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LETTER

A novel BaEVRless-LV packaging system for the production of lentiviral vectors for clinical-grade CAR-NK cell manufacturing

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Chimeric antigen receptor natural killer (CAR-NK) cell therapy is an alternative immunotherapy that provides robust tumor-eliminating effects without inducing life-threatening toxicities and graft-versus-host disease. CAR-NK cell therapy has enabled the development of "off-the-shelf" products that bypass the lengthy and expensive cell manufacturing process[1]. Lentiviral and γ-retroviral vector systems are the most frequently used gene delivery vehicles for the generation of clinical-grade CAR-NK cells. According to the current literature, both lentiviral and retroviral systems can efficiently transduce primary NK cells[1]. However, both viral vectors have prompted safety concerns in clinical application, because of the risk of oncogenic insertion, whereas lentiviral vectors have been reported to be less risky[1,2]. Lentiviruses (LVs) exhibit a lower preference for viral integration near transcriptional start sites than γ-retroviruses, and therefore might decrease the risk of mutagenesis[3]. On the basis of these considerations, we selected the lentiviral vector system as the gene delivery method for NK cell transduction in this study. To substantially improve NK cell transduction efficiency, we used the mutant baboon envelope pseudotyped LV (BaEVRless-LV) as an attractive alternative to the traditional vesicular stomatitis virus G protein LV (VSV-G-LV), because this novel envelope protein targets the alanine/serine/cysteine transporter-1 and -2 (ASCT1 and ASCT2, *SLC1A4* and *SLC1A5*) receptors broadly expressed on NK cells[4,5]. Unfortunately, transfecting 293T cells with the BaEVRless envelope protein induces fatal cell-to-cell fusion and causes 293T cells to immediately detach from the culture plate. This issue significantly decreases BaEVRless pseudotyped lentiviral productivity and limits its application in adoptive NK cell therapy[6].

In this study, knocking out the ASCT2-encoding gene *SLC1A5* on 293T cells substantially alleviated BaEVRless-induced 293T syncytia formation and increased lentiviral productivity, thus supporting CAR transgene transduction in CAR-NK clinical applications. The detailed methods used in this research are listed in the **Supplementary material**.

BaEVRless-induced 293T cell-cell fusion leads to limited BaEVRless-LV productivity

First, to verify the BaEVRless-induced 293T syncytia formation issue, we transfected 293T cells with the VSV-G packaging system (VSV-G envelope plasmid, pLP1 and pLP2 packaging plasmids) and BaEVRless packaging system (BaEVRless envelope plasmid, pLP1 and pLP2 packaging plasmids). Both systems contained an identical transfer plasmid, pLenti-blue fluorescent protein (BFP), which was used to produce the

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LV-BFP virus. Microscopic imaging of the 293T cells transfected with the BaEVRless-LV packaging system exhibited very large syncytia and a disrupted cell structure after 24 h, whereas the 293T cells transfected with the VSV-G packaging system maintained their typical cell morphology even after 72 h (**Figure S1A**). Accordingly, the titers of the BaEVRless packaging group harvested at 48 and 72 h post-transfection were approximately 10-fold and 50-fold lower, respectively, than those of the VSV-G packaging group (**Figure S1B**).

SLC1A5 knockout (KO) alleviates 293T syncytia formation and upregulates lentiviral production

Envelope glycoproteins bind the cell surface receptors on host cells with high affinity, thereby inducing cell-to-cell fusion in host cells[7,8]. Because ASCT1 and ASCT2 are surface receptors of the BaEVRless glycoproteins[5], we hypothesized that knocking out one of the surface receptors on 293T cells might alleviate the syncytia formation issue. The immunoblotting results indicated that the expression level of ASCT2 on 293T cells was significantly higher than that of ASCT1; consequently, we hypothesized that ASCT2 might be the primary receptor inducing syncytia formation (**Figure S2**).

To verify this hypothesis, we generated *SLC1A5* KO 293T cells by electroporation of Cas9/ribonucleoprotein (RNP) followed by single-cell sorting with a flow cytometer. The KO efficiency of single-cell cloning was subsequently determined with Sanger sequencing, and further verified with flow cytometry and immunoblotting (**Figure 1A**). Four candidate single clones based on the results of Sanger sequencing were chosen for further analysis (**Figure 1B**). Clone *SLC1A5* KO #30 (#30) exhibited complete disruption of *SLC1A5* expression (**Figures 1C and S3A**) and was used in subsequent experiments. Because ASCT2 is a neutral amino acid transporter in cells, we first investigated whether *SLC1A5* KO might affect the survival and proliferation of 293T cells. The growth kinetics and viability of #30 cells were comparable to those of wild-type (WT) 293T cells after recovering from cryopreservation (**Figure S3B, C**). More importantly, we did not observe large syncytia formation in #30 cells, even at 120 h after BaEVRless plasmid transfection, whereas in the WT 293T cells, nearly all cells detached from the plate and died (**Figure 1D, E**). This finding suggested that ASCT2 was the primary cause of BaEVRless-induced cell membrane fusion. Next, we measured the viral titer harvested from #30 and WT 293T cells with BaEVRless-LVs to determine whether alleviating syncytia formation led to recovery of BaEVRless-LV productivity. We harvested BFP-encoding BaEVRless-LVs from #30 293T cells after 48, 72, 96, and 120 h of transfection, and observed significantly higher viral titers than those of WT 293T cells, as determined by flow cytometry (**Figure 1F**). Therefore, *SLC1A5* KO #30 might serve as an ideal alternative tool for high BaEVRless-LV production.

SLC1A5 KO 293T cells significantly increase CAR-encoding LV packaging efficiency for CAR-NK cell therapy

Because large CAR-encoding transgenes are inefficient in transducing primary NK cells, increasing lentiviral output is essential for optimal CAR-NK cell therapy applications[9]. We designed 2 large transgenes containing CAR constructs to verify whether *SLC1A5* KO 293T cells might be amenable to large transgene BaEVRless-LV packaging (**Figure 2A**). shNKG2A was designed to enhance CAR-NK cell function by expressing a short hairpin RNA of *KLRC1* to silence the expression of the inhibitory receptor NKG2A[10]. The 1915NGFR design included aCD19CAR and soluble IL-15 constructs. We collected the virus-containing supernatants from WT and #30 293T cells that packaged LV-shNKG2A and LV-1915NGFR at 48, 72, and 96 h for titer analysis. After purification and concentration, all viral titers harvested from #30 293T cells at each point were significantly higher than those from WT 293T cells (**Figure 2B, C**). We extended the titer analysis by concentrating the supernatants harvested from 3 time points. The titers of LV-shNKG2A and LV-1915NGFR were 5-fold greater in #30 293T cells than WT 293T cells (**Figure 2D, E**). Moreover, we did not observe any apparent 293T syncytia during the LV-producing process in #30 293T cells under microscopic imaging (**Figure S4A, B**). These results demonstrated robust packaging of large transgenes by *SLC1A5* KO 293T cells, thus suggesting potential for substantially increasing CAR-encoding LV productivity.

We then assessed the transduction efficiency of LV-shNKG2A and LV-1915NGFR in primary NK cells. The NK cells were isolated from cord blood units with the approval of ethics committee of the Second Affiliated Hospital Zhejiang University School of Medicine (Approval No. 2024-1378). We transduced the NK cells with the LVs produced by #30 and WT 293T cells at a multiplicity of infection of 8. The CAR expression of NK cells transduced by LVs produced by #30 293T cells was much higher

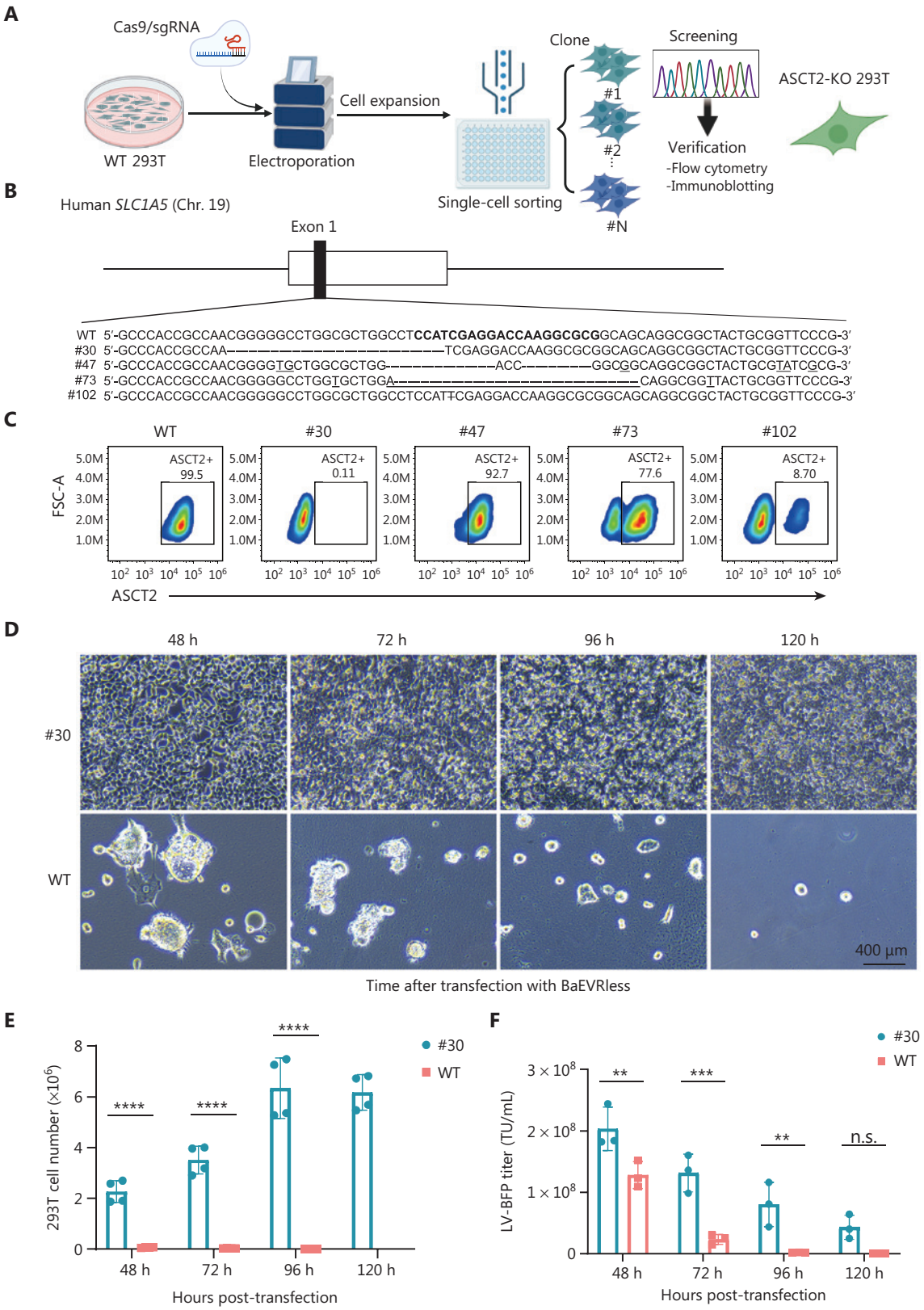


Figure 1 Use of *SLC1A5* KO 293T cells increased the viral titer of BaEVRless pseudotyped LVs. (A) Pipeline for generating *SLC1A5* KO 293T cells by electroporation of Cas9/RNP. The plot was created in BioRender.com. (B) Sanger sequencing results of ASCT2 on WT 293T cells as

a control (top) and different *SLC1A5* KO cell clones (bottom). Bold text indicates the single-guide RNA sequence. (C) Flow cytometry plot of ASCT2 expression levels on WT 293T cells and different *SLC1A5* KO cell clones. (D) Microscopic images of #30 (top) and WT (bottom) 293T cells transfected with BaEVRless plasmids at the indicated times. (E) Bar plot summarizing the cell number of WT 293T cells and #30 293T cells during the BaEVRless-based LV-BFP synthesis procedure at the indicated times after plasmid transfection (sample size n = 4, each dot represents 1 independent experimental replicate). Statistical significance was calculated with 2-way ANOVA and is indicated as $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; bars represent mean values with standard deviation (SD). (F) Bar plot showing the viral titers produced by WT 293T cells and #30 293T cells after transfection with the BaEVRless-based LV-BFP synthesis system at the indicated times (sample size n = 3, each dot represents 1 independent experimental replicate). Statistical significance was calculated with 2-way ANOVA and is indicated as $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, n.s., not significant; bars represent mean values with SD.

than that produced by WT 293T cells on day 5 post-transduction (**Figure 2F**). To determine the activity of the shNKG2A and 1915NGFR transgenes, we determined the expression of NKG2A and NGFR, respectively, with flow cytometry (**Figure S4C, D**). The IL-15 secretion level of the 1915NGFR-transduced NK cells was determined with an ELISA (**Figure S4E**). In agreement with findings from a prior study[11], CAR expression levels on the shNKG2A-transduced NK cells and the 1915NGFR-transduced NK cells both slightly decreased during long-term *ex vivo* expansion (**Figure 2G, H**). Overall, these results indicated that *SLC1A5* KO 293T cell-produced BaEVRless-LVs were able to transduce large-sized transgenes.

The novel BaEVRless-LV packaging system optimizes the clinical CAR-NK cell manufacturing process

Given its outstanding packaging and improved large-sized transgene transduction efficiency, we applied this novel *SLC1A5* KO-293T cell-based BaEVRless-LV packaging system to prepare CAR-NK cells in our current clinical study (NCT05472558). In the clinical study, we generated 1915-CAR NK cells containing the aCD19CAR molecule (derived from FMC63) and a secretory IL-15 construct (**Figure 2I**). To facilitate clinical implementation of the CAR-NK therapy, we further engineered a streamlined *SLC1A5* KO-293T cell-based BaEVRless-LV packaging system by using a 10× biofactory for large-scale production of the 1915-CAR lentivirus, as illustrated in **Figure 2I**. The optimized BaEVRless-LV production increased the viral harvesting time from a single time point (48 h) to 3 time points (48, 72, and 96 h) in all subsequent experiments, thereby decreasing the cost and labor associated with lentiviral production. In addition, the viral yield for each batch increased with the 10× biofactory. The viral titers of LV-1915-CAR generated with this packaging system were markedly greater than those generated with the WT 293T-based packaging system (#30 *vs* WT: 1.37×10^8 *vs* 5.35×10^7 TU/mL average, 1.05×10^9 *vs* 1.53×10^8 TU total; **Table S1**, **Figure 2J**). Correlation analysis confirmed that the high-titer lentiviruses generated by the novel packaging system significantly increased the transduction efficiency of the primary NK cells in clinical production of CAR-NK cells (**Figure 2K**). Hence, the data suggested that transducing the CAR-NK cells with high-titer lentiviruses generated by the #30 293T cell-based LV packaging system might increase CAR expression levels and facilitate the mass production of CAR-NK cells. Using the #30 *SLC1A5* KO 293T cells as an innovative LV packaging system for clinical purposes optimized BaEVRless-LV output and enhanced transduction efficacy.

In summary, we generated *SLC1A5* KO 293T cells as an ideal alternative to WT 293T cells for synthesizing BaEVRless-based LVs. With *SLC1A5* KO 293T cells, BaEVRless-induced 293T cell fusion and detachment were alleviated, and the lentiviral titers significantly increased. We further optimized the lentiviral packaging process to increase the LV yield. This novel LV production system boosted NK cell transduction efficiency and facilitated clinical CAR-NK manufacturing.

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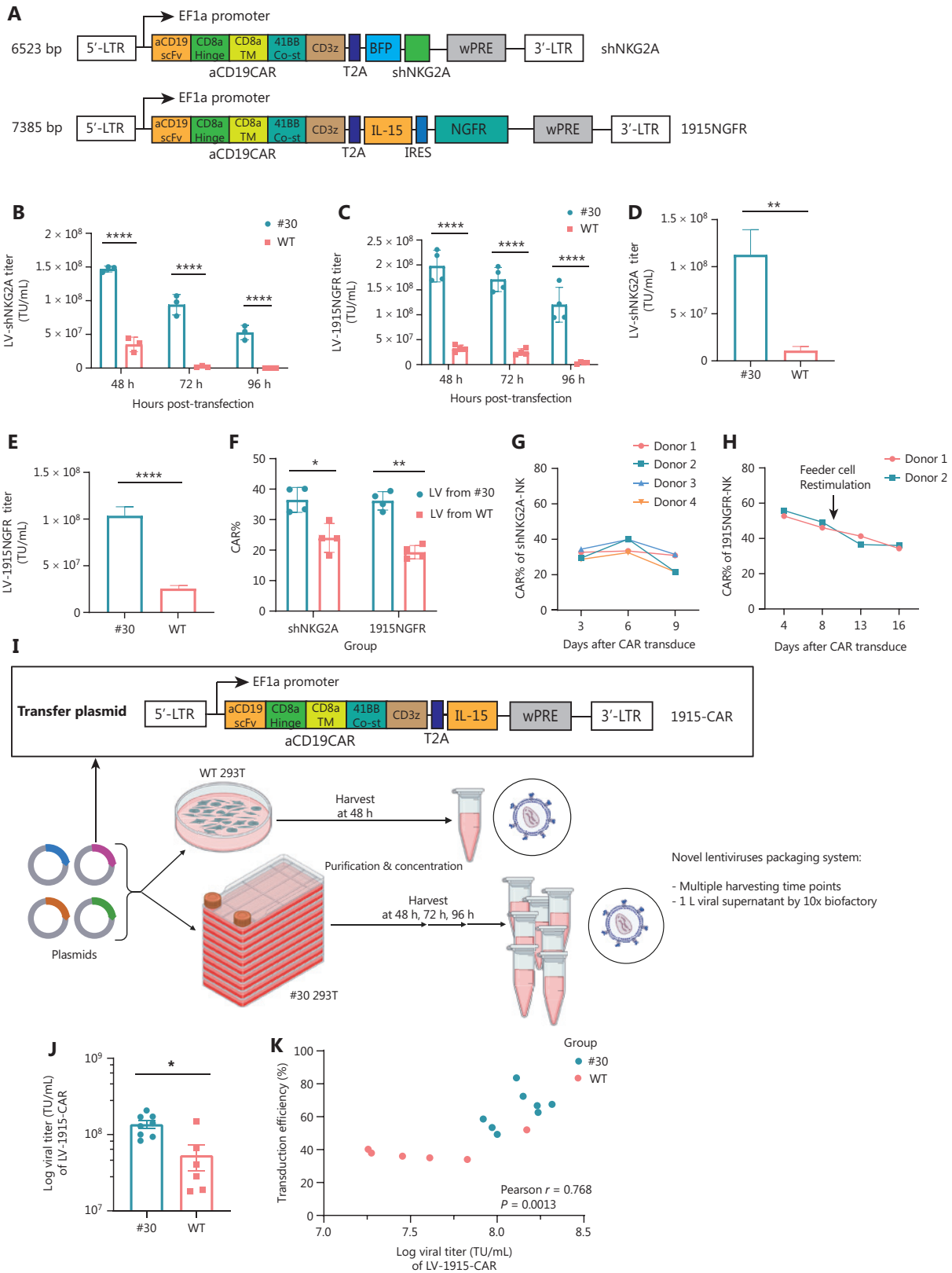


Figure 2 Using *SLC1A5* KO 293T cells in a BaEVRless-based LV package system increases LV productivity and facilitates CAR-NK cell production. (A) Schematic diagram representing the shNKG2A and 1915NGFR structures used to transduce primary NK cells. The left numbers

indicate transgene length. scFv, single-chain variable fragment; TM, transmembrane domain; Co-st, co-stimulation domain. (B) Bar plot showing the LV-shNKG2A titers derived from WT 293T cells and #30 293T cells after transfection with the BaEVRless-based LV-shNKG2A synthesis system at the indicated times (sample size n = 3, each dot represents an individual experimental replicate). Statistical significance was calculated with 2-way ANOVA and is indicated as ****$P \leq 0.0001$; bars represent mean values with SD. (C) Bar plot showing the LV-1915NGFR titers derived from WT 293T cells and #30 293T cells after transfection with the BaEVRless-based LV-1915NGFR synthesis system at the indicated times (sample size n = 4, each dot represents an individual experimental replicate). Statistical significance was calculated with 2-way ANOVA and is indicated as ****$P \leq 0.0001$; bars represent mean values with SD. (D, E) The supernatants harvested from WT and #30 293T cells at the indicated time points described above were mixed and concentrated, to evaluate the overall LV yield. The bar plot shows the overall LV-shNKG2A (D) and LV-1915NGFR (E) titers produced by WT 293T cells and #30 293T cells (D: sample size n = 3, each dot represents an individual experimental replicate; E: sample size n = 4, each dot represents an individual experimental replicate). Statistical significance was calculated with 2-tailed Student's t-test and is indicated as **$P \leq 0.01$, ****$P \leq 0.0001$; bars represent mean values with SD. (F) Bar plot summarizing the mean CAR expression level of primary NK cells transduced with LV-shNKG2A and LV-1915NGFR produced from WT 293T cells and #30 293T, respectively (sample size n = 4, each dot represents an individual donor). Statistical significance was calculated with 2-tailed Student's t-test for each group and is indicated as *$P \leq 0.05$, **$P \leq 0.01$; bars represent mean values with SD. (G) Dot-line plot showing the CAR expression level of shNKG2A-NK cells derived from 4 individuals evaluated every 3 days post-transduction by flow cytometry. (H) Dot-line plot showing the CAR expression level of 1915NGFR-NK cells derived from 2 individuals evaluated at the indicated times after CAR transduction. (I) Top: Schematic diagram representing the 1915-CAR plasmid used in the clinical trial. scFv, single-chain variable fragment; TM, transmembrane domain; Co-st, co-stimulation domain. Bottom: Schematic diagram of the BaEVRless-LV production pipeline using WT 293T cells and #30 293T cells. The plot was created in BioRender.com. (J) Bar plot of log-transformed titers of the individual LV-1915-CAR batch produced with the WT 293T cell-based (sample size n = 6, each dot represents an individual donor) or #30 293T cell-based (sample size n = 8, each dot represents an individual donor) BaEVRless-LVs production system. The data distribution of the viral titers was found to be log-normal with the Shapiro-Wilk test. Statistical significance was calculated with a 2-tailed Student's t-test after log transformation and is indicated as *$P \leq 0.05$; bars represent mean values with SD. (K) After transduction of primary NK cells with the individual LV-1915-CAR viral batch shown in (J), Pearson correlation analysis between the transduction efficiency of the infected NK cells and the corresponding log-transformed viral titers was conducted (r = 0.768, P = 0.0013). Each dot represents an individual donor.

Conflict of interest statement

No potential conflicts of interest are disclosed.

Author contributions

Conception, design, and manuscript writing: Wenbin Qian, Wen Lei, Wenhai Deng, and Yinyin Zhang.
Collection, analysis, and interpretation: Yinyin Zhang, Minghuan Zhang, Mengyuan Li, Lihong Zong, and Qian Ye.

Data availability statement

The data generated in this study are available upon request from the corresponding authors.

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