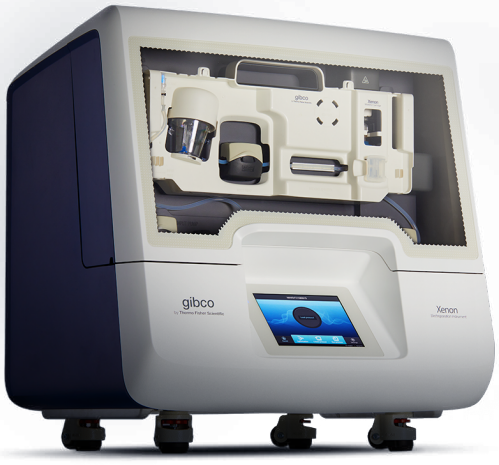
Cell therapy

Optimization and scale-up of genetically modified NK cells

Keywords
CAR NK cell therapy, gene editing, gene delivery, electroporation, cell therapy manufacturing, allogeneic cell therapy

Introduction

Natural killer (NK) cells are part of the innate immune system and have been shown to be responsible for rapidly identifying and killing foreign, virally infected, and tumorigenic cells. Like T cells, NK cells can be genetically modified to increase specificity and potency for cell therapy applications. However, compared to T cells, genetic modification of primary NK cells is more difficult. Viral transduction can be inconsistent, even at high multiplicity of infection (MOI), and plasmid transfection has limited efficiency for expressing genes of interest [1,2]. To obtain robust genetic modification of NK cells, Kararoudi et al. reported that electroporation with Cas9 RNP followed by transduction with an adeno-associated virus resulted in efficient, stable, and functional editing of NK cells [3]. Here we describe screening and optimization experiments we conducted to create a genetic modification protocol for generating CAR NK cells using upstream and downstream cell processing with the Gibco™ CTS™ Rotea™ Counterflow Centrifugation System and a combination of viral and nonviral payload delivery methods with the Gibco™ CTS™ Xenon™ Electroporation System.



CTS Xenon Electroporation System

Materials and methods

Cell isolation and electroporation

The NK cell genome editing process (Figure 1) started with separation of peripheral blood mononuclear cells (PBMCs) from healthy donor leukopaks using the CTS Rotea Counterflow Centrifugation System, followed by isolation of CD56+ NK cells from PBMCs using labeled beads. Isolated NK cells were then expanded in Gibco™ CTS™ NK-Xpander™ Medium, both before and after electroporation. Isolated, expanded NK cells underwent gene knockout with Gibco™ CTS™ TrueCut™ Cas9 Protein and the Invitrogen™ TrueGuide™ sgRNA RNP system, via electroporation. To generate CAR-NK cells, NK cells were transduced with AAV6 virus carrying anti–mesothelin 3 CAR DNA under the control of an EF1 alpha promoter 30 minutes after Cas9 RNP electroporation. AAV6 virus was produced using the Gibco™ AAV-MAX Helper-Free AAV Production System Kit. Genetically modified cells were analyzed using the Invitrogen™ Attune™ NxT Flow Cytometer. See the application notes "**The CTS Rotea system: automation and consistency in PBMC separation—a comparison of protocols**" for detailed protocols for PBMC separation using the CTS Rotea system, and "**Closed, automated wash and concentration of expanded human natural killer (NK) cells**" for detailed protocols for NK cell expansion using CTS NK-Xpander Medium.

Electroporation (EP) parameters were optimized for small-volume (10 µL) editing of NK cells by testing 24 preset protocols on Invitrogen™ Neon™ systems. The five top-performing protocols were then tested at small scale (10 µL and 100 µL in the Neon Transfection and Neon NxT Electroporation Systems) and a larger scale (1 mL in the CTS Xenon system). Different systems were compared side by side using the highest-performing EP protocol, and that protocol was then used to test high-density electroporation and ultimately generate anti-meso3 CAR NK cells. This anti-meso3 CAR targets region III of mesothelin, a cell surface protein that has been identified in many different tumor types and is known to play a role in the regulation of proliferation, growth, and adhesion signaling.

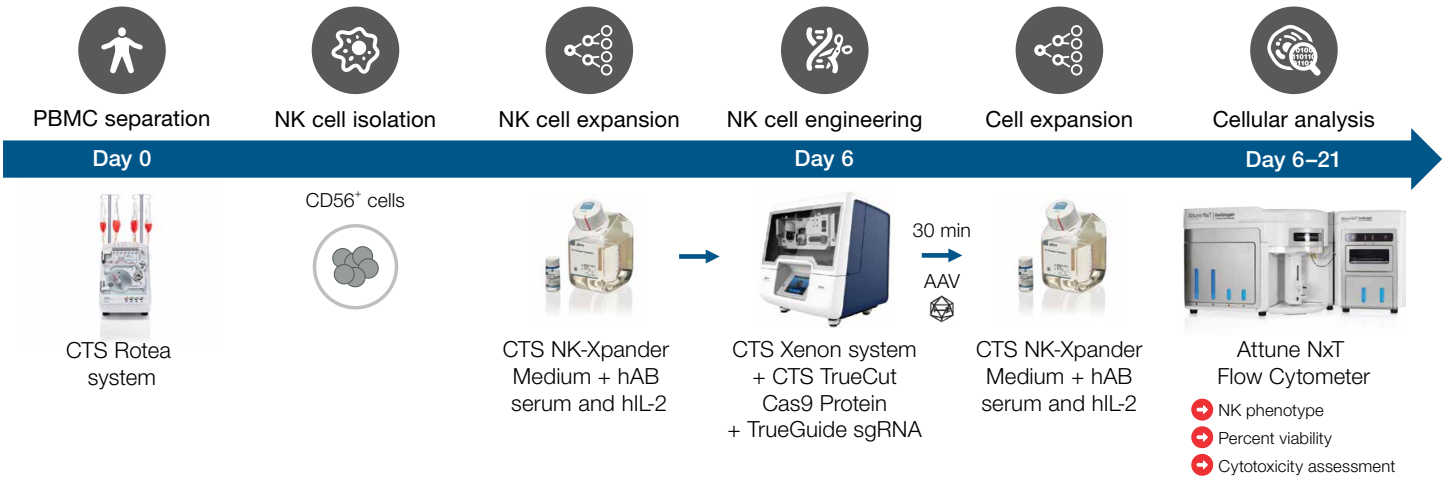


Figure 1. NK cell genome editing workflow using Gibco CTS products.

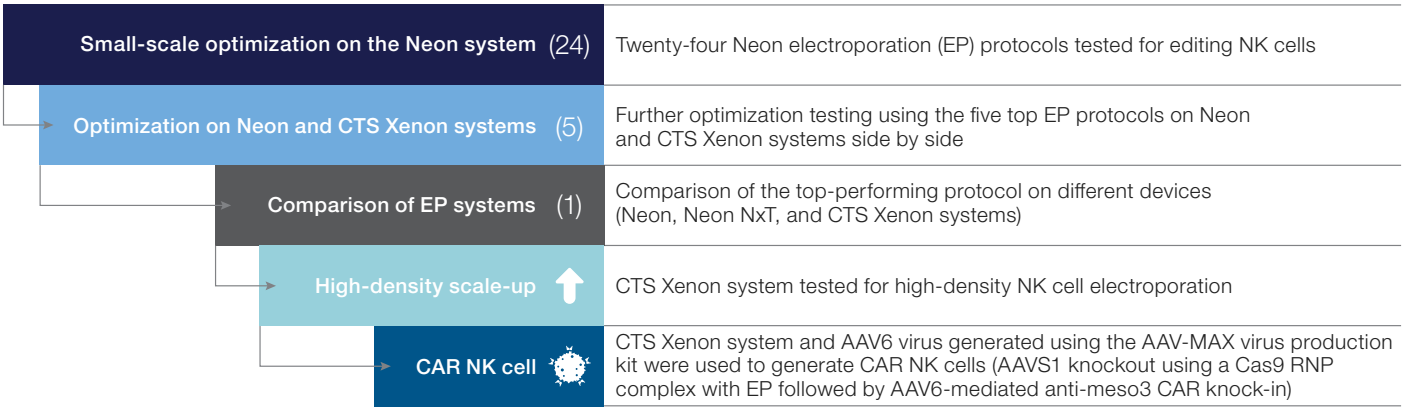


Figure 2. Genome editing optimization steps. Twenty-four potential electroporation (EP) protocols were tested and optimized to find the best-performing protocol, which was used to test high-density electroporation and ultimately used to manufacture anti-meso3 CAR NK cells.

NK cell engineering

Several EP experiments were conducted in this study, with details for each listed in Table 1. For all experiments, unless otherwise noted, cultured NK cells were concentrated to 50 x 10^6 cells/mL in Gibco™ CTS™ Xenon™ Genome Editing (GE) buffer on day 6. The Cas9 RNP complex was prepared at 120 µg/mL CTS TrueCut Cas9 Protein and 30 µg/mL TrueGuide sgRNA, added to cells, and electroporated following preset optimization programs.

Table 1. Electroporation parameters for all experiments (initial screening, scale-up, and CAR NK manufacturing).

Experimental parameter	Details
Common parameters for each electroporation experiment	
Cell source separation and isolation	PBMCs were separated from cryopreserved leukapheresis products from healthy donors, using the CTS Rotea system. NK cells were then isolated from the PBMCs via CD56 negative-selection magnetic beads.
Cell concentration during EP*	50 x 10^6 cells/mL in CTS Xenon GE buffer 1:20 RNP:cells (50 µL CRISPR-Cas9 RNP for every 1 mL EP reaction)
Payload (Cas9-sgRNA RNP complex)**	CTS TrueCut Cas9 Protein (120 µg per 1 mL of EP reaction) Custom β2M TrueGuide sgRNA (30 µg per 1 mL of EP reaction)
Initial screening on the Neon electroporation system	
Electroporation system	Neon system (10 µL)
Samples	N = 1 donor No-EP control
Electroporation protocol†	Neon system, following preset programs 1–24 using AAVS1 sgRNA for knockout
Scale-up screening on Neon and CTS Xenon systems	
Electroporation system	Neon system (100 µL) CTS Xenon system (1 mL, Gibco™ CTS™ Xenon™ SingleShot Electroporation Chamber)
Samples	N = 3 donors No-EP control
Electroporation protocol†	Neon system preset programs 5, 9, 16, 24 CTS Xenon system OPT 1–3 and 5–6 using β2M sgRNA for knockout
Side-by-side comparison of three different electroporation systems	
Electroporation system	Neon system (100 µL) Neon NxT system (100 µL) CTS Xenon system (1 mL, CTS Xenon SingleShot chamber)
Samples	N = 3 donors No-EP control
Electroporation protocol†	Neon/Neon NxT system preset program 5 (1,700 V, 20 ms pulse width, 1 pulse) CTS Xenon system OPT 1 (1,700 V, 20 ms pulse width, 1 pulse) using β2M sgRNA for knockout
High-density scale-up on the CTS Xenon system	
Electroporation system	Neon system (100 µL) CTS Xenon system (1 mL, CTS Xenon SingleShot chamber)
Samples	N = 2 donors No-EP control
Cell concentration during EP	50 x 10^6 cells/mL in CTS Xenon GE buffer for scale-up using Neon and Xenon systems 100 x 10^6 cells/mL in CTS Xenon GE buffer for high-density scale-up using the CTS Xenon system 1:20 RNP:cells (50 µL CRISPR-Cas9 RNP for every 1 mL EP reaction)
Electroporation protocol†	Neon system preset program 5 CTS Xenon system OPT 1
Generation of CAR NK cells	
Electroporation system	Neon system (100 µL) CTS Xenon system (1 mL, CTS Xenon SingleShot chamber)
Samples	N = 3 donors No-EP control
Payload	CTS TrueCut Cas9 Protein (120 µg/mL) Custom AAVS1 TrueGuide sgRNA (30 µg/mL) AAV6–anti-meso3 CAR (1 x 10^5 multiplicity of infection (MOI), transduced 30 minutes post-EP)
Electroporation protocol†	Neon system preset program 5 CTS Xenon system OPT 1

* Cell concentration during EP is consistent in all experiments except high-density scale-up in the CTS Xenon system. Cell concentration details are listed in the high-density scale-up section of the table.

** Payload for generation of CAR NK cells is different from those of the other experiments, with details listed in the "Generation of CAR NK cells" section of the table.

† Details of all EP protocols are also listed in Table 2.

In the initial small-scale screening, 24 preset protocols were tested on the Neon Transfection System (Table 2). The four top-performing programs, plus one additional program that has been used for successful editing of T cells (CTS Xenon system OPT 6), were selected for additional screening and protocol confirmation and optimization on the Neon and CTS Xenon systems. The top-performing program from the second screening (CTS Xenon system OPT 1/Neon program 5) was used for all remaining experiments—side-by-side comparison of CTS Xenon system vs. Neon system vs. Invitrogen™ Neon™ NxT Electroporation System, high-density scale-up in CTS Xenon system, and creation of anti-meso3 CAR NK cells.

Expansion following genetic modification

Following every EP reaction, cells were plated in Gibco™ CTS™ NK-Xpander Medium with human AB serum and IL-2, following recommendations in the **user guide**. Cells were cultured according to recommendations and analyzed for viability, phenotype including EP efficiency, and functionality (CAR NK cytotoxicity assessment).

Table 2. Electroporation protocols tested in various systems. Twenty-four protocols were tested in the Neon or Neon NxT system. Five progressed to testing in the CTS Xenon system, and one protocol was tested in all three systems. CTS Xenon system preset protocol OPT 1 combined with Neon/Neon NxT protocol program 5 is the recommended starting protocol when working with NK cells. **Note:** Variability among donors, user-specific payload type, or genomic loci may necessitate further optimization of EP protocols to obtain optimal editing efficiencies.

CTS Xenon system protocol	Neon/Neon NxT program	Voltage (V)	Pulse width (ms)	Pulse number	Pulse interval (ms)
Protocols tested for small-scale screening in the Neon system, which did not progress to testing in the CTS Xenon system					
Not preset	Program 1	Control without electroporation			
Not preset	Program 2	1,400	20	1	–
Not preset	Program 3	1,500	20	1	–
Not preset	Program 4	1,600	20	1	–
Not preset	Program 6	1,100	30	1	–
Not preset	Program 7	1,200	30	1	–
Not preset	Program 8	1,300	30	1	–
Not preset	Program 10	1,000	40	1	–
Not preset	Program 11	1,100	40	1	–
Not preset	Program 12	1,200	40	1	–
Not preset	Program 13	1,100	20	2	1,000
Not preset	Program 14	1,200	20	2	1,000
Not preset	Program 15	1,300	20	2	1,000
Not preset	Program 17	850	30	2	1,000
Not preset	Program 18	950	30	2	1,000
Not preset	Program 19	1,050	30	2	1,000
OPT 4	Program 20	1,150	30	2	1,000
Not preset	Program 21	1,300	10	3	1,000
Not preset	Program 22	1,400	10	3	1,000
Not preset	Program 23	1,500	10	3	1,000
Protocols selected for scale-up screening in CTS Xenon system after showing success in the Neon system					
OPT 2	Program 9	1,400	30	1	–
OPT 3	Program 16	1,400	20	2	1,000
OPT 5	Program 24	1,600	8 (CTS Xenon system) 10 (Neon system)	3	1,000
OPT 6	Not preset	2,300	3	4	500 (CTS Xenon system) 1,000 (Neon system)
Recommended starting protocol for NK cell electroporation (tested in the CTS Xenon, Neon, and Neon NxT systems)					
OPT 1	Program 5	1,700	20	1	–

NK cell phenotype and viability characterization

For the primary screening of 24 different EP protocols, the Invitrogen™ GeneArt™ Genomic Cleavage Detection (GCD) Kit was used to measure AAVS1 knockout (KO) efficiency. For all other EP experiments, the phenotype of expanded and genetically modified NK cells was assessed by flow cytometry on the Attune NxT Flow Cytometer by gating for live cells using the Invitrogen™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit, and measuring expression of CD56, β2M, and anti-meso3 CAR (V5+) using appropriate antibodies.

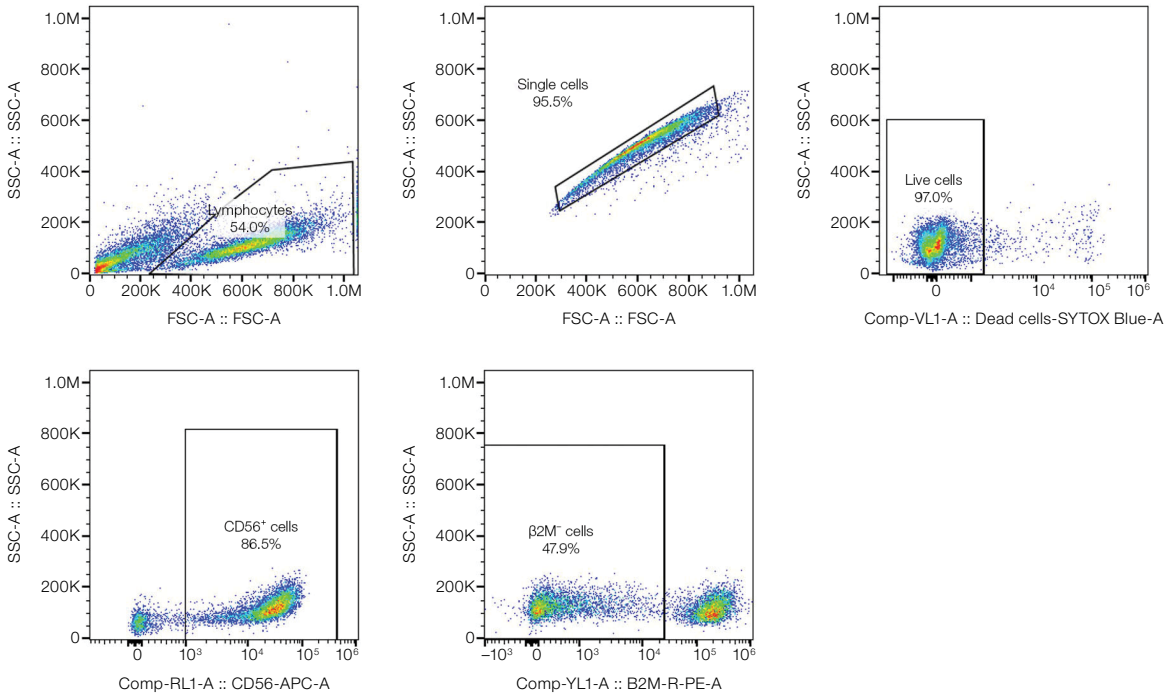
Functional CAR NK cells kill tumor cells *in vitro*

Genetically modified NK cells were co-incubated with SKOV3-GFP (target cells) at effector-to-target cell ratios of 0.625:1, 1.25:1, 2.5:1, 5:1, and 10:1 for 6 hours. Following incubation, cytotoxicity of NK and CAR NK cells was assessed by measuring SKOV3 cell death on the Attune NxT system by gating for GFP-expressing cells and measuring the percentage of dead cells using the LIVE/DEAD stain kit. Cells were imaged using an Invitrogen™ EVOS™ imaging system.

Gating strategies

Gene editing efficiency for both β2M KO and anti-meso3 CAR knock-in was calculated as a percentage of viable CD56⁺ single cells, with gates set according to the no-EP control (Figure 3).

A β2M KO



B Anti-meso3 CAR NK cells

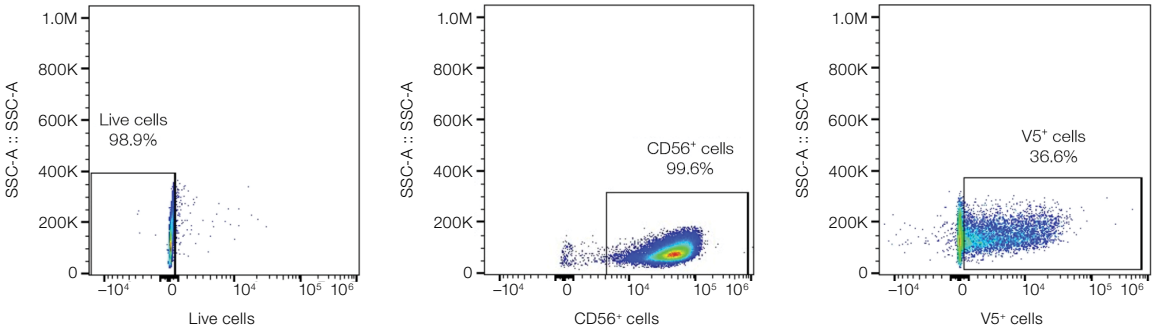


Figure 3. Gene editing efficiency for (A) β2M KO and (B) anti-meso3 CAR NK cells were calculated as a percentage of viable CD56⁺ cells, with gates set according to the no-EP control.

Results

Initial screening on the Neon and Neon NxT systems

Of the 24 preset protocols screened on both Neon and Neon NxT systems, four protocols (5, 9, 16, and 24) reached an acceptable threshold of cutting efficiency using the GeneArt GCD assay and were advanced to scale-up screening on the CTS Xenon system (Figure 4). Best practices would also include data confirmation on a more quantitative platform.

Scale-up screening on the Neon and CTS Xenon systems

In addition to the four protocols chosen in the initial NK cell EP screening, one additional protocol was selected for scale-up screening on the CTS Xenon system, as it has shown good performance for editing T cells. Protocols OPT 6 and OPT 1 worked well on both the Neon and CTS Xenon systems, resulting in the highest β2M KO% and highest total viable edited NK cells on day 3 post-EP (Figure 5). OPT 6 had slightly higher KO% yet slightly lower viability and expansion, due to a higher voltage used. Based on these results, Neon OPT 6 and OPT 1 protocols are good small-volume starting points to consider.

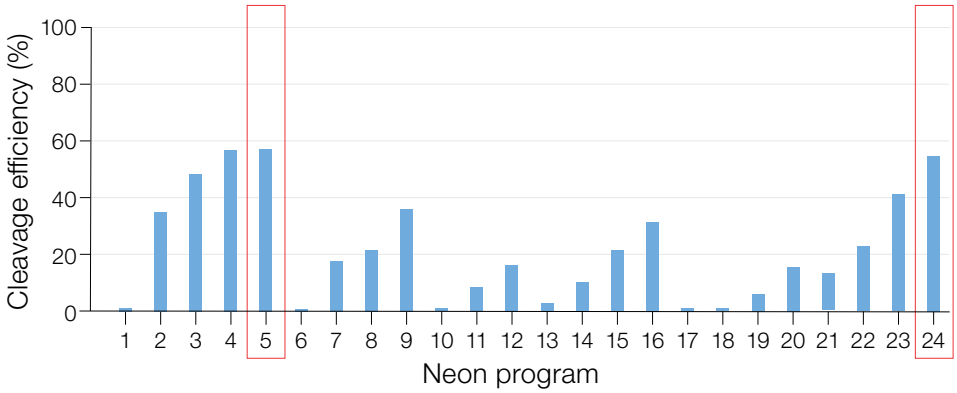


Figure 4. AAVS1 gene KO efficiency using 24 different preset Neon system protocols, 72 hr post-electroporation, as measured using the GeneArt GCD assay.

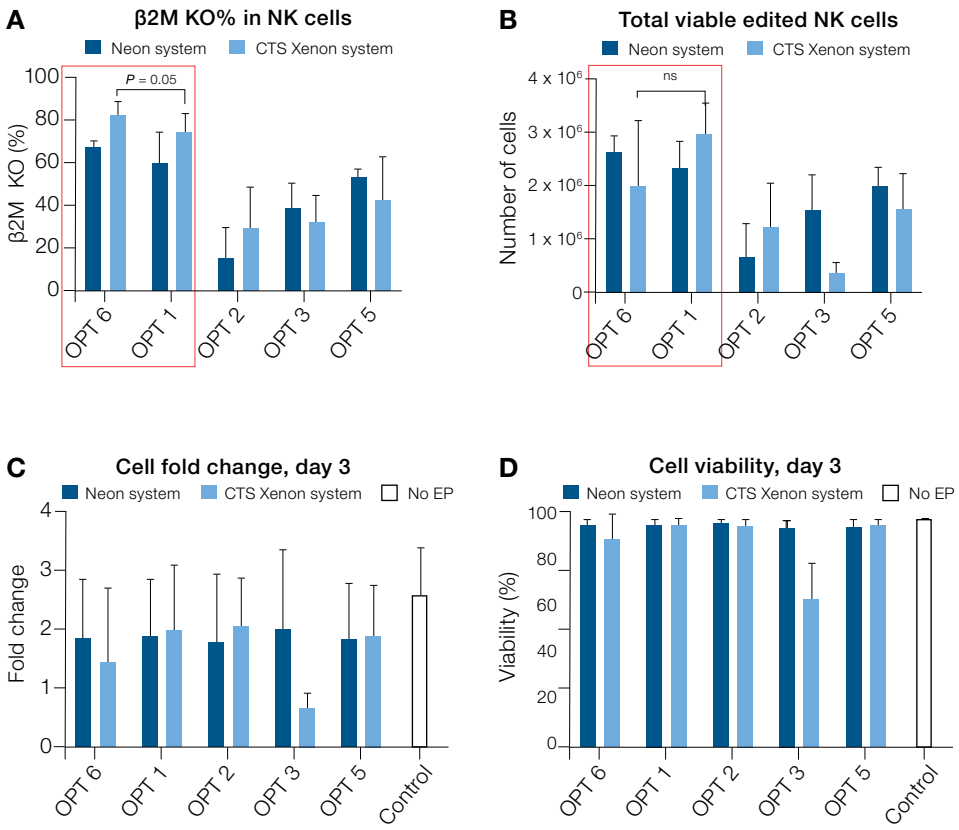


Figure 5. EP efficiency as measured by (A) β2M KO% in NK cells, (B) total viable edited NK cells, (C) NK cell fold change 3 days post-EP, and (D) cell viability 3 days post-EP. The OPT 1 and OPT 6 protocols for the Neon and CTS Xenon systems performed well, as demonstrated by β2M knockout and total viable edited cells. The OPT 6 protocol provided a higher voltage, while OPT 1 was a gentler protocol that still generated high editing efficiency. N = 3 leukopaks.

Comparison of electroporation systems

To compare the two small-volume and one large-volume EP systems, one program was tested (voltage at 1,700 V, 20 milliseconds, and 1 pulse) using three different donors on all three systems: the CTS Xenon, Neon, and Neon NxT systems. Across the three systems there was 61–85% KO efficiency. Edited NK cells maintained their phenotypes, as the cell population ranged from 75–88% CD56⁺ cell with 81–99% cell viability (Figure 6). The CTS Xenon system consistently showed higher KO.

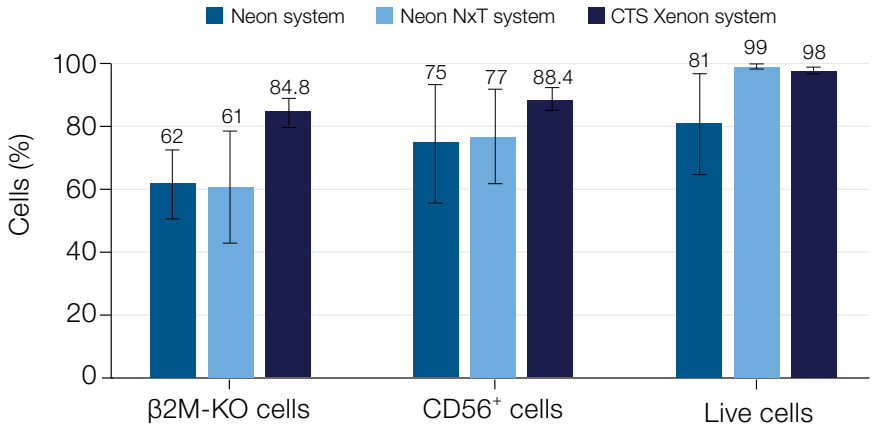


Figure 6. Side-by-side comparison of Neon, Neon NxT, and CTS Xenon electroporation systems (n = 3 donors) using the same electroporation parameters. The CTS Xenon system consistently shows higher KO.

High-density scale-up

To test high-density electroporation compared to the standard density of 50 x 10^6 cells/mL, 100 x 10^6 cells/mL were electroporated on the CTS Xenon system using the same protocol (CTS Xenon system OPT 1). All densities showed acceptable KO efficiency (73–84% β2M KO), phenotype (93% CD56⁺), and viability (94–97% viability), as shown in Figure 7.

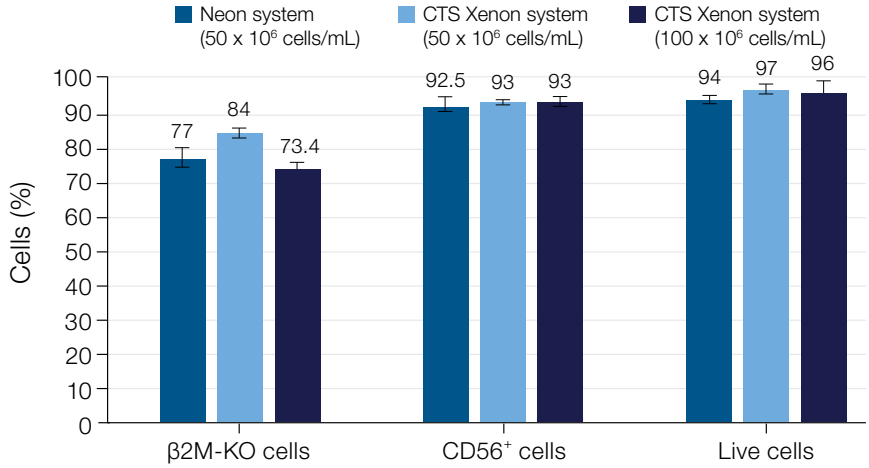


Figure 7. High-density electroporation in CTS Xenon system. The low (standard) cell density (50 x 10^6 cells/mL) was tested on both the Neon and CTS Xenon systems, and the high cell density (100 x 10^6 cells/mL) was tested on the CTS Xenon system only (n = 2 donors for all experiments), using the same electroporation parameters.

Generation of CAR NK cells

Generation of CAR NK cells by delivery of AAV6–anti-meso3 CAR to NK cells through viral transduction alone (no EP, negative control) proved unsuccessful, as can be seen by lack of V5⁺ expression in the top histogram (Figure 8A). To address this, a combination of viral and nonviral genetic modification methods was explored. A Cas9-AAVS1 RNP complex was delivered to NK cells using either the Neon or the CTS Xenon electroporation system to knock out the AAVS1 locus (Figure 8A, middle and bottom panels), followed by transduction with AAV6–anti-meso3 CAR at an MOI of 10^5, 30 minutes post-EP (Figure 8B). The anti-meso3 CAR construct is under the control of an exogenous EF1 alpha promoter with a V5 tag. Cells showed stable expression of anti-meso3 CAR, were able to expand, and maintained CD56⁺ phenotype and high viability (Figure 8B–E).

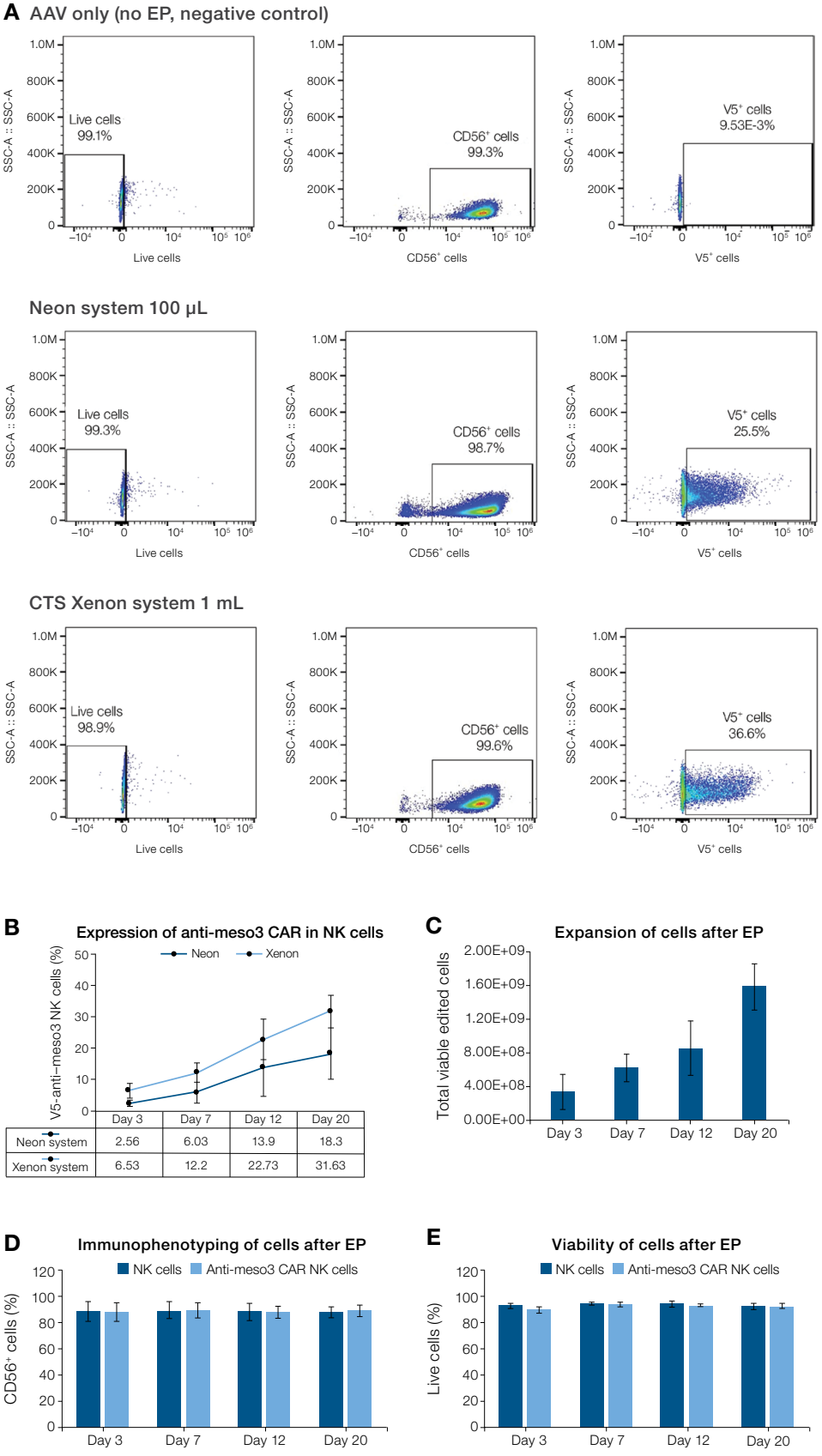


Figure 8. CAR NK cell generation studies. (A) Delivering anti-meso3 CAR by AAV6-mediated viral transduction alone (AAV only, no RNP via EP) showed no anti-meso3 CAR knock-in, as confirmed by no significant V5⁺ cells. Delivery of Cas9 RNP via electroporation (middle and bottom panels), followed by **(B)** transduction with AAV6–anti-meso3 CAR, resulted in stable expression of anti-meso3 CAR in edited NK cells. **(C–E)** These cells were able to expand and maintain a CD56⁺ phenotype and high viability.

Assessing the function of CAR NK cells

The anti-meso3 CAR NK cells generated using the protocols above showed improved killing efficiency of SKOV3-GFP cells compared to unmodified NK cells during a 6 hr co-culture (Figure 9).

Discussion and conclusion

NK cells have potential as an effective allogeneic source of therapeutic cells armed to fight many diseases. However, difficulty in growing and genetically modifying NK cells has hindered progress toward their adoption in the clinic. Here we have presented a combination of viral and nonviral strategies to genetically modify NK cells efficiently, using commercially available products developed specifically for use in cell therapy manufacturing.

The Neon or Neon NxT system coupled with the CTS Xenon system provides a solution for seamless scalability from research- to clinical-scale genetic modification. In addition, the CTS Xenon system can help further scale up manufacturing by enabling efficient electroporation at high cell densities.

The CTS suite of products when used together are powerful tools—closed modular instruments supported by robust reagents—that can further the efforts toward creating an efficient NK cell therapy manufacturing process.

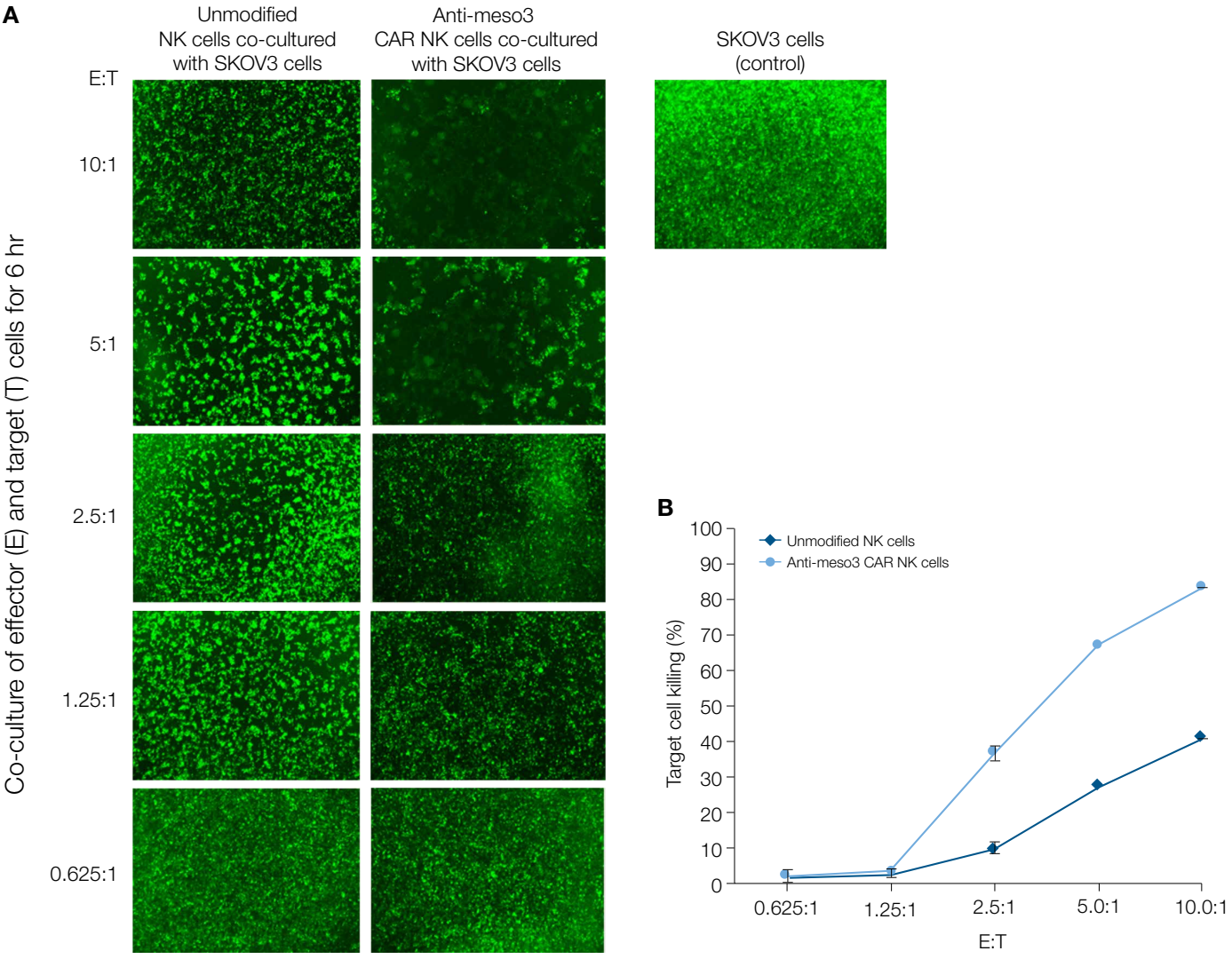


Figure 9. Functional efficiency of anti-meso3 CAR NK cells: CAR NK cells elicit cytotoxicity against the SKOV-3 tumor cell line. (A) Live field images for different effector to target cell ratios. **(B)** Engineered anti-meso3 CAR NK cells have higher killing efficiency toward the target tumor cell line than the unmodified NK cells in this *in vitro* assay. E: effector cells; T: target tumor cell line.

References

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