Lipid Nanoparticle-Mediated Gene Editing of Human Primary T Cells and Off-Target Analysis of the CRISPR-Cas9 Indels

Booth #317

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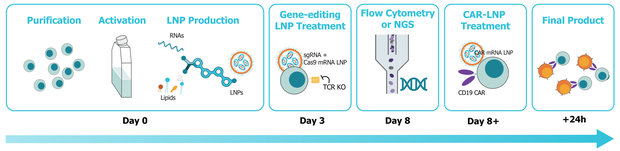
Introduction

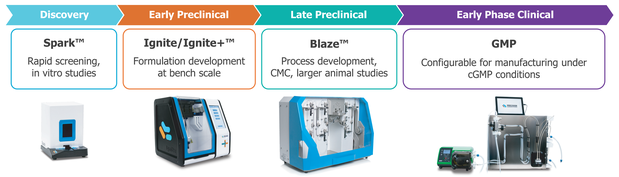
- Engineering T cells to express chimeric antigen receptor (CAR) redirects these cells to target tumours, making this a promising cell-based cancer therapy [1].
- To engineer universal CAR T cells for allogeneic cell therapy, gene editing can be used to remove risks associated with graft-versus-host disease [2,3].
- However, conventional delivery methods such as viral vectors and electroporation have performance and manufacturing shortcomings that limit their utility for the multi-step engineering of T cells [4].
- Lipid nanoparticles (LNPs) use endogenous uptake pathways to deliver therapeutic RNAs, making this technology gentle to cells. LNPs are fully synthetic and can be rapidly scaled-up using microfluidics [5,6].
- Here we report on the use of a novel LNP reagent in an optimized protocol to achieve successful multi-step engineering of primary T cells with high efficiency and high cell viability.
- Further, we used the rhAmpSeq™ CRISPR Analysis system for targeted next-generation sequencing (NGS) to evaluate multi-target performance of Cas9-mediated editing in the LNP treated T cells

Materials and Methods



Materials

Lipid nanoparticle reagent, GenVoy-ILM™ T Cell Kit for mRNA, Precision NanoSystems ULC, 1000683 and 1001144 • CleanCap® Cas9 mRNA (5moU), Trilink, L-7206 • CleanCap® CD19-CAR mRNA (wt), Trilink, custom order • CleanCap® WT or HiFi mRNA (wt bases), Trilink, custom order • sgRNAs Alt-R® CRISPR-Cas9 sgRNA, Integrated DNA Technologies, custom targets • Quant-iT™ RiboGreen® RNA Assay Kit,Thermo Fisher Scientific, R11490 • Cryopreserved T cells, STEMCELL Technologies, 70024 • ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator, STEMCELL Technologies, 10970 • ImmunoCult™-XF T Cell Expansion Medium, STEMCELL Technologies, 10981, • Human Recombinant IL-2, STEMCELL Technologies, 78036 • Recombinant Human Apo E4, Peprotech, 350-04 • rhAmpSeq™ CRISPR Analysis System, Integrated DNA Technologies



Equipment

NanoAssemblr® Spark™ (NIS0003) and Ignite™ (NIN0001), Precision NanoSystems ULC • BioTek™ Synergy™ H1 plate reader, Agilent • CytoFLEX V3-B3-R0, Beckman Coulter, C09747 • NextSeq™ 2000 Sequencing System, 20038897

Results

1. Lipid nanoparticles were produced with consistent size and high encapsulation efficiency

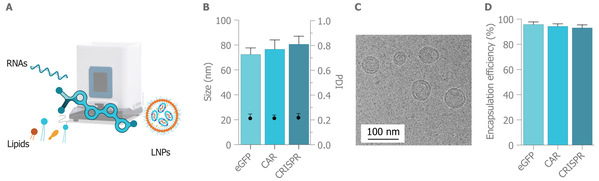


Figure 1. A) Schematic of LNP production using microfluidics. **B)** Size and polydispersity Index (PDI) of LNPs with different RNA payloads: left axis (bars) for size, right axis (dots) for PDI. **C)** Cryo-TEM image of mRNA loaded LNPs. **D)** Encapsulation efficiency (%EE) of LNPs with different mRNA payloads. For B, D: error bars represent standard deviation. GFP n=25 LNPs, CAR n=18 LNPs, CRISPR-Cas9 n=12 LNPs.

2. Dose response shows the tunable nature of LNP treatment

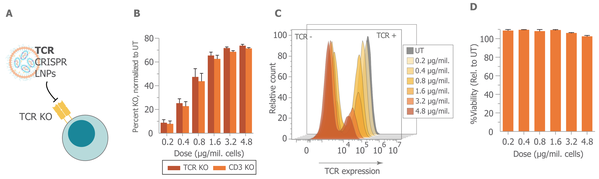


Figure 2. B) Percent TCR (red) or CD3 (orange) knockout of TCR sgRNA treated samples at the indicated 0.2 – 4.8 µg RNA / million cells dose. **C)** Surface expression of TCR as detected by flow cytometry at the indicated RNA dose. **D)** Percent cell viability normalized to untreated (UT) for LNP treated samples at the indicated doses. Cas9 mRNA and sgRNA were co-encapsulated at a 1:1 wt. ratio. Error bars represent standard deviation of n=2 technical and n=2 biological replicates.

3. Next generation sequencing shows non-significant off-target effects with LNP-based editing

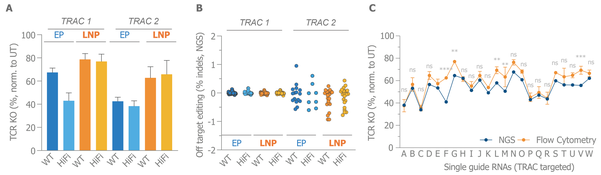


Figure 3. A) Percent TCR knockout of samples treated with TRAC 1 or TRAC 2 sgRNAs, and with wild type or high fidelity Cas9 mRNA, delivered either by electroporation (EP) or with LNPs at a dose of 3.2 µg RNA/million cells. **B)** Off-target editing analysis was performed for both TRAC 1 and TRAC 2 sgRNAs sites using IDT's rhAmpSeq analysis platform. Most off-target sites for TRAC 1 and TRAC 2 were below detection limits of 0.5%. **C)** LNPs were used to screen various TRAC targeting sgRNAs. NGS (rhAmpSeq) was compared with flow cytometry for on-target editing analysis. Error bars represent standard deviation of n=2 technical and n=2 biological replicates.

4. Knockout of both CD52 and TCR achieved in a multiplex gene editing approach

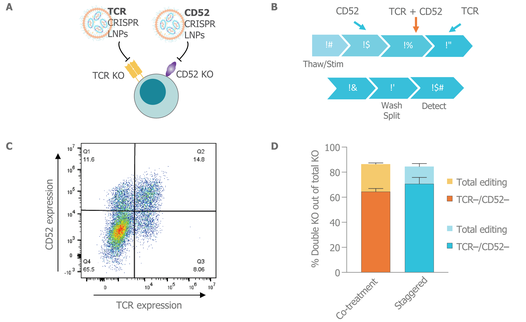


Figure 4. Cells were treated with TCR and CD52 LNPs simultaneously and sequentially **A)** Timeline for double knockout cell workflow. **B)** 2D quadrant analysis of knockout efficiency of both TCR (X-axis) and CD52 (Y-axis) before selection. **C)** Double KO efficiency was determined by dividing Q4/(Q1+Q4+Q3). Error bars represent standard deviation of n=2 technical and n=2 biological replicates.

5. Scalable Microfluidic Technology Allows for Discovery to Pre-clinical Translation

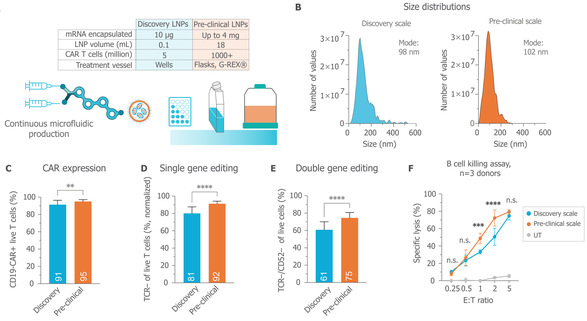


Figure 5. A) Seamless scale-up allows for production of discovery scale LNPs at 10 µg RNA, to pre-clinical scale at 4 mg RNA. **B)** Nanoparticle Tracking Analysis (NTA) shows agreeable size distribution between the two scales. **C)** Percent anti-CD19 CAR expression. **D)** TCR Knockout **E)** TCR/CD52 double knockout efficiency was assessed by flow cytometry **F)** Functional killing of CD19+ B cells (SUP-B15) by either UT or gene-edited TCR−/CAR+ T cells at the indicated effector to target ratios (E:T). For all, a dose of 3.2 µg RNA/million cells was applied. % Specific Lysis determined by normalizing target cell viability in co-culture to untreated target B cell controls. Error bars represent standard deviation. Statistical significance was evaluated using t-tests or one-way ANOVA among the shown groups

6. Seamless Scale-up of Cell Culture Process from Discovery to Pre-clinical Applications

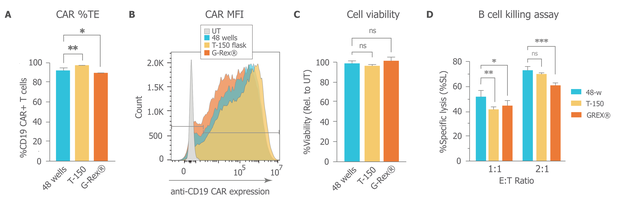


Figure 6. Scalability of cell culture and treatment parameters exemplified with various vessel types and sizes tested in-house. **A)** CAR transfection efficiency in primary T cells, 48 h post-LNP treatment and **B)** representative CAR expression histograms. **C)** Corresponding cell viability relative to the untreated cells. **D)** 24 h post-CAR mRNA-LNP treatment, T cells were evaluated for tumor-specific cytotoxicity. Anti-CD19 CAR T cells (effector, E) were co-cultured with SUP-B15 (target, T) at indicated E:T ratios. % Specific lysis was determined by normalizing target cell viability in co-culture to untreated target B cell controls. For all: Error bars represent standard deviation of n=2 technical and n=2 biological replicates. T cells treated at a 1.6 µg RNA/million T cells. Significance evaluated using t-tests (Figs A-C) or one-way ANOVA with multiple comparisons (Fig D).

Conclusions

- The GenVoy-ILM™ T Cell Kit for mRNA is a versatile LNP reagent for gene expression, gene editing, or the combination of both in a multi-step approach.
- LNPs show exceptional performance with 81.5 ± 4.3% CD19 CAR expression and 92.3 ± 3.0% TCR and 74.5 ± 6.1% TCR−/CD52− gene knockout in primary T cells. LNPs are gentle and maintain >90% cell viability.
- LNPs are easily scalable using PNI's microfluidic technology, where both the discovery and preclinical LNPs show excellent performance.
- High-throughput NGS analysis showed good agreement to flow cytometry for on-target editing and negligible off-target editing.

References

[1] Feins, S., et al., An introduction to chimeric antigen receptor (CAR) T-cell immunotherapy for human cancer. Am J Hematol, 2019. 94(S1): p. S3-S9. [2] Rafiq, S., C.S. Hackett, and R.J. Brentjens, Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol, 2020. 17(3): p. 147-167. [3] Zhao, J., et al., Universal CARs, universal T cells, and universal CAR T cells. J Hematol Oncol, 2018. 11(1): p. 132. [4] Atsavapranee, E.S., M.M. Billingsley, and M.J. Mitchell, Delivery technologies for T cell gene editing: Applications in cancer immunotherapy. EBioMedicine, 2021. 67: p. 103354. [5] Absalon, J., K. Koury, and W.C. Gruber, Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. Reply. N Engl J Med, 2021. 384(16): p. 1578. [6] Baden, L.R., et al., Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. N Engl J Med, 2021. 384(5): p. 403-416.

Acknowledgments

We thank the contributions of Rebecca De Souza, Andrew Brown and Helena Son during the early development of the T Cell Kit. We further thank the guidance of Vicki Stronge throughout the development process, and Suraj Abraham for assistance in the design of the CAR construct. Additionally, Ghinwa Darwish for conducting the NTA measurements.