Highly Efficient Single and Multi-gene Knockout with CRISPR-Cpf1 in T Cells for the Development of Improved Cell Therapies

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Introduction

Genome editing using RNA-guided nuclease technology has gained widespread attention for its potential to improve current cell therapies. The CRISPR-Cpf1 (also known as Cas12a) system is complementary to Cas9 with several distinct differences. Cpf1 features a single ~40 nucleotide crRNA and can target T- and C- rich PAMs with the WT and engineered PAM variants (1). The expanded targeting space, when compared to the purine rich PAMs of Cas9, makes it an attractive addition to enable broader targeting opportunities (2). Unlike SpCas9, Cpf1 makes a staggered cut in the DNA leaving behind a 4–5 nucleotide 5'-overhang, which could result in different editing outcomes.

We screened multiple loci of therapeutic interest in T cells with AsCpf1 and its engineered RR and RVR PAM variants and optimized multiple components of the Cpf1 RNP assembly and nucleofection process to improve editing efficacy without compromising cell viability. Robust single (>95% KO) and multiplexed (80-90% double KO) gene disruption was observed with Cpf1 enzymes when delivered as an RNP.

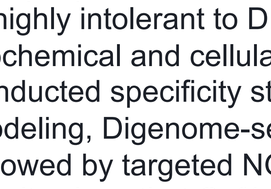
Multiple published studies have shown that Cpf1 is highly intolerant to DNA:RNA mismatches in biochemical and cellular assays (3). We conducted specificity studies using *in silico* modeling, Digenome-seq, and GUIDE-seq, followed by targeted NGS sequencing. The results show that Cpf1 is a highly specific enzyme. Taken together, these data suggest that Cpf1 is both a robust and specific technology for developing T cell-based medicines.

Background

Cpf1 Expands Targeting Space for Gene Editing

Variant	PAM	Frequency (bp)
SpCas9	NGG	1 in 8
SaCas9	NNGRRT	1 in 32
SaCas9 KKH	NNNRRT	1 in 8
AsCpf1	TTTV	1 in 43
AsCpf1 RR	TYCV/CCCC	1 in 18
AsCpf1 RVR	TATV	1 in 43

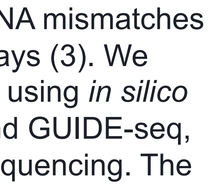
Cas9



Separate crRNA and trRNA that can be linked (~100 nt)

Predominantly blunt DNA cut or 1 nt overhang

Cpf1 (Cas12a)

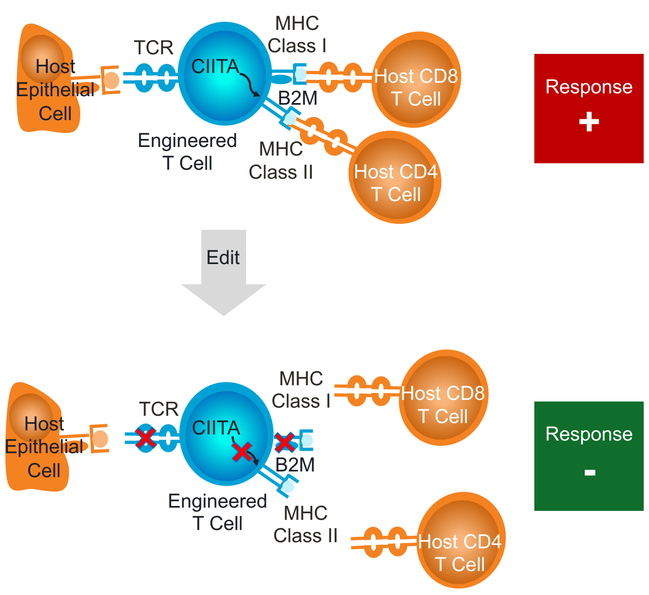


Naturally occurring ~40 nt single guide RNA

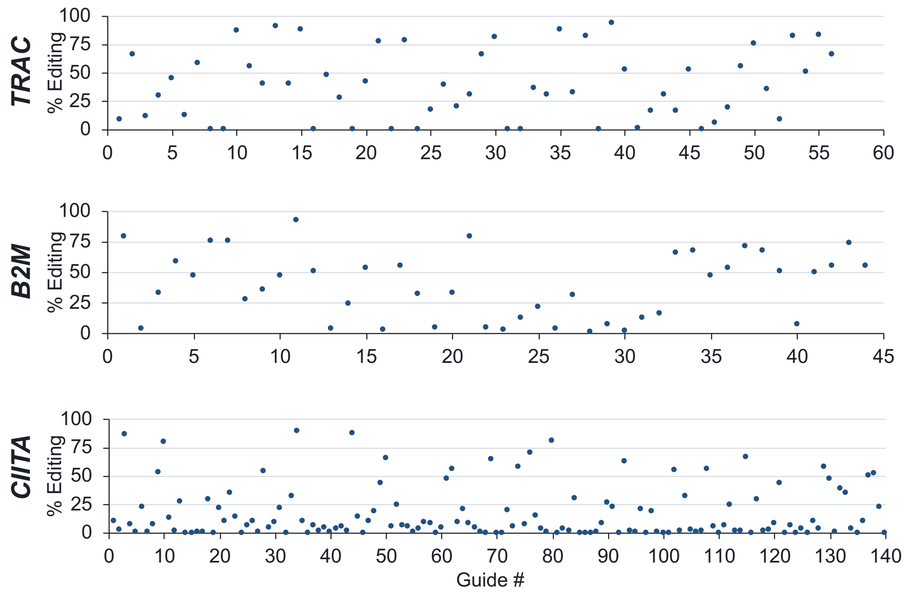
5' staggered DNA cut with 4 nt overhangs

Screening of T Cell Allogeneic-enabling Targets with Cpf1 Reveals Several Promising Hits

(a) **Multiple Gene Edits Allow the Generation of an Allogeneic "Off-the-Shelf" T Cell Product**

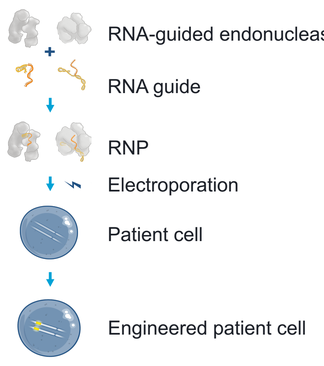


(b) **Full Screen of T Cell Target Loci with AsCpf1 and Engineered PAM Variants Led to Several Hits**

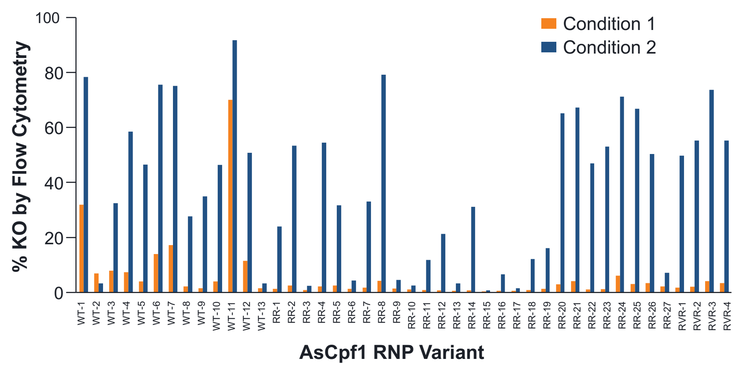


Efficient Single and Multi-gene Knockout in Primary Human T Cells with Cpf1 RNPs

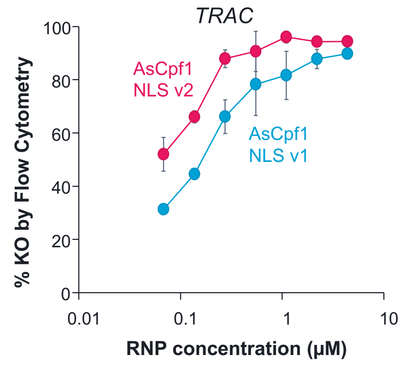
(a) **Workflow for RNP Delivery to Make a Gene Edited Cell Therapy**



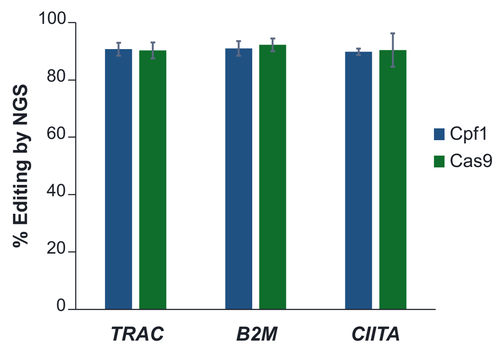
(b) **Optimisation of Electroporation Conditions Improved Maximal Editing in T Cells**



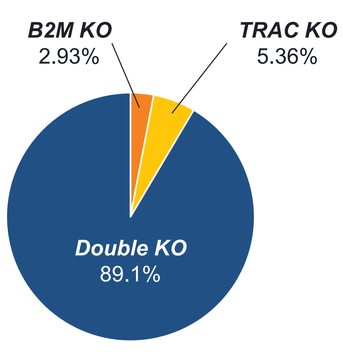
(c) **Optimisation of NLS Configuration Improved Potency in T Cells**



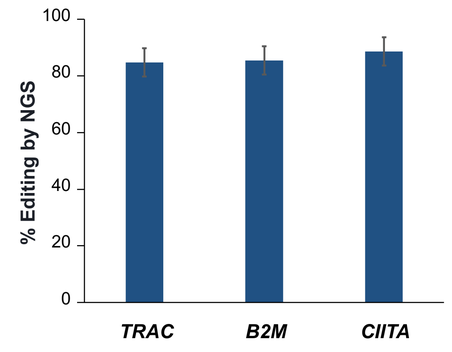
(d) **Single KO Efficiency Comparable to SpCas9 with a Cpf1 RNP**



(e) **Efficient Double KO with a Single Delivery of Two Cpf1 RNPs**

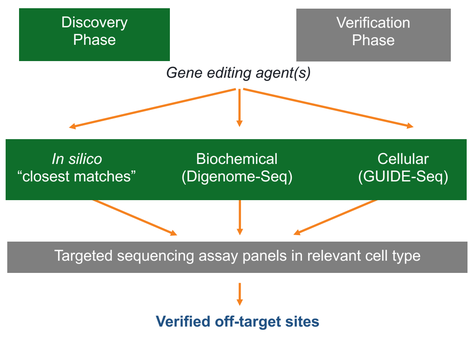


(f) **Efficient Triple KO with a Single Delivery of Three Cpf1 RNPs**



No Detectable Off-targets for Cpf1 TRAC, B2M, and CIITA Lead Candidate Guides in T Cells

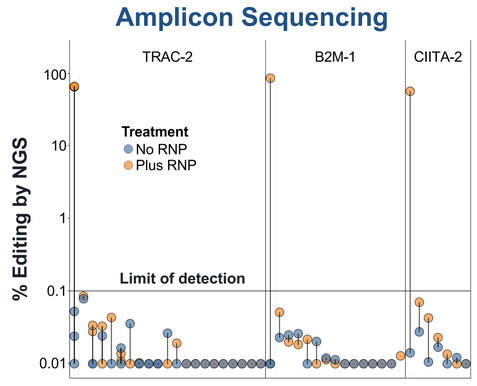
(a) **Editas Workflow for Identification and Verification of Potential Off-targets**



(b) **Specificity Assays Performed for Top Cpf1 Candidates Guides for T Cell Targets**

Guide (variant)	% Editing in T cells	*In silico* (off-by 1, 2, 3)	GUIDE-seq off-targets	Digenome-seq off-targets	Amp-seq verified off-targets
B2M-1 (WT)	98.3	0, 1, 23	0	6	0
B2M-2 (RR)	90.0	0, 0, 13	0	0	0
TRAC-1 (WT)	94.5	0, 2, 14	0	0	0
TRAC-2 (RR)	97.8	0, 2, 18	0	9	0
CIITA-1 (WT)	81.0	0, 1, 10	0	TBD	0
CIITA-2 (RR)	88.9	0, 2, 21	0	TBD	0
Control (SpCas9)	93.0	1, 4, 39	12	98	2

(c) **No Detectable Off-targets Found by Targeted Amplicon Sequencing**



References

1. Zetsche, Gootenberg et al. (2015) *Cell* 163:1117
2. Gao et al. (2017) *Nature Biotechnology* 35:789
3. Kleinstiver, Tsai et al. (2016) *Nature Biotechnology* 34:869

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Conclusions

- Optimisation of the Cpf1 protein and the electroporation conditions for RNP delivery led to improved editing at T cell target loci.
- Robust single, double, and triple KO of *TRAC*, *B2M*, and *CIITA* was achieved with the CRISPR-Cpf1 genome editing platform with comparable editing efficacies to those achieved in the field with the SpCas9 nuclease.
- There were no detectable off-targets observed by targeted amplicon sequencing of potential off-target sites from the *in silico*, Digenome-seq, and GUIDE-seq off-target assays.
- Taken together, these data suggest that Cpf1 (Cas12a) is both a robust and specific genome editing platform for the development of potential T cell-based medicines.