Efficient *In Vivo* Gene Editing of T Cells Utilizing Novel Targeted Lipid Nanoparticles

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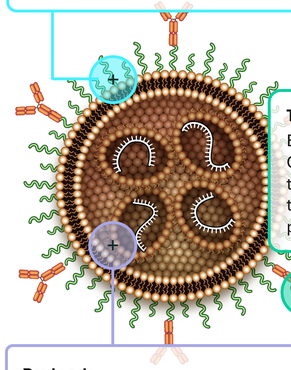
Background

Immunotherapy, and T cell therapy in particular, have revolutionized the oncology field and carry promise in other indications. Nevertheless, novel, scalable technologies to engineer immune cells *in vivo* are needed. In this study, we present results from a novel T cell-targeting lipid nanoparticle (tLNP) designed to deliver gene editing (GE) payloads to T cells, with potential applicability including next-generation immunotherapies. These tLNPs is a product of Capstan's CellSeeker™ platform technology, which comprises a novel LNP conjugated with a recombinant protein binder designed to deliver RNA payloads to specific cell types *in vivo*.

LNP Delivery Vehicle

Capstan's proprietary lipid nanoparticle is a non-viral system demonstrated in preclinical models to have rapid biodegradation affording the ability to re-dose.



Targeting Binders

Binding moieties, such as anti-CD8 antibodies, conjugated to the nanoparticle surface enable tLNPs to preferentially deliver payloads to CD8⁺ cells.

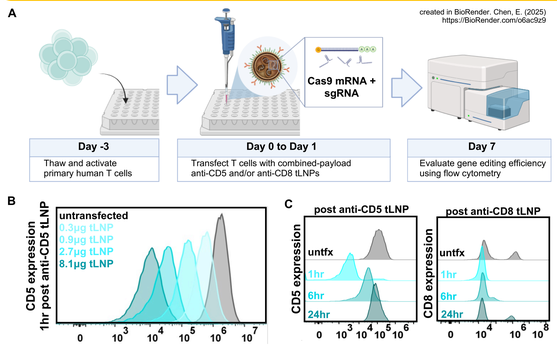
Payloads

RNA payloads comprise gene-editing components such as single guide RNA (sgRNA) and mRNA-encoded CRISPR/Cas9.

Methods

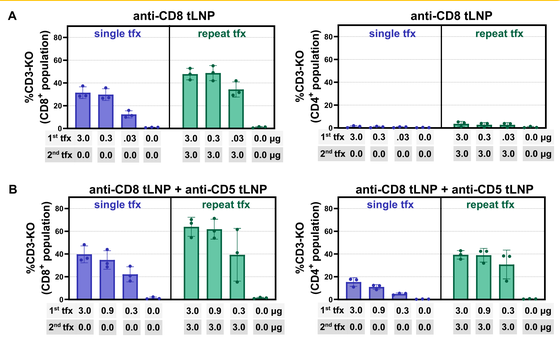
Gene editing payloads, consisting of an mRNA encoding Cas9 and an sgRNA targeting T-cell receptor alpha constant (TRAC), were encapsulated separately (single-payload) or co-encapsulated (combined-payload). tLNPs were targeted to either pan-T cells via an anti-CD5 antibody, or a T cell subset via an anti-CD8 antibody. The dosage listed in this study refers to the total amount of RNA. Single- and combined-payload tLNPs were administered to primary human T cells in vitro and intravenously (IV) to NCG mice engrafted with human T cells. Primary T cells were isolated from human leukopaks and activated with CD3/CD28 beads prior to tLNP transfection. T cell gene editing efficiencies in vitro and *in vivo* were evaluated using flow cytometry.

Figure 1. Combined-payload anti-CD8 and/or anti-CD5 tLNPs for gene editing (GE)



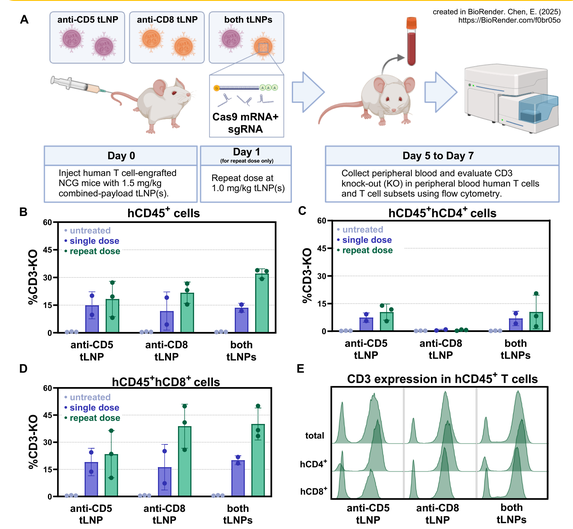
A) Schematic of in vitro transfection of primary human T cells using combined-payload anti-CD5 or anti-CD8 tLNP delivering Cas9 mRNA and TRAC-targeting sgRNA. **B)** Histogram showing CD5 expression on primary human T cells 1 hour after transfection with 0.3, 0.9, 2.7, or 8.1 µg of RNA in anti-CD5 tLNP. **C)** Histogram of CD5 expression (left) or CD8 expression (right) on human primary T cells at 1, 6, or 24 hours post anti-CD5 or anti-CD8 tLNPs, showing antigen recovery timeframe for possible repeat transfection.

Figure 2. Specific and efficient GE achieved by combined-payload anti-CD8 and/or anti-CD5 tLNPs



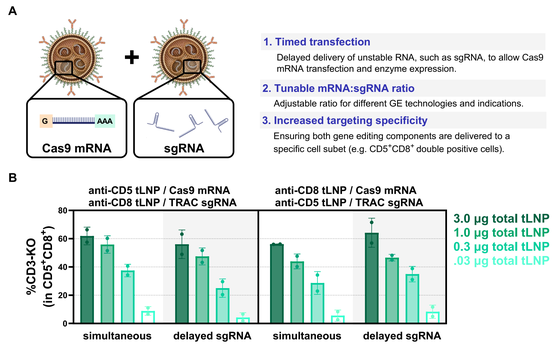
A) Percentage of CD3 knock-out (CD3-KO) in vitro in human primary T cells 7 days after a single transfection (tfx) or a repeat transfection (performed 24 hours after the first transfection) with combined-payload anti-CD8 tLNPs. **B)** Percentage of CD3 knock-out (CD3-KO) in human primary T cells 7 days after a single transfection or a repeat transfection with a combination of anti-CD8 tLNP and anti-CD5 tLNPs. CD3 expression was measured by flow cytometry (FC), and data are presented as mean ± SD (n=3 donors).

Figure 3. Efficient gene editing of human T cells *in vivo* using combined-payload tLNPs



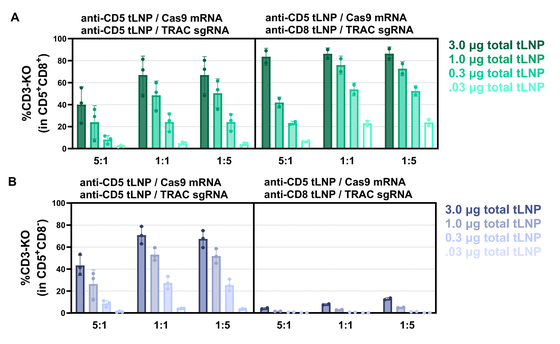
A) Schematic of the *in vivo* study in human T cell-engrafted NCG mice. Mice were IV injected with 1.5 mg/kg tLNP(s) on Day 0. For repeat dose, a 2nd dose of 1.0 mg/kg was administered on Day 1. **B)** Day 5 percentage of CD3-KO in hCD45⁺ T cells in peripheral blood (PB) after treatment(s) with combined-payload tLNPs: anti-CD5 tLNP, anti-CD8 tLNP, or a combination of both tLNPs. **C)** Percentage of CD3-KO in hCD45⁺hCD4⁺ T cells in PB on Day 5. **D)** Percentage of CD3-KO in hCD45⁺hCD8⁺ T cells in PB on Day 5. **E)** Representative histogram of CD3 expression in T cell subsets on Day 7. CD3 expression was measured by FC, and data are presented as mean ± SD (n=2-3 mice).

Figure 4. Single-payload anti-CD5 and/or anti-CD8 tLNPs allow tunable transfection



A) Schematic of single-payload tLNPs: one tLNP delivering Cas9 mRNA and one tLNP delivering TRAC-targeting sgRNA. The benefits of single-payload tLNPs include timed transfection, tunable mRNA:sgRNA ratio, and increased specificity. **B)** Percentage of CD3-KO in human primary CD5⁺CD8⁺ T cells using single-payload tLNPs delivering mRNA or sgRNA simultaneously or with a 6-hour delay in sgRNA delivery. Data are presented as mean ± SD (n=2).

Figure 5. Single-payload tLNPs allow tuning RNA ratios for increased GE efficacy and specificity



A) Percentage of CD3-KO in human primary CD5⁺CD8⁺ T cells using various mRNA:sgRNA ratios. Cas9 mRNA was delivered using anti-CD5 tLNP, while sgRNA was delivered using either anti-CD5 or anti-CD8 tLNPs. **B)** Percentage of CD3-KO in human primary CD5⁺CD8⁻ T cells using anti-CD5 tLNP delivering Cas9 mRNA and using either anti-CD5 or anti-CD8 tLNP delivering TRAC-sgRNA. CD3 expression was measured by flow cytometry, and data are presented as mean ± SD (n=2-3 donors).

Results and Conclusions

This proof-of-concept study supports the potential of our CellSeeker™ platform through precise and efficient gene editing of T cells *in vivo* or *ex vivo*. Using both single- or combined-payload tLNPs, we achieved robust gene knockout, with efficiencies reaching up to 90% KO in vitro and 40% *in vivo* (Figures 2-5). Additionally, our data showed that split-payload tLNPs enable controlled transfection timing (Figure 4), tunable mRNA:sgRNA ratios and enhanced specificity for distinct T cell subsets (e.g. CD5⁺CD8⁺ double-positive cells) (Figure 5). These results highlight the Cellseeker™ platform's effectiveness and specificity in delivering GE components directly to T cells, with broad potential applicability to 1) gene knock out to modulate immune pathological processes and enable immunotherapy in general, or 2) provide a springboard for the development of next-generation durable *in vivo* CAR therapies.