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 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) Screening for top gRNAs in primary T cells

B) PD-1 Deletion does not significantly change T cell subpopulation composition

C) Generation of >90% PD-1 KO using D10A nickase pairs

Assessment of lead gRNA specificity

GUIDE-seq Protocol

Table 1: Results for 6 lead gRNAs

<table>
<thead>
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<th>gRNA Type</th>
<th>Of 6 targets</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>AAV51 KO</td>
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<td>0</td>
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<tr>
<td>PD-1 KO</td>
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<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>D10A</td>
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<td>1</td>
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<tr>
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</table>

The specificity of lead gRNAs was assessed in primary T cells by GUIDE-seq. 4 independent gRNA samples derived from 4 separate experiments are summarized in Table 1. All off-targets were scored based on the presence or absence of 2 of the 4 samples. To confirm the GUIDE-seq results, Amplicon-seq was performed on 6 independent gRNAs from T cells treated by CRISPR 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) CD8 T cells

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

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+PD-1- 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.

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 labeled target cells (CD19 or MNC) and loss of targets was measured over 72 hours via lysis assay. Representative of n=4 experiments. (B) CAR T cell proliferation in (A) were labeled with MNC trace violet and proliferation was measured at 96 hours via FACs. (C) Supernatants from the experiment in (A) were isolated at the cultures at 24 hours and cytokines were measured via mesoscale cytokine analysis. Results representative of n=3 experiments.