An all-Prime Editing, one-step approach for non-viral generation of a multiplex-edited CAR-T cell drug product

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Background

Multiplex Prime Editing to generate an allogeneic off-the shelf cell drug product may be able to address current limitations of CAR-T cell therapy:

- Manufacturing time, costs, yield, and efficacy associated with autologous cell therapy cell quantity and quality issues
- Safety risks associated with semi-random integration & double strand breaks at multiple genomic loci

Current strategies for delivery and expression of CAR transgenes are limited by:

- Semi-random integration via lentivirus or transposons risks unintended gene disruption of activation of proto-oncogenes and may lead to variable efficacy
- Targeted integration using nuclease + template for HDR limited by low efficiency and risks associated with DSB induction (e.g., chromothripsis, p53 activation)

Limitations of current gene editing strategies for multiplex editing

- Targeted gene disruption at multiple loci simultaneously with nucleases carries a risk of chromosomal rearrangements
- Base editing to disrupt splicing or introduce pmSTOP codons is limited in scope, risks pmSTOP readthrough, and cannot support targeted integration

PASSIGE™ in combination with multiplex Prime Editing (PE) maybe be able to overcome these challenges to create a potentially best-in-class allogeneic CAR-T cell product

PASSIGE strategy to integrate CAR at T cell receptor locus (T Cell Receptor Alpha or *TRAC*):

- ✓ **Antigen specific T cell receptors** created to eliminate pathogenic cells
- ✓ **Potential to prevent graft-versus-host disease** by knocking out endogenous TCR expression

Prime Editing to KO MHC Class I

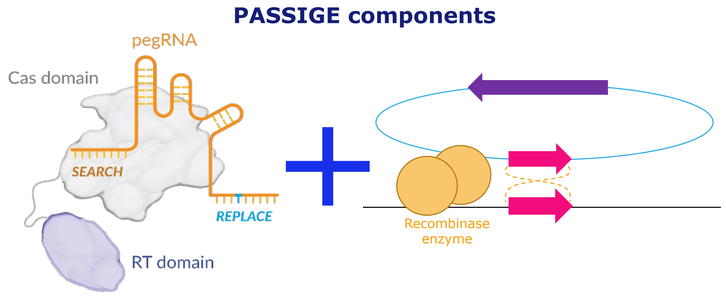
- ✓ Edited cells may **evade patient immune system**
- ✓ Allows for **repeat administration** if needed

Multiplex with other indication-specific Prime Edits

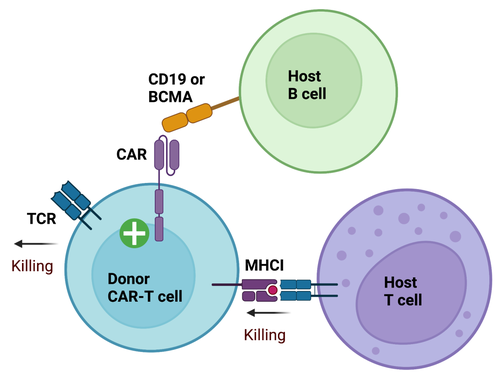
Prime Editing Assisted Site-Specific Integrase Gene Editing (PASSIGE):
Prime Editing in combination with integrases or recombinases for targeted integration of gene-sized DNA

- ✓ Targeted integration of DNA in a **single delivery step**
- ✓ **No double strand break (DSB)** as integrase catalyzes recombination directly
- ✓ **Integration can be irreversible:** attL and attR products are distinct from initial attB and attP sequences

PASSIGE components



Model of Multiplex Edited CAR-T

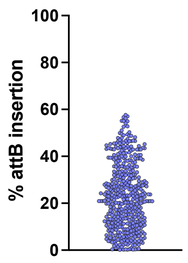


Results

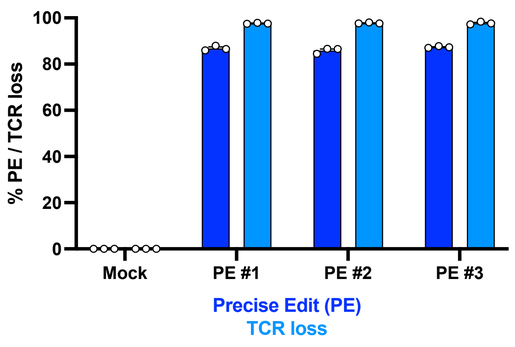
1 High performing Prime Editors precisely insert attB sequence at *TRAC* locus with >80% efficiency

- ✓Prime Editors screened using automated HTS in cell line
- ✓Targeted exon 1 to make use of endogenous promoter
- ✓Top performing Prime Editors tested in human T cells
- ✓Top performing Prime Editors optimized in human T cells
- ✓Achieved >80% precise attB insertion

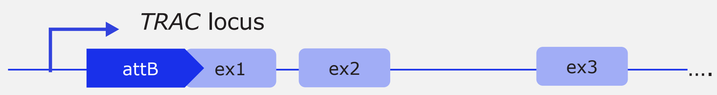
Prime Editors screened in cell line

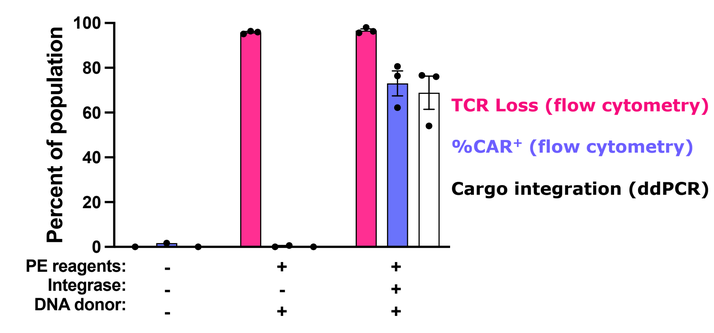


Top 3 Prime Editors validated in human T cells (n=3 donors)



2 Non-viral PASSIGE delivery supports integration of >3.5 kb CD19 CAR construct at *TRAC* locus in >70% of T cells

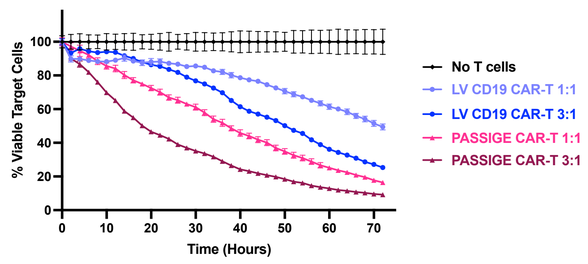




- ✓ Loss of endogenous TCR with attB insertion in *TRAC* exon 1
- ✓ Use of endogenous *TRAC* promoter allows for tuned regulation of expression
- ✓ Promoter-less cargo will not express if integrated elsewhere in genome

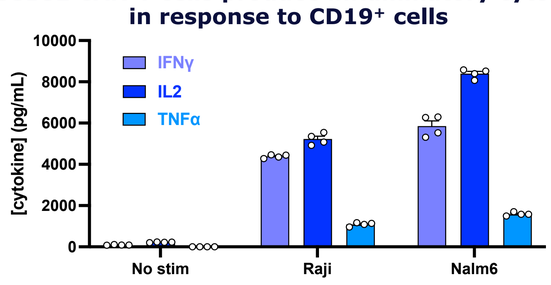
3 In vitro assays to evaluate CAR-T cell antigen specificity and functionality

PASSIGE CAR-T cells kill >90% of CD19⁺ cells



PASSIGE CAR-T: CD19-CAR at *TRAC;* **Lentiviral vector (LV) CAR-T**: CD19-CAR. PASSIGE- and LV-generated cells were ~30% CAR⁺ at time of plating.

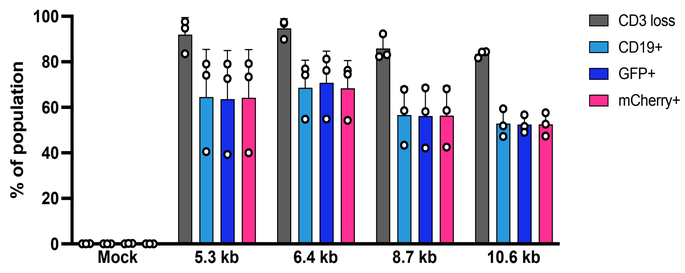
PASSIGE CAR-T cells produce inflammatory cytokines in response to CD19⁺ cells



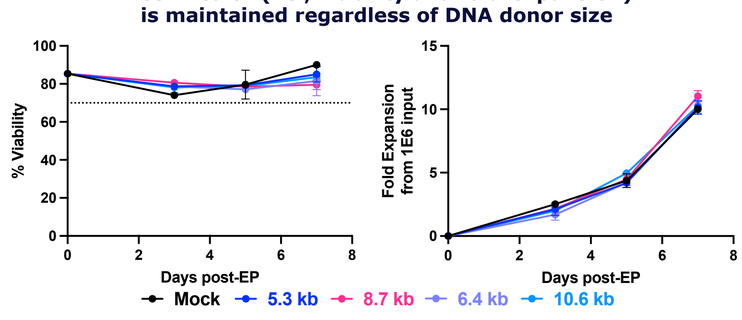
PASSIGE CAR-T: MND-CD19-CAR at *TRAC;* CAR-T cells co-cultured with CD19⁺ tumor cell lines (Raji or Nalm6) for 24 h

4

Expression of all 3 reporter cargos is consistent across DNA donor sizes (n=3 T cell donors)



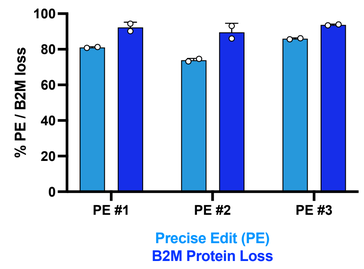
T cell health (i.e., viability and fold-expansion) is maintained regardless of DNA donor size



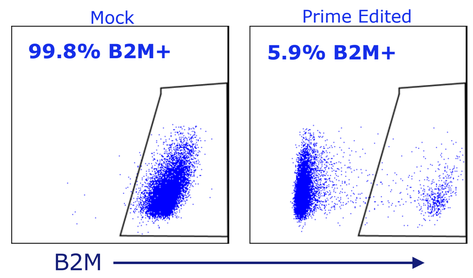
5 High performing Prime Editors precisely disrupt *B2M* expression with >90% efficiency in T cells

- ✓Prime Editors screened in cell line
- ✓Targeted early exon coding region
- ✓Compared efficacy of multiple edit types (e.g., insertion, deletion, STOP codon)
- ✓Top performing Prime Editors tested in human T cells
- ✓Top performing Prime Editors optimized in human T cells
- ✓Achieved >93% B2M protein loss

Top 3 Prime Editors validated in human T cells (n=2 donors)

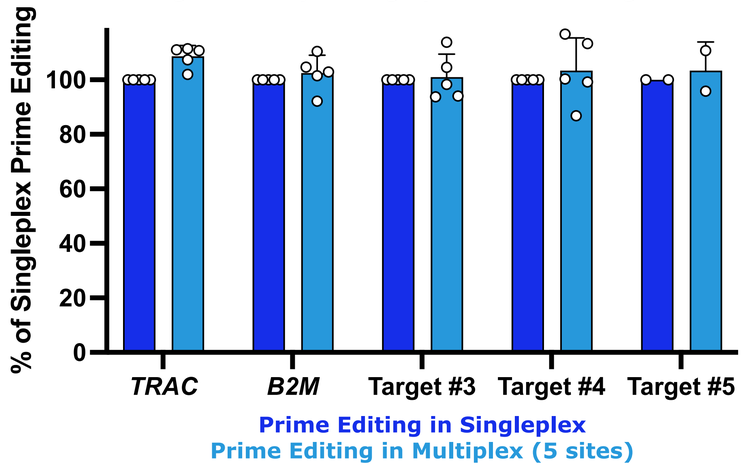


Top performing Prime Editors knock out B2M protein expression in >90% of T cells



6 Prime Editing efficiency is maintained in multiplex context at 5 target sites in T cells

Percent precise Prime Editing in multiplex normalized to Prime Editing efficiency in singleplex for each target site.



Conclusions

- Prime Editing precisely introduced recombinase target sequence at *TRAC* locus in >80% of T cells
- Achieved >70% site-specific integration of CD19-targeting CAR through systematic PASSIGE component and process optimization
- PASSIGE can be multiplexed with Prime Editing at other target sites in a single delivery step with no loss of efficiency observed
- PASSIGE-generated CAR-T cells are healthy and show potent antigen-specific function and cytotoxicity

Abbreviations. TCR: T Cell Receptor; CAR: Chimeric Antigen Receptor; PASSIGE: Prime Editing Assisted Site-Specific Integrase Gene Editing; PE: Prime Editing, rime Edited, or Precise Edit; UI: unintended edits; ddPCR: droplet digital Polymerase Chain Reaction; LV: Lentivirus; EP: electroporation; KO: Knockout; MHC: Major Histocompatibility Complex; DSB: Double Strand Break; L: Left; R: Right; Ex: Exon; SA: Splice Acceptor; bGH: bovine Growth Hormone;