Successful Generation of CAR⁺PD-1⁻ Primary T Cells Using Cas9-Mediated Genome Editing

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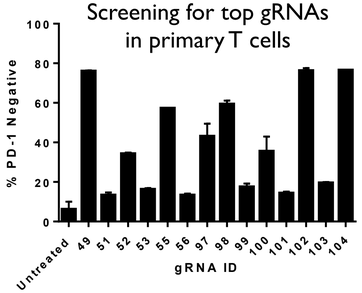
Background

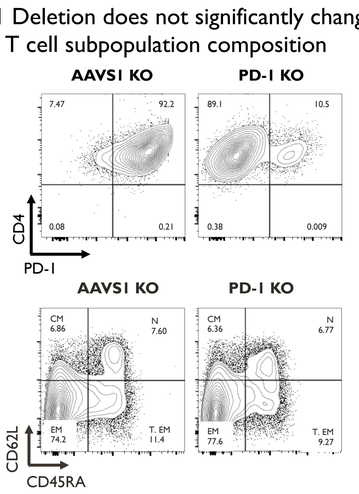
Engineered T cells that are programmed to attack tumors via chimeric antigen receptors (CARs) have shown promise in early clinical trials. The PD-1/PD-L1 axis, however, may dampen the effectiveness of CAR T therapy in certain cancer types. One way to alleviate this restraint on T cell function is to eliminate PD-1 expression on CAR-expressing T cells using CRISPR/Cas9-based gene editing.

Targeting of PD-1 in primary T cells results in ~90% knockout

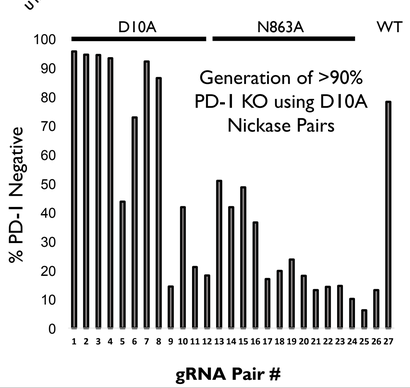
A)



B)



C)



(A) T cells were electroporated with RNPs comprising the labeled gRNA. (B) Top: T cells were electroporated with RNPs comprising the labeled gRNA. Bottom: CD8+ T cells were stained for CD62L and CD45RA to identify relevant T cell subpopulations CM: Central Memory; N: Naïve; EM: Effector Memory; T. EM: Terminally Differentiated Effector Memory (C) Primary T cells were transfected with 12 different gRNA pairs using either D10A Cas9 nickase protein or the N863A Cas9 nickase enzyme. Samples 25 and 26 are D10A and N863A with a single gRNA while 27 is the wt Cas9 with a single gRNA. In all cases, PD-1 expression was assessed after re-stimulation

Assessment of lead gRNA specificity

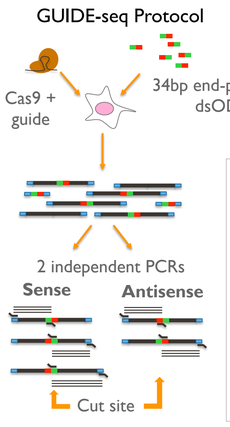
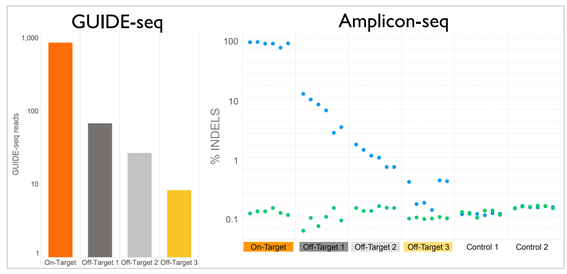


Table 1: Results for 6 lead gRNAs

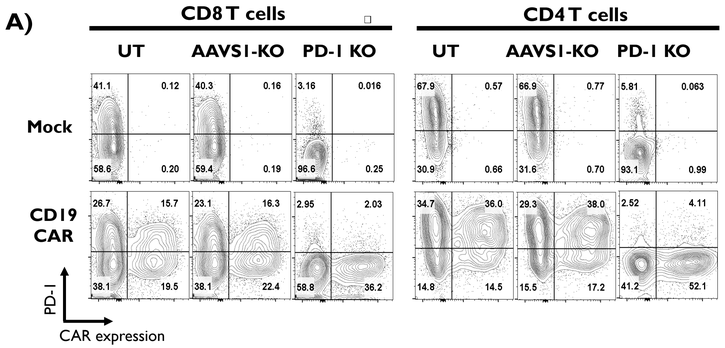
gRNA	49	55	97	98	102	104
# of off-targets	4	2	0	1	5	3
# exonic	1	0	0	0	1	0
# intronic	0	1	0	0	2	2
Reads in Experiments (M)	10.5	7.9	12	9.5	9.9	8



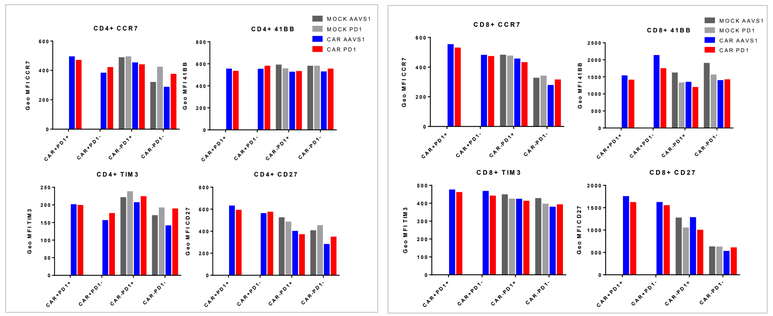
The specificity of lead gRNAs was assessed in primary T cells by GUIDE-seq. 4 independent gDNA samples derived from 2 separate experiments is summarized in Table 1. An off target was called if bidirectional reads were present in at least one of the 4 samples or unidirectional reads were present in at least 2 of the 4 samples. To confirm the GUIDE-seq results, Amp-seq was performed on 6 independent gDNAs from T cells treated by PD49 RNP. Not only were the off targets identified by GUIDE-seq confirmed but also the rank order was the same.

Characterization of CAR⁺/PD-1⁻ T cells

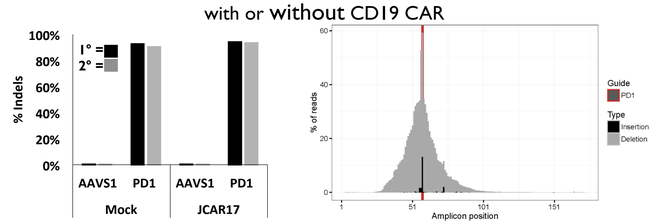
A)



B) PD-1 KO does not affect the phenotype of CD19 CAR T cells



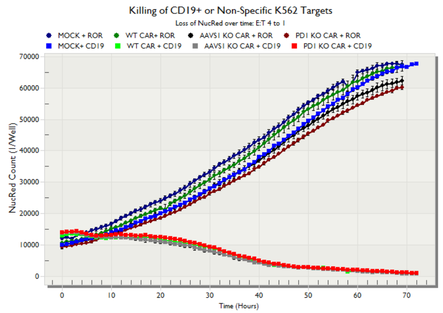
C) MiSeq analysis shows ~90% editing in PD-1 RNP treated T cells, with or without CD19 CAR



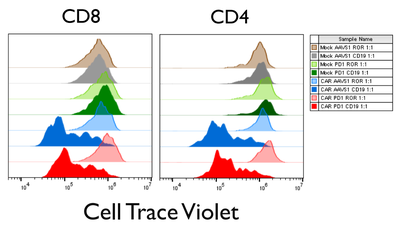
(A) CD4⁺ and CD8⁺ T cells were transduced with CD19 CAR lentivirus and then electroporated with RNPs comprising the indicated gRNA. PD-1 expression was assessed with CAR expression on day 12 after transfection following a 24 hour re-stimulation with anti-CD3/CD28 beads. (B) Cells were gated for PD-1 and CAR expression and the levels of the indicated phenotypic and activation markers were measured. Additional markers evaluated with no differences observed; CD45RA, CD45RO, Lag3, CD62L, CD25 and CD69. (C) T cells were isolated at day 20 post primary expansion (black) or 10 days after a secondary expansion (gray) and the percent of indels (left) and position (right) was measured via MiSeq.

PD-1 Knock out CAR T cells are functional in vitro

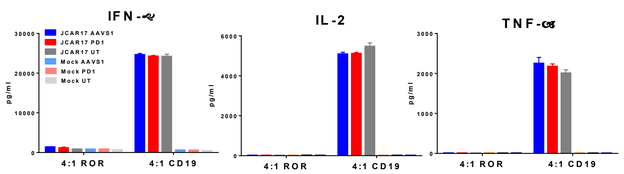
A) PD-1 KO CAR T cells kill CD19+ targets



B) PD-1 KO CAR T cells proliferate normally in response to target cells



C) PD-1 KO CAR T cells produce cytokines in response to target cells



(A) PD-1 KO or control CAR T cells were incubated at a 4 to 1 ratio with NucRed Target cells (CD19 or ROR +) and loss of targets was measured over 70 hours via Incucyte assay. Representative of n=4 experiments. (B) CAR T cells from experiment in (A) were labeled with cell trace violet and proliferation was measured at 96 hours via FACS. (C) Supernatants from the experiment in (A) were isolated from the cultures at 24 hours and cytokines were measured via mesoscale cytokine analysis. Results representative of n=3 experiments.

Conclusions

- Delivery of a *PDCD1* targeting Cas9 RNP to primary T cells resulted in the deletion of PD-1 expression in ~90% of cells.
- Delivery of a pair of PDCD1 targeting Cas9 D10A RNP complexes to primary T cells resulted in the deletion of PD-1 expression in ~90% of cells.
- GUIDE-seq was used to locate off target sites in primary T cells and can be used to prioritize gRNAs in terms of specificity.
- gRNAs with no detectable off-target cutting were identified and characterized.
- This approach was successful in generating >50% CAR+/PD-1⁻ primary T cells from multiple donors.
- PD-1 KO does not affect CAR transduction levels as CAR+ cells always have equal PD-1 KO compared to CAR- cells.
- CAR+/PD-1⁻ T cells have normal phenotype and functionality, and can efficiently kill antigen positive target cells in a specific manner.