Strategies for multi-gene editing and reduction of translocations with CRISPR-Cpf1 in T cells for the development of improved cell therapies

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Introduction

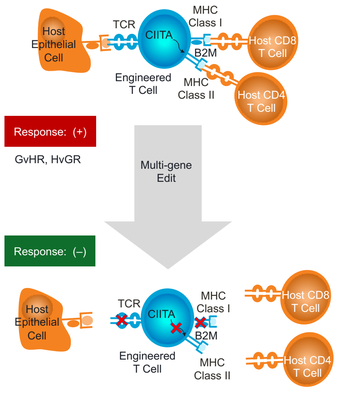
Gene editing using RNA-guided nuclease technology has gained widespread attention for its potential application to current cell therapies. The CRISPR-Cpf1 system (also known as Cas12a) is complementary to Cas9 with several distinct differences. Cpf1 uses a single ~40 nucleotide crRNA and can target T- and C- rich PAMs with the WT and engineered PAM variants. The expanded targeting space, when compared to the purine rich PAMs of Cas9, makes it an attractive addition to enable broader targeting opportunities. 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 targeted *TRAC*, *B2M*, and *CIITA* in primary human CD4+ and CD8+ T cells with Cpf1 and its engineered RR and RVR PAM variants with these different enzymes delivered as ribonucleoproteins (RNPs). We were able to consistently achieve robust multiplexed (>80% triple KO) gene disruption. To examine the genomic consequences of multiplexed editing, we applied a set of detection technologies including targeted and genome-wide methods to quantitate editing efficiency and genomic rearrangements within and between target loci.

Methods

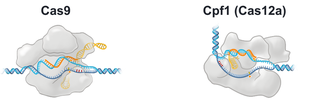
- Used Cpf1 or SpCas9 to KO single or multiple combinations of *TRAC*, *B2M*, and *CIITA* in a single delivery of RNP to CD4+ and/or CD8+ T cells as measured by flow cytometry and NGS
- Conducted specificity studies on top RNPs using GUIDE-seq, Digenome-seq, and *in silico* analyses followed by NGS
- Applied a set of translocation detection technologies including targeted and genome-wide methods to quantitate editing efficiency and genomic rearrangements within and between target loci
- Developed strategies to reduce translocation frequencies that were observed in a multi-editing context

Results

Multiple Gene Edits Allow for the Generation of an "Off-the-shelf" Allogeneic T Cell Product



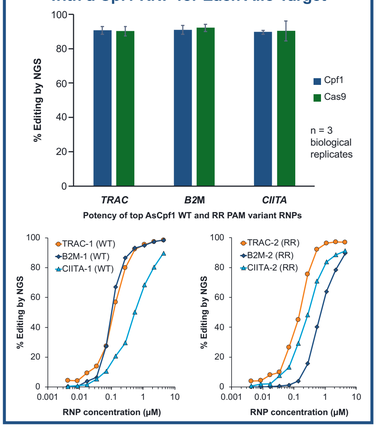
Cpf1 Offers Several Potentially Advantageous Features for the Genome Editing Tool Kit



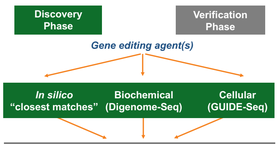
Cas9	Cpf1 (Cas12a)
Targets G-rich PAMs	Targets T- and C-rich PAMs
Separate crRNA and trRNA that can be linked (~100 nt)	Naturally occurring ~40 nt single guide RNA
Predominantly blunt DNA cut or 1 nt overhang	5' staggered DNA cut with 4 nt overhangs

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

Single KO Efficiency Comparable to SpCas9 with a Cpf1 RNP for Each Allo Target

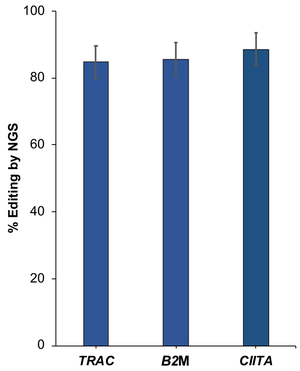


Top Cpf1 RNPs Appear to be Specific by Multiple Orthogonal Specificity Assays



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)	89.7	0, 1, 10	0	14	0
CIITA-2 (RR)	91.3	0, 2, 21	0	2	0
Control (SpCas9)	93.0	1, 4, 39	12	98	2

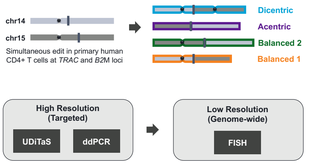
Efficient Multi-Gene KO in T cells with Cpf1 When Using a Single Delivery of Three RNPs



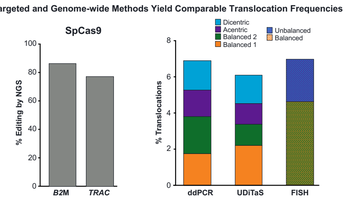
n = 3 biological replicates

Translocation Detection in the Context of Multiplexing with CRISPR Nucleases

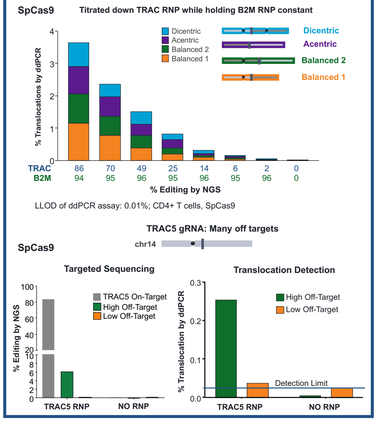
Develop detection methods for accurate quantitation of genome editing-induced structural rearrangements.



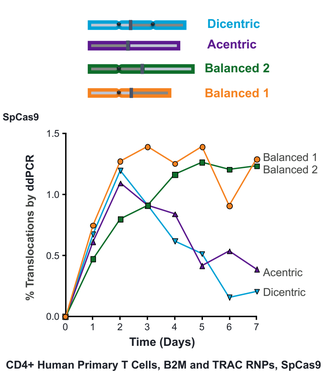
Targeted and Genome-wide Methods Yield Comparable Translocation Frequencies



Translocation Rates Follow Editing Rates for Both On-Target and Off-Target Edits

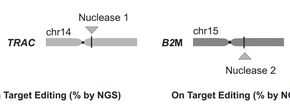


Balanced Translocations Persist While Unbalanced Translocations Do Not



CD4+ Human Primary T Cells, B2M and TRAC RNPs, SpCas9

Strategies to Reduce Translocations Using Cpf1 in a Multi-gene Edit



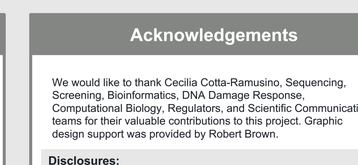
On Target Editing (% by NGS)

Locus	Enzyme		Percent
	B2M	TRAC	
TRAC	SpCas9	SpCas9	89
	AsCpf1	AsCpf1	93
	SpCas9	AsCpf1 RR	96
	AsCpf1	SpCas9	91
	AsCpf1	AsCpf1 RR	96

On Target Editing (% by NGS)

Locus	Enzyme		Percent
	B2M	TRAC	
B2M	SpCas9	SpCas9	95
	AsCpf1	AsCpf1	92
	SpCas9	AsCpf1 RR	96
	AsCpf1	SpCas9	89
	AsCpf1	AsCpf1 RR	93

Translocation Frequency with Different CRISPR Nuclease Combinations



Conclusions

- Multi-gene editing of T cell loci with either the CRISPR-Cas9 or CRISPR-Cpf1 system resulted in translocations which were detected by multiple orthogonal methods. These translocations were dependent on editing rates and persisted over time.
- Multi-gene editing with a CRISPR-Cas9/CRISPR-Cpf1 or CRISPR-Cpf1 with engineered Cpf1 PAM variants reduced translocation frequencies compared to multiplexing with only CRISPR-Cas9.
- For the development of T cell-based medicines, these data suggest that CRISPR-Cpf1 is both robust, specific, and capable of reducing genomic rearrangements when making multiple gene edits compared to the CRISPR-Cas9 system alone.

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