinical Immunology* 142, no. 2 (2018): 605–617.

58. F. Gao, M. C. Mora, M. Constantinides, et al., "Feeder Cell Training Shapes the Phenotype and Function of *In Vitro* Expanded Natural Killer Cells," *MedComm* 5, no. 10 (2024): e740.

59. H. Tang, C. Li, L. Wang, H. Zhang, and Z. Fan, "Granzyme H of Cytotoxic Lymphocytes Is Required for Clearance of the Hepatitis B Virus Through Cleavage of the Hepatitis B Virus X Protein," *Journal of Immunology* 188, no. 2 (2012): 824–831.

60. Q. Hou, T. Zhao, H. Zhang, et al., "Granzyme H Induces Apoptosis of Target Tumor Cells Characterized by DNA Fragmentation and Bid-Dependent Mitochondrial Damage," *Molecular Immunology* 45, no. 4 (2008): 1044–1055.

61. Q. Ren, Y. Zu, H. Su, et al., "Single VHH-Directed BCMA CAR-NK Cells for Multiple Myeloma," *Experimental Hematology & Oncology* 12, no. 1 (2023): 98.

62. S. Shimode, R. Nakaoka, H. Shogen, and T. Miyazawa, "Characterization of Feline ASCT1 and ASCT2 as RD-114 Virus Receptor," *Journal of General Virology* 94, no. Pt 7 (2013): 1608–1612, https://doi.org/10.1099/vir.0.052928-0.

63. R. Yoshikawa, J. Yasuda, T. Kobayashi, and T. Miyazawa, "Canine ASCT1 and ASCT2 Are Functional Receptors for RD-114 Virus in Dogs," *Journal of General Virology* 93, no. 3 (2012): 603–607, https://doi.org/10.1099/vir.0.036228-0.

64. J. C. Sun and L. L. Lanier, "Natural Killer Cells Remember: An Evolutionary Bridge Between Innate and Adaptive Immunity?," *European Journal of Immunology* 39, no. 8 (2009): 2059–2064.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.