Poster #279

CRISPR/Cas9 Enables the Efficient Production of Allogeneic CAR-T Cells Engineered to Contain Multiple Genome Edits to Enhance Therapeutic T Cell Function

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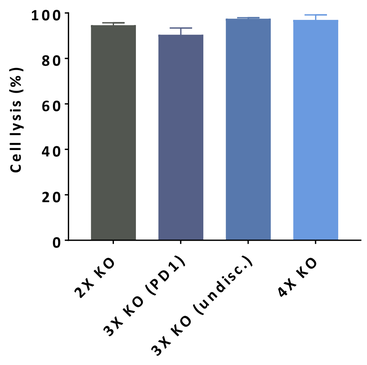
Abstract

The CRISPR/Cas9 system allows for rapid assessment of the consequences of perturbing many genes while at the same time deriving potentially lead molecules for cell and gene therapies. We have applied this technology to discover the following in the allogeneic CAR-T cell setting:

(1) multiple edits (>5) can be applied efficiently to produce stable non-transformed CAR-T cells;
(2) the effects of single and multiple edits on CAR-T function can be examined efficiently to determine gene edits that improve CAR-T cell function;
(3) the consequence of these effects for on-target and off-target activity can be used to rapidly generate lead gRNAs;
(4) next-generation cell therapies can be defined towards targeting solid tumors with allogeneic CAR-T cells.

Here we show the effects *in vitro* and *in vivo* of knocking-out multiple genes singly and in combination, including the response to multiple antigen challenges and the ability to overcome PD-L1 induced resistance. Producing CAR-T cells with multiple edits could be an important step towards enhancing the ability of this therapeutic class to tackle solid tumors with improved efficacy over the current therapeutics.

Figure 1: Anti-CD70 CAR-T Cells with Multiple KOs Show High Cytotoxic Activity Against an RCC Tumor Cell Line



Descriptions of KOs

2X KO – *TRAC* and *ß2M* KO, with CAR inserted into *TRAC* locus

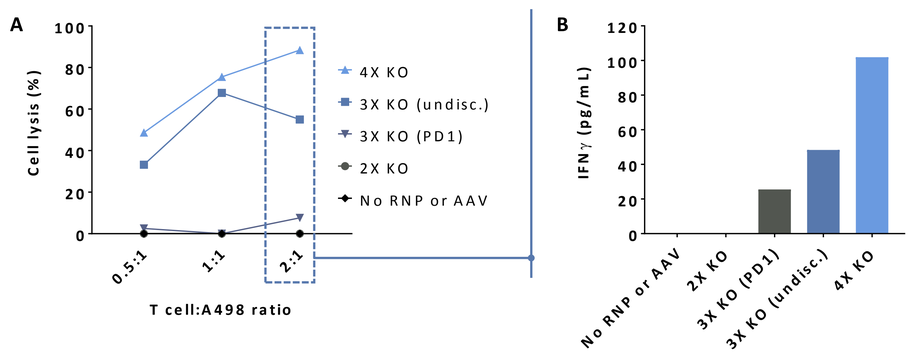
3X KO (PD1) – *TRAC*, *ß2M*, and PD1 KO, with CAR inserted into *TRAC* locus

3X KO (undisc.) – *TRAC*, *ß2M*, and undisclosed KO, with CAR inserted into *TRAC* locus

4X KO – *TRAC*, *ß2M*, PD1, and undisclosed KO, with CAR inserted into *TRAC* locus

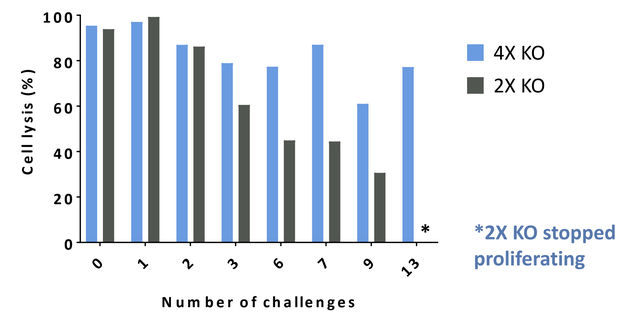
Allogeneic anti-CD70 CAR-T cells with two, three, or four knock-outs (KOs) efficiently kill the A498 renal cell carcinoma (RCC) tumor cell line in a 24-hour cell kill assay, shown here at a 2:1 T cell:A498 ratio.

Figure 2: Additional KOs Confer Superior Cytotoxic Activity Against an RCC Tumor Cell Line Overexpressing PD-L1



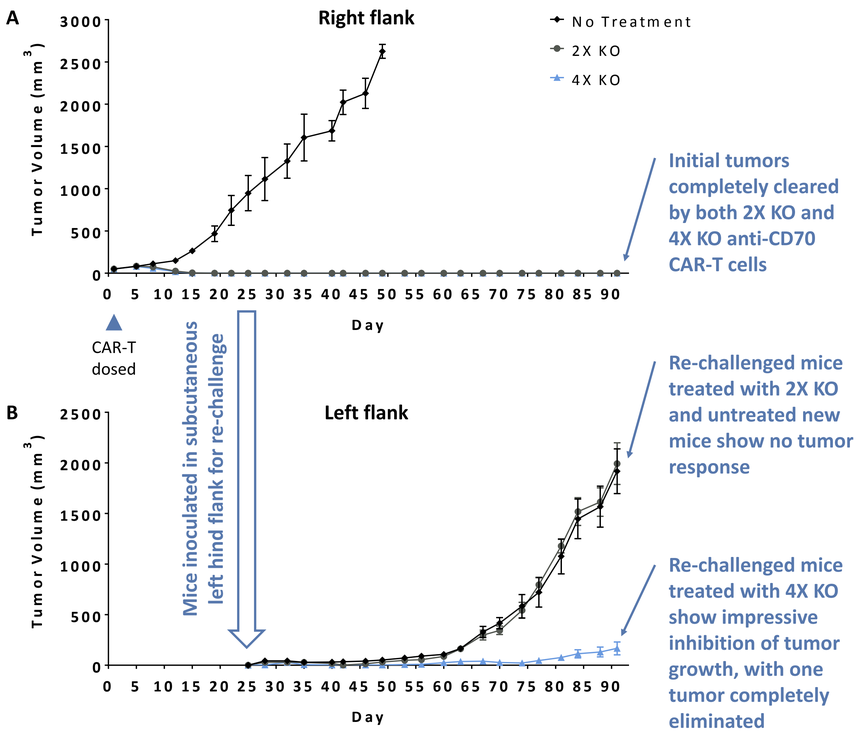
(A) 4X KO or undisclosed 3X KO anti-CD70 CAR-T cells show higher cytotoxicity than 2X KO or PD1 3X KO anti-CD70 CAR-T cells against A498-PD-L1, an RCC cell line overexpressing PD-L1 made by infecting A498 cells with lentivirus encoding a PD-L1 cDNA. **(B)** Secretion of the T cell activation cytokine INFγ by CAR-T cells in response to A498-PD-L1 shows the same trend, as does IL-2 secretion (data not shown).

Figure 3: Additional KOs Confer Superior Cytotoxic Activity During *In Vitro* Re-Challenge



Upon 13 repeated challenges *in vitro* with A498, 4X KO anti-CD70 CAR-T cells retain cytotoxicity, while 2X KO anti-CD70 CAR-T cells lose their ability to mount a cytotoxic response and eventually stop proliferating. 2:1 T cell:A498 ratio.

Figure 4: 2X KO and 4X KO Anti-CD70 CAR-T Cells Both Eliminate an RCC Tumor Model *In Vivo*, but 4X KO CAR-T Cells Exhibit Superior Efficacy Following *In Vivo* Re-Challenge

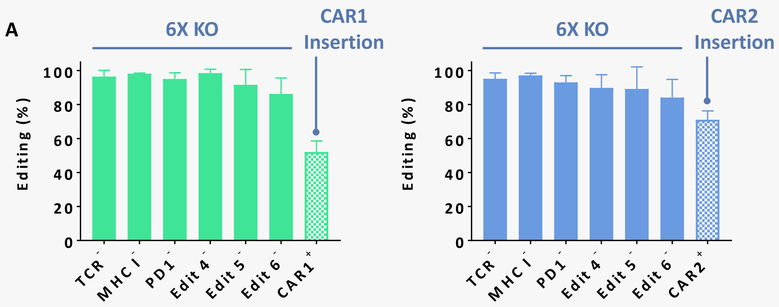


(**A**) 4X and 2X KO anti-CD70 CAR-T cells both eradicate tumors in a subcutaneous A498 xenograft tumor model. $5x10^6$ A498 cells were injected subcutaneously into NOG mice in the right hind flank, followed by CAR-T cells intravenously when mean tumor size reached 25-75 mm^3 (target of 50 mm^3). n=5 for each group. (**B**) 4X KO anti-CD70 CAR-T cells severely reduce tumor regrowth after *in vivo* re-challenge, while 2X KO anti-CD70 CAR-T cells fail to prevent tumor regrowth. On day 25 following CAR-T cell administration, $5x10^6$ A498 cells were injected subcutaneously into NOG mice in the left hind flank. No additional CAR-T cells were administered. n=5 for each group.

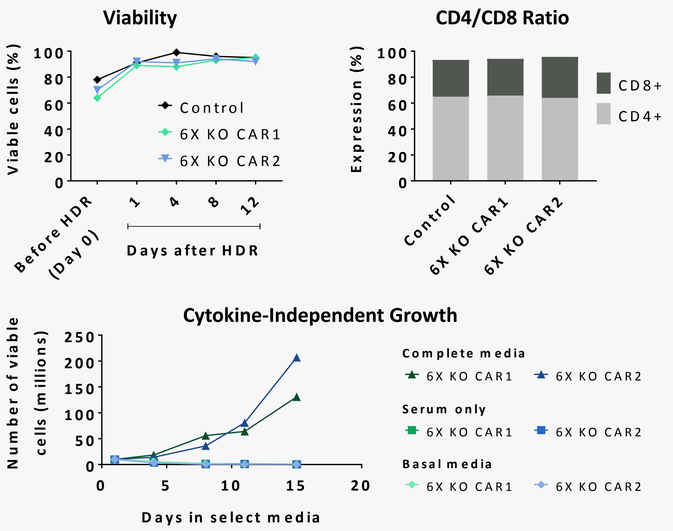
Conclusions

- The efficiency of the CRISPR/Cas9 system enables rapid screening of a number of different target genes to identify those that improve CAR-T function in a model solid tumor immunosuppressive environment
- Multi-edited CAR-T cells containing these edits show impressive efficacy in both *in vitro* and *in vivo* re-challenge models
- 7 edits (6 knock-outs and 1 knock-in) can be performed in a single experiment, generating functional and non-transformed CAR-T cells
- These septuple-edited CAR-T cells show high efficiency and specific cytotoxicity and cytokine response
- The CRISPR/Cas9 system enables the selection and incorporation of edits that can help overcome the immunosuppressive environment of solid tumors

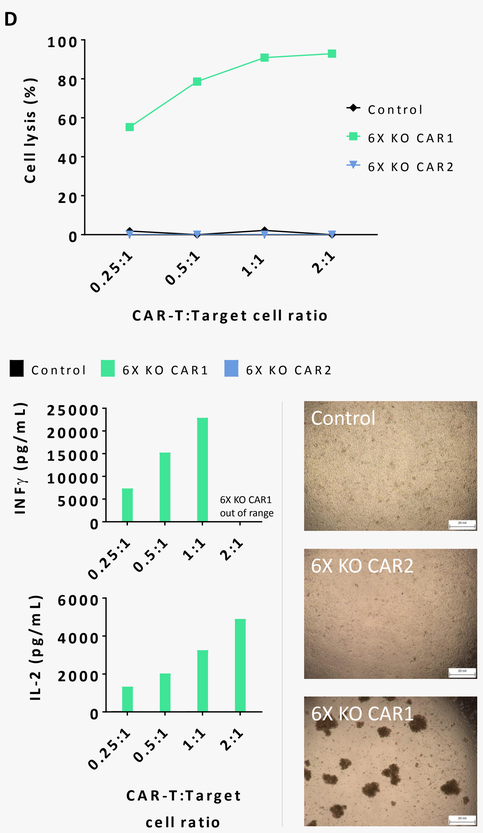
Figure 5: High Efficiency Sextuple KO Plus CAR Insertion by CRISPR/Cas9 to Produce CAR-T Cells that Exhibit Good Health and Target-Specific Cytotoxicity



(**A**) Sextuple CRISPR/Cas9 multi-editing of *TRAC*, *ß2M*, PD1, and Edits 4-6 results in near-complete elimination of surface expression of TCR, MHC I, PD1, and Edits 4-6. More than 50% and 70% of T cells contain the CAR1 and CAR2 insertions, respectively. n=3 donors.



(**B**) The viability and CD4/CD8 subset ratios (assessed 1 week after HDR) of sextuple KO CAR-T cells remain similar to unedited controls. (**C**) Sextuple KO CAR-T cells still remain dependent on cytokines for growth and survival following multi-editing, suggesting no oncogenic transformation has occurred.



(**D**) Sextuple KO CAR1 CAR-T cells show potent cytotoxicity against a target-positive tumor cell line. The CAR-T cells also secrete the cytokines IFNγ and IL-2 in a dose-dependent manner and exhibit expansion and clustering around target-positive tumor cells. Sextuple KO CAR2 CAR-T cells specific for a different target do not exhibit these characteristics.