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Multiplexing of up to 10 gene edits using CRISPR/Cas9 to generate CAR-T cells with improved function

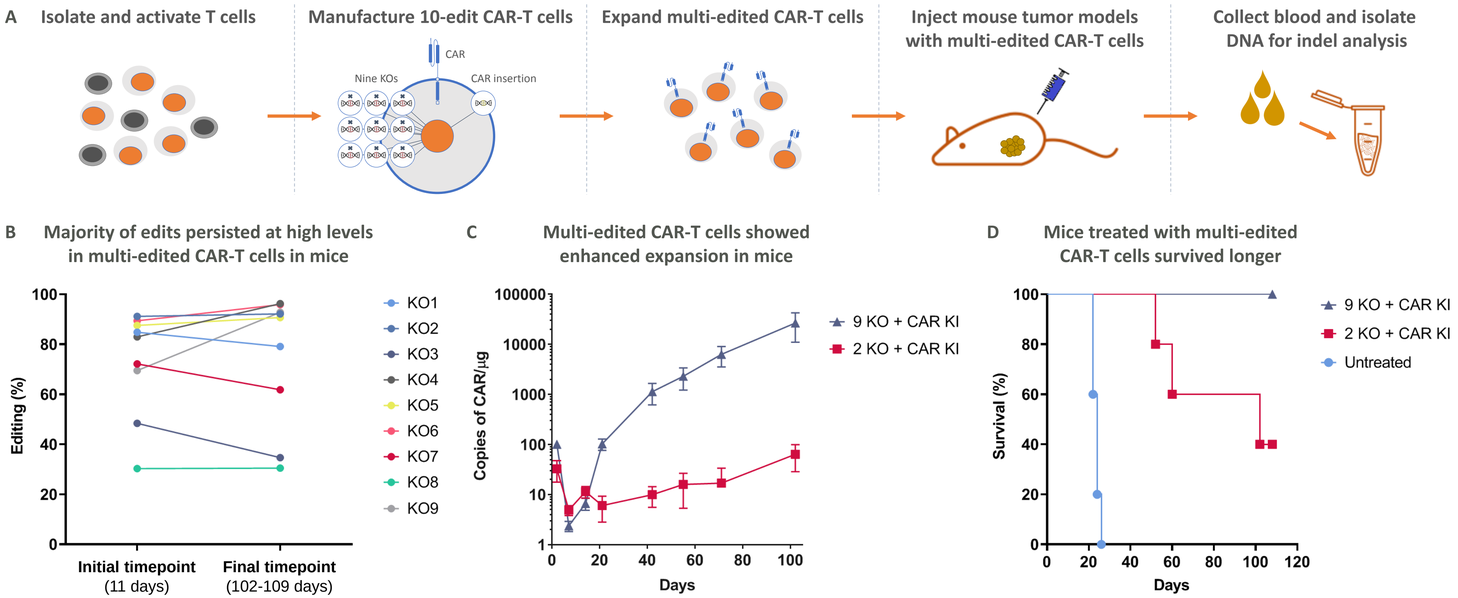
Brigid McEwan*, Zinkal Padalia*, Andrew Dunn, Konstantinos Karagiannis, Meghna Kuppuraju, Sarah Spencer, Henia Dar, Daniel Henderson, Minh Thu Pham, Jason Sagert, Jonathan Terrett, Demetrios Kalaitzidis

CRISPR Therapeutics, 610 Main Street, Cambridge, MA, USA 02139 *Equal contribution

Abstract

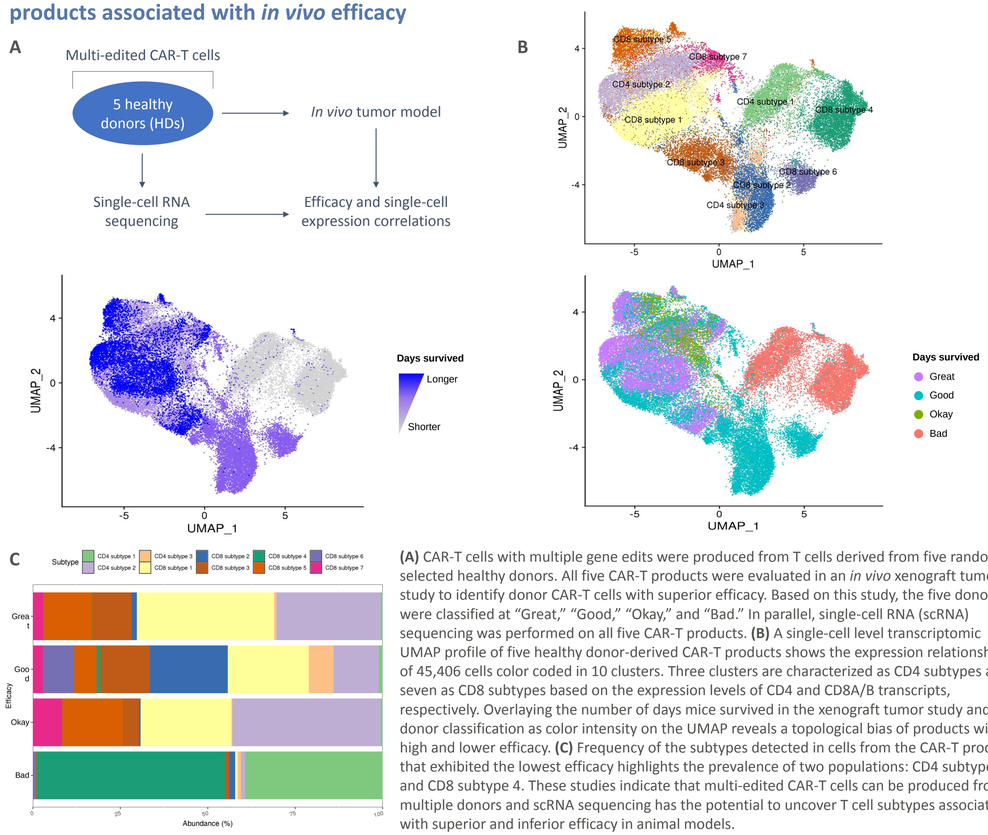
Autologous chimeric antigen receptor (CAR) T cell therapies are currently approved for use in humans with relapsed/refractory B-cell malignancies. These products contain a mixture of T cell subtypes in differing cell states and have the CAR expression construct inserted randomly via viral vectors. Despite remarkable responses in many patients, significant variability in both CAR-T product quality and the responses in different hematological malignancies has been observed. In addition, there are few reports of clinical activity of CAR-T cells in solid tumors. CAR-T products may benefit from gene editing, such as gene knockouts and knock-ins, to enable more efficacious CAR-T cells with enhanced abilities to proliferate, survive, persist, and evade immune-suppressive environments. Here we show that CRISPR/Cas9 can be used to generate CAR-T cells with up to 10 edits. These edits can improve many of the desirable CAR-T properties noted above, as evidenced by CAR-T activity *in vitro* and *in vivo* in mouse models. Furthermore, we have generated highly edited CAR-T cells using starting material from multiple healthy donors. Characterization of the resulting CAR-T cells using single-cell RNA sequencing revealed numerous T cell subtypes and heterogeneity across donors. Taken together, this work shows the possibilities of using CRISPR/Cas9 to improve the potency of CAR-T products and demonstrates that the impact of these changes can be further understood using single-cell sequencing methods.

Figure 1: Allogeneic CAR-T cells with up to 10 gene edits and exhibit enhanced antitumor function in mice



(A) Allogeneic CAR-T cells were produced with nine genetic disruptions, as well as the CAR construct inserted at the TRAC locus. These highly multi-edited CAR-T cells were generated by introducing RNPs targeting nine different loci and an AAV vector to deliver CAR transgene donor template into human T cells derived from a healthy donor. **(B)** The cells were used in a xenograft tumor study and insertion/deletions (indels) at each gene disruption were tracked over time in mice by Sanger sequencing. While most edits persisted at high levels in the CAR-T cells after over 100 days in mice, some edits showed diminished prevalence, suggesting these knockouts may have a deleterious effect on the CAR-T cells, while others increased in prevalence. Many of the edits resulted in enhanced CAR-T cell function, as indicated by **(C)** greatly increased expansion in mice, as assessed by droplet digital PCR, and **(D)** prolonged survival of mice treated with these cells in a xenogeneic tumor model. These studies demonstrate that CRISPR/Cas9 can be used to produce complex CAR-T products that can persist *in vivo* in mice for several months and show enhanced potency against xenograft tumors.

Figure 2. Single-cell protein and RNA sequencing reveal multiple cell states in multi-edited CAR-T products associated with *in vivo* efficacy



(A) CAR-T cells with multiple gene edits were produced from T cells derived from five randomly selected healthy donors. All five CAR-T products were evaluated in an *in vivo* xenograft tumor study to identify donor CAR-T cells with superior efficacy. Based on this study, the five donors were classified at "Great," "Good," "Okay," and "Bad." In parallel, single-cell RNA (scRNA) sequencing was performed on all five CAR-T products. **(B)** A single-cell level transcriptomic UMAP profile of five healthy donor-derived CAR-T products shows the expression relationship of 45,406 cells color coded in 10 clusters. Three clusters are characterized as CD4 subtypes and seven as CD8 subtypes based on the expression levels of CD4 and CD8A/B transcripts, respectively. Overlaying the number of days mice survived in the xenograft tumor study and the donor classification as color intensity on the UMAP reveals a topological bias of products with high and lower efficacy. **(C)** Frequency of the subtypes detected in cells from the CAR-T product that exhibited the lowest efficacy highlights the prevalence of two populations: CD4 subtype 1 and CD8 subtype 4. These studies indicate that multi-edited CAR-T cells can be produced from multiple donors and scRNA sequencing has the potential to uncover T cell subtypes associated with superior and inferior efficacy in animal models.

Summary & Conclusions

- CRISPR/Cas9 can generate multi-edited CAR-T cells (nine disruptions plus one knock-in)
- These multi-edited CAR-T cells can have enhanced function, exhibiting increased *in vivo* potency in mice
- scRNA sequencing of multi-edited CAR-T cell products can identify favorable T cell subtypes associated with improved efficacy