Efficient Non-Viral Genome Engineering Produces Functional, Expandable CAR-NK Cells

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Abstract

Introduction: Natural killer (NK) cells have intrinsic cytotoxicity and a strong safety profile, making them ideal for allogeneic immunotherapies. Gene editing can be used to enhance the function and persistence of NK cells in vivo. TcBuster™ is a commercially available non-viral transposase system that can deliver large payloads efficiently into NK cells.

Methods: NK cells purified from peripheral blood were grown in serum-free media and edited via electroporation using TcBuster-M mRNA with a multicistronic CD19-CAR plasmid. NK cell phenotype and CD19 CAR expression were determined by flow cytometry. Genomic integration of the CAR was quantified by dPCR. Cytotoxicity and cytokine secretion were measured using luciferase-based target cell assays and Simple Plex™ automated ELISA.

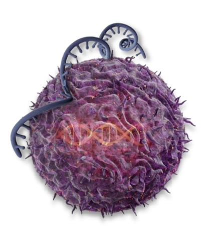
Results: NK cells edited with TcBuster demonstrated high CD19-CAR expression without compromising expansion and viability. Gene editing enabled tumor-specific killing by NK cells and enhanced cytokine secretion while leaving intrinsic cytotoxicity intact. Edited NK cells displayed equivalent phenotypic marker expression compared to unmodified controls.

Conclusion: The TcBuster transposon system provides a practical non-viral path to potent allogeneic CAR-NK therapies.

Materials and Methods

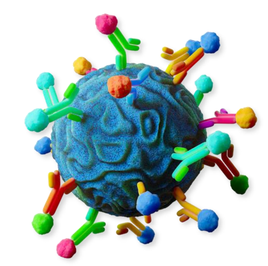
Media and Culture

NK cells were isolated from PBMCs of 5 independent donors and seeded at a density of 1.5 x 10^6 cells/cm^2 into G-Rex® bioreactors (ScaleReady™). NK cells were activated with irradiated feeder cells and expanded with ExCellerate™ Human NK Cell Expansion media supplemented with 50 IU/mL IL-2. Cells were cultured for 4 days before editing. After editing, NK cells were rested for 2 days, then restimulated with irradiated feeder cells weekly. NK cells were cultured for 16 days post editing for a total culture time of 20 days.



NK Cell Engineering with TcBuster

NK cells were edited using TcBuster-M mRNA and a 5.8 kb multicistronic DNA transposon encoding CD19-CAR and eGFP. The Lonza® 4D Nucleofector® was used to electroporate 5 x 10^6 NK cells with 1 µg of mRNA and 7.5 µg of transposon in 100 µL Nucleocuvettes using program CM-138. Three biological replicates were used for each of the 5 donors with a single No EP control per donor.



NK Cell Analysis

Cell counts and viability were assessed using a NucleoCounter® NC-200™ cell counter. Phenotype analysis was performed by flow cytometry using LIVE/DEAD™ Fixable Aqua, a CD19 protein fluorokine, and antibodies against CD56, CD3, CD57, CD16, CD158, NKp46, NKp44, NKp30, NKG2A, NKG2C, and NKG2D. Transposition efficiency was percent of CD56$^+$ CD3$^-$ cells expressing CD19-CAR$^+$. Genomic integration of CD19-CAR insert was quantified by QIAcuity Digital PCR (dPCR) system.

Potency Assays

NK cells were co-cultured with K562 tumor cells for 4 hours, or CD19$^+$ Nalm-6 cells for 24 hours in 96-well plates. Supernatants were reserved following the incubation periods. Luminescence was measured after the addition of assay buffer and substrate. Killing was calculated as % Killing = [1-(Sample Luminescence/ Control Luminescence)] x 100. Wells containing only target cells served as controls. Supernatants from Nalm-6 co-cultures were analyzed using Simple Plex™ multi-analyte cartridges to detect human IFN-γ, TNF-α, Granzyme B, and Perforin on the Ella™ Instrument.

Table 1: Key R&D Systems Reagents

Material	Catalog Number
ExCellerate™ Human NK Cell Expansion Media, ACF	CCM037
Recombinant Human IL-2 GMP Protein, CF	BT-002-GMP
TcBuster-M Transposase mRNA	TCB-001.1
TcBuster Transposon CD19CAR-DHFR-eGFP	TCBP001-100
Recombinant Human CD19 Protein, Atto 647N Conjugate	ATM9269-020
Simple Plex Cell Activation Panel 2	ST01C-CS-007366

TcBuster Non-Viral Engineering System

The TcBuster system requires three components: TcBuster-M mRNA, a DNA transposon containing the gene of interest (GOI) flanked by TcBuster-specific inverted terminal repeats (ITRs), and an electroporation system.

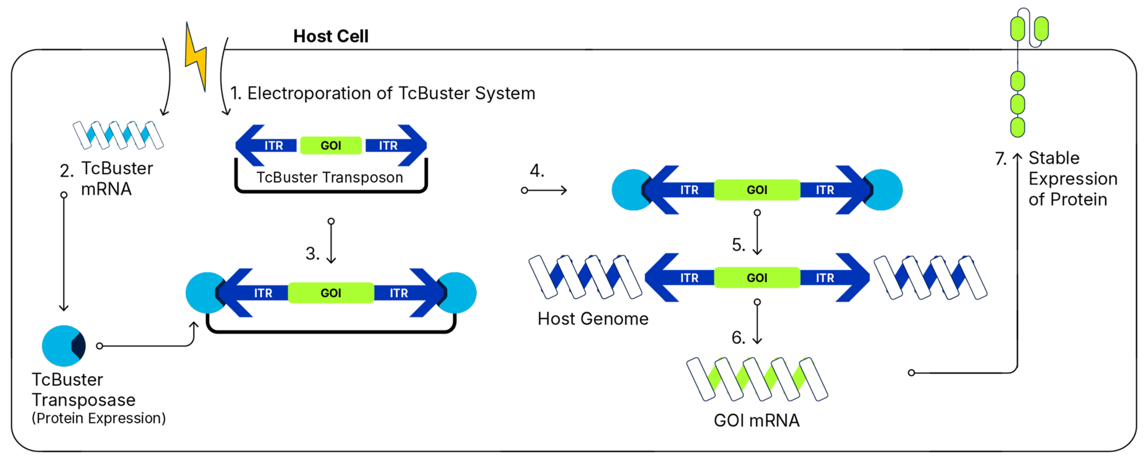


Figure 1: The TcBuster transposition mechanism. Electroporation is used to introduce the TcBuster components into the cell. TcBuster mRNA is translated into the transposase enzyme, which binds to ITRs on the DNA transposon. TcBuster then excises the GOI from the transposon and inserts it into the host genome, allowing stable expression of the GOI.

Results

NK cells engineered with TcBuster achieve high transposition efficiency and undergo robust expansion.

NK cells from five donors were expanded for 4 days then edited with TcBuster to express a CD19-CAR. Cells were then grown for 16 days post editing for a total culture period of 20 days. Expression of the CD19-CAR was ≥ 60% in all donors while copy number remained below 6 (**Figure 2, A&B**). Fold expansion at harvest ranged between 13,000-fold and 500,000-fold depending on donor (**Figure 2C**). Viability was high for all donors, averaging over 92% viability for TcBuster-edited samples (**Figure 2D**).

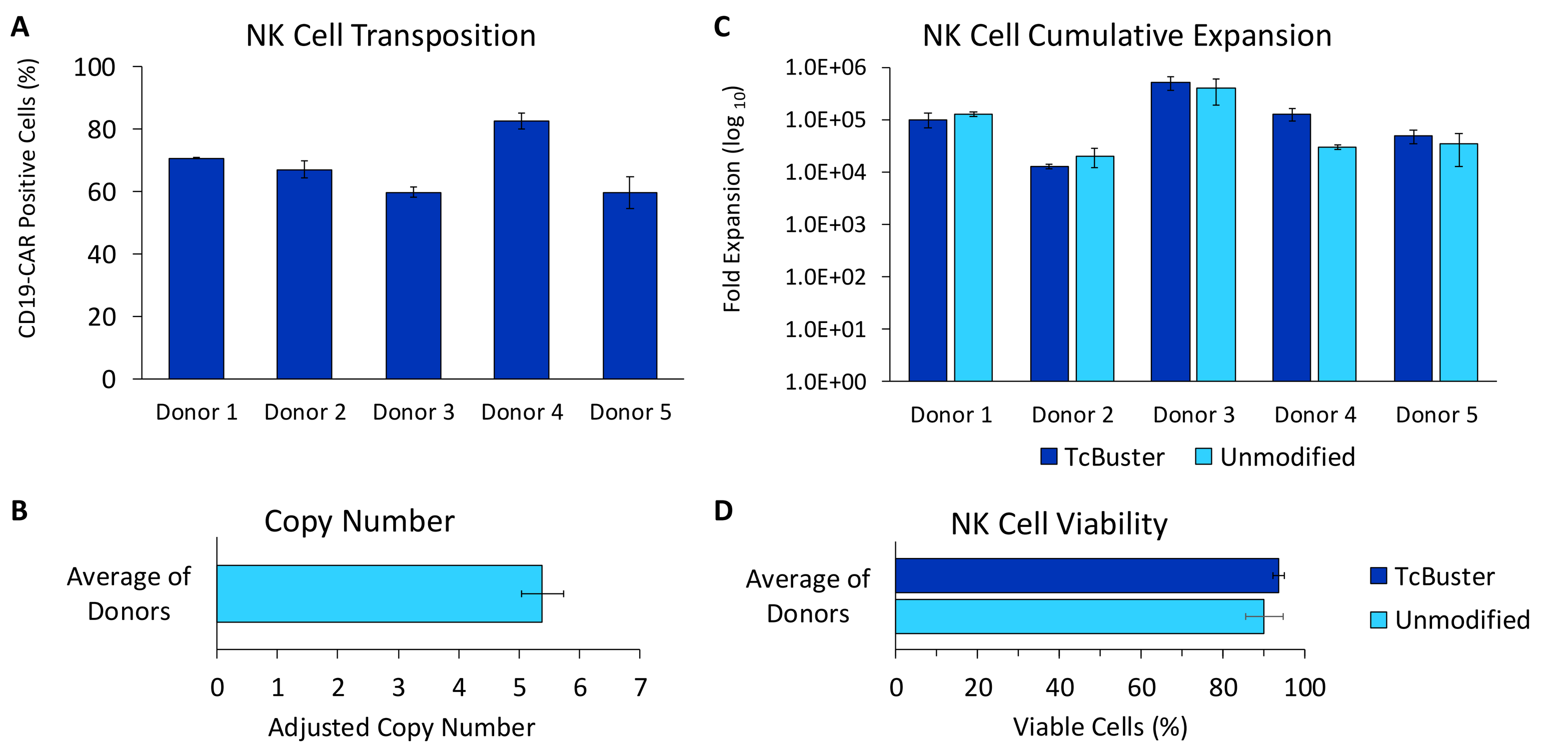


Figure 2: NK cells on day 20 of culture. **(A)** Percentage of NK cells in culture expressing CD19-CAR. **(B)** Average genomic vector copy number quantified by dPCR. **(C)** Cumulative expansion of CD56$^+$ CD3$^-$ NK cells in TcBuster-edited cells and unmodified growth controls. Expansion between donors is variable; note log_{10} axis. **(D)** Total cell viability in culture. Data is the average of 5 donors with 3 biological replicates ±SD.

NK cells edited with TcBuster express a favorable phenotype.

NK cells were evaluated by flow cytometry to detect common NK cell markers. Gene editing had a negligible impact on expression when compared to unmodified controls. High expression of CD16 and NKG2A characterize functionally competent NK cells, with low CD57 expression indicating reduced terminal differentiation and greater proliferative potential.

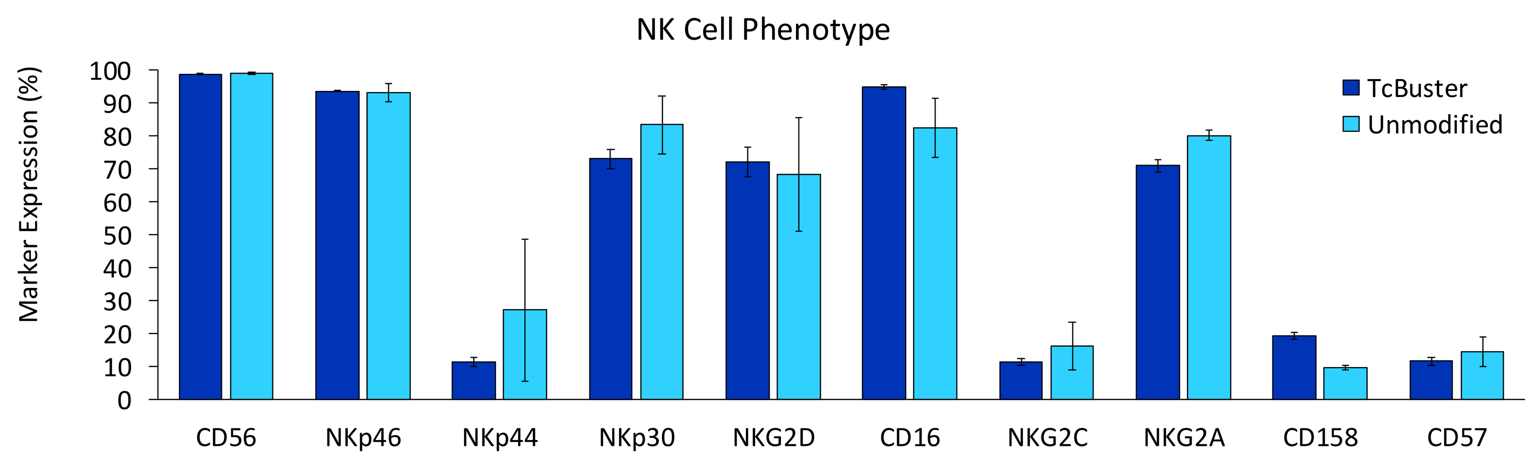


Figure 3: Representative expression of common NK cell markers at day 20 of culture. Data is the average of biological triplicates in one donor ±SD.

NK cells engineered with TcBuster show enhanced immune effector function and maintain innate cytotoxicity.

Day 20-cryopreserved NK cells were thawed and co-cultured with CD19$^+$ Nalm-6 cells or K562 cells. CD19-CAR-NK cells exhibit potent antigen-specific activity, clearing 80% of Nalm-6 target cells at a 1:1 ratio of NK cells to tumor cells. Innate cytotoxicity was preserved in edited NK cells, with K562 target cell killing comparable to unmodified cells (**Figure 4**). Secretomes from Nalm-6 cytotoxicity assays were analyzed using Simple Plex multi-analyte cartridges. CAR-NK cells show enhanced secretion of pro-inflammatory cytokines IFN-γ and TNF-α compared to unmodified controls. Granzyme B and Perforin secretion match unmodified NK levels (**Figure 5**). Combined with the enhanced killing ability of CD19-CAR-NK cells, this demonstrates targeted efficacy of the existing cytolytic granule pool in comparison to unmodified cells.

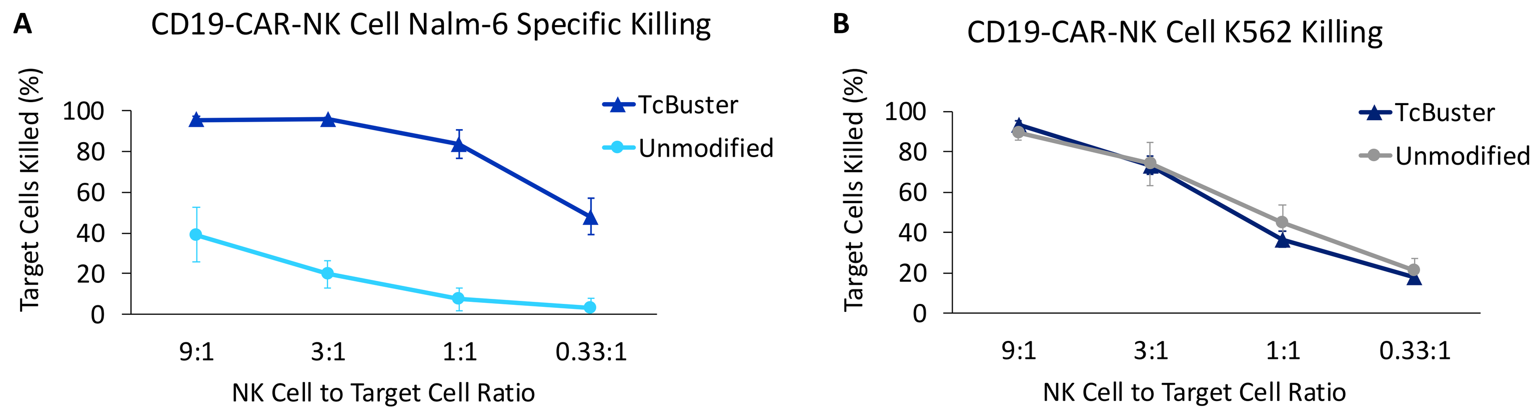


Figure 4: (A) Percentage of CD19$^+$ Nalm-6 target cells killed by NK cells. NK cell numbers were not adjusted for CAR percentage; results include all NK cells in culture. **(B)** Percentage of K562 tumor cells killed by NK cells. Data is the average of 5 donors, each with biological triplicates ±SD.

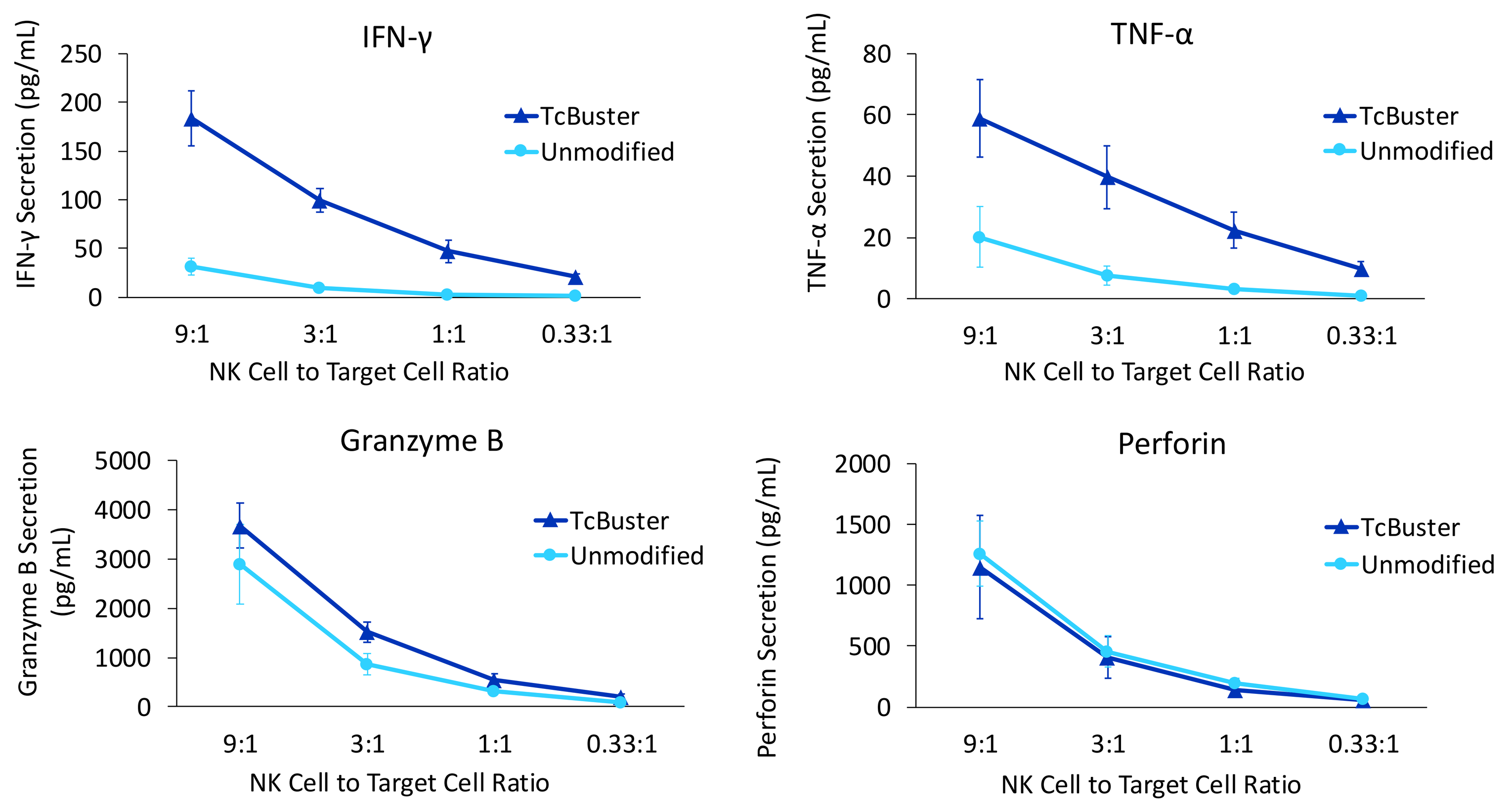


Figure 5: Supernatants from Nalm-6 cytotoxicity assays were analyzed on the Simple Plex Ella instrument. Levels of secreted IFN-γ, TNF-α, Granzyme B and Perforin are shown for both TcBuster-edited and unmodified NK cells. Data is the average of 4 donors ±SD. Note one donor was excluded due to 3-fold higher secretion levels than the other donors.

Conclusions

- Here we leveraged the TcBuster non-viral transposon system to generate CAR-NK cells with high transposition efficiency and low copy number while achieving robust expansion and viability.
- CD19-CAR-NK cells show enhanced cytotoxic potential toward specific targets while retaining innate function and phenotype.
- The TcBuster transposon system efficiently integrates large gene editing cassettes and provides an effective tool for the generation of therapeutic CAR-NK workflows.

References

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Scan the QR code to learn more about the TcBuster transposon platform with R&D Systems at www.rndsystems.com/tcbuster