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Allogeneic CAR-T cell products containing 10 gene edits using CRISPR/Cas9 can retain full functionality *in vivo* and *in vitro*

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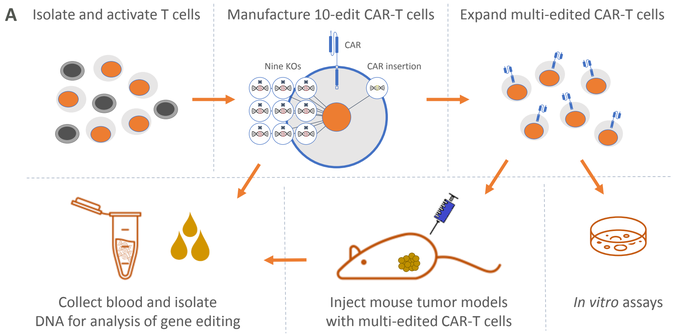
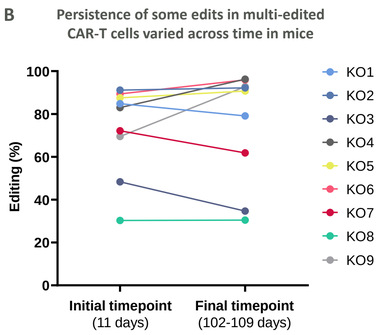
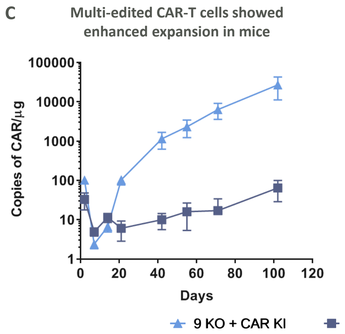
Abstract

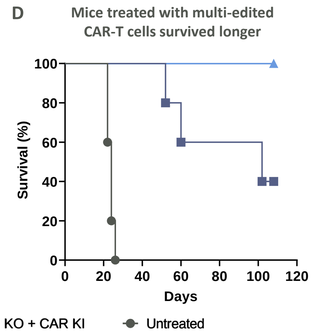
Hematologic malignancies have proven most responsive to CAR-T therapies. However, there is significant variability in the depth and duration of response to autologous CAR-T cells. Furthermore, CAR-T responses in solid malignancies have been minimal. T cells from cancer patients can be compromised by the disease and prior treatments, and the use of allogeneic CAR-T products could potentially provide a more robust therapy. However, even CAR-T cells from healthy donors may benefit from additional gene edits that could enhance effector function, increase durability, evade immune mechanisms and/or combat the tumor microenvironment. These types of additional CAR-T features are readily enabled by CRISPR/Cas9 technology. While recent clinical data has demonstrated the persistence of therapeutic T cells that had been edited with CRISPR, the extent of gene editing that therapeutic T cells can tolerate has not been fully described. Herein, we have generated CAR-T cells with 10 gene edits. Loss of function alleles were produced in 9 genes and a CAR cassette was knocked into the *TRAC* locus via homology-directed repair. CD19-directed CAR-T cells with these 10 gene edits were fully functional *in vitro*, as shown by their ability to expand and kill target cells and secrete cytokines while remaining non-transformed. These cells were also highly efficacious in an *in vivo* model of CD19⁺ malignancy. Importantly, these edits, which produced insertions and deletions in target genes, could be detected in the blood of mice several months post injection. These data demonstrate that CRISPR/Cas9 can be used to create highly edited allogeneic CAR-T cells, unlocking the potential to enhance many functions relevant to CAR-T health and anti-tumor activity.

Figure 1: Performing up to 10 gene edits using CRISPR/Cas9 can improve allogeneic CAR-T function





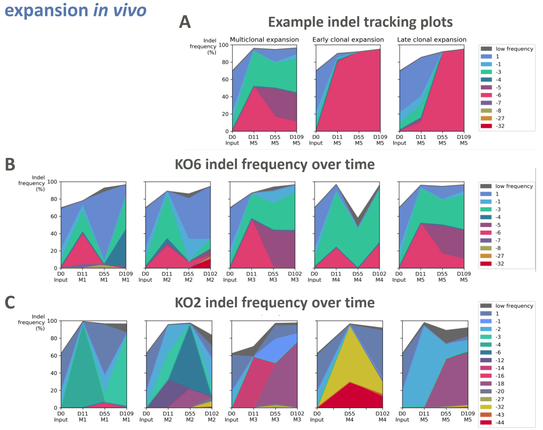




9 KO + CAR KI 2 KO + CAR KI Untreated

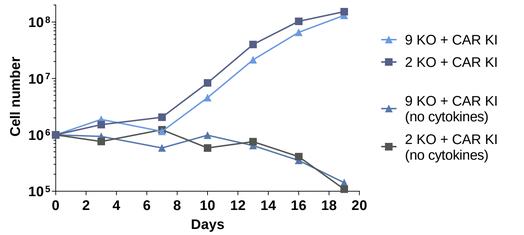
(A) Allogeneic CAR-T cells were produced with nine genetic disruptions and the CAR construct inserted at the *TRAC* locus. These 10-edit CAR-T cells were generated by introducing RNPs targeting nine different loci into human T cells derived from a healthy donor along with an AAV vector to deliver CAR transgene donor template. **(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: 10-edit allogeneic CAR-T cells show polyclonal expansion *in vivo*



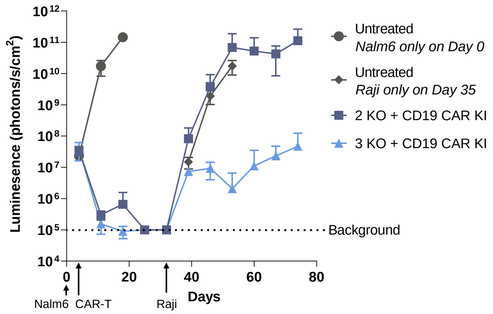
(A) Example plots for tracking indel patterns over time *in vivo*. Examples of polyclonal and monoclonal expansion are shown including early and late clonal expansion overtaking the CAR-T compartment. **(B,C)** Indel patterns were assessed from two genes in input 10-edit CAR-T cells and tracked in mouse blood over the indicated days in five separate mice (M1-M5) bearing Nalm6 leukemia (as described in Figure 1). Indel patterns fluctuated over the length of the study with no specific pattern overtaking the CAR-T compartment. These data suggest multi-clonal contribution to CAR-T function *in vivo* and dynamic fluctuations in these clones. The lack of monoclonal expansion suggests that the process of producing 10-edit CAR-T cells with CRISPR/Cas9 does not produce transformed cells *in vivo* over this observation period.

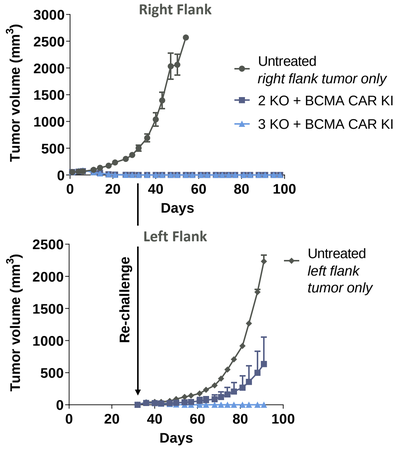
Figure 3: 10-edit allogeneic CAR-T cells do not grow in the absence of cytokines

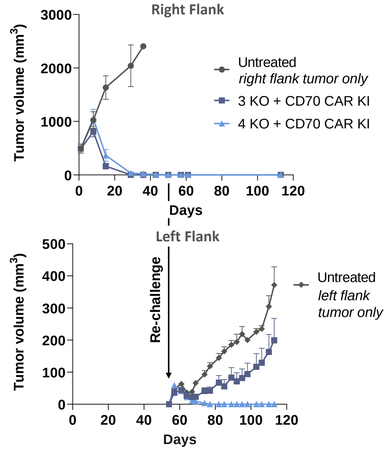


Allogeneic CAR-T cells were produced and placed in media that contained serum and cytokines or serum alone (no cytokines). The total number of cells was assessed over the indicated days in culture. Cells that contained up to 10 edits remained cytokine dependent, indicative of a lack of aberrant outgrowths due to the editing process or specific genes edited.

Figure 4: Specific edits identified can enhance the potency and persistence of CAR-T cells *in vivo*







Allogeneic CAR-T cells bearing additional, potency-enhancing edits identified in the experiment described in Figures 1-3 allow mice to withstand aggressive cancer re-challenges in liquid and solid tumor xenograft models. **(A)** In a disseminated CD19⁺ Nalm6 (luciferase expressing) leukemia model, anti-CD19 CAR-T cells with and without an additional edit rapidly cleared the primary leukemia, as assessed by total body luminescence. 35 days after CAR-T infusion the mice were re-challenged with CD19⁺ Raji (luciferase expressing) lymphoma cells delivered intravenously. Mice that had received allogeneic CAR-T cells with the additional edit exhibited control of lymphoma growth, while all other mice rapidly developed lethal lymphoma. Similar clearance of aggressive tumors upon re-challenge was observed in subcutaneous xenograft models of **(B)** multiple myeloma (MM) and **(C)** renal cell carcinoma (RCC) using CAR-T cells targeting BCMA and CD70, respectively, and engineered with an additional edit.

Conclusions from preclinical studies

- CRISPR/Cas9 can be used to readily produce highly multiplexed (10 edits) allogeneic CAR-T cells with favorable attributes
- 10-edit allogeneic CAR-T cells maintain functionality *in vitro* and *in vivo*
- 10-edit allogeneic CAR-T cells do not show signs of oncogenic transformation *in vitro* or *in vivo*
- Multiplex edited CAR-T cells can be engineered for enhanced potency and persistence *in vivo*