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Expanding the Scope of Multiplex Engineering for Superior Generation of Efficient CAR T-cells

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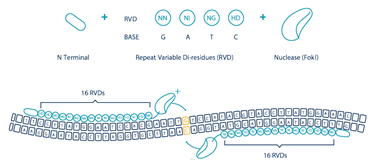
CAR T-cell therapies have revolutionized the way we can treat hematological malignancies. In the meanwhile, the presence of additional physiological and biological barriers imposed by the microenvironment has limited the ability to target solid tumors. In recent years, advances in genomic-based cellular engineering are bringing us a step closer to conquer solid tumors. This glimpse of success also demonstrated that we need to be able to creatively modify and equip CAR T-cells to target these tumors. A caveat to this is that increasing the number of genetic modifications at the cellular level could compromise the level of editing efficiency as well as lead to genomic toxicities. Here, we show that we can use our state-of-the-art TALEN® technology to precisely edit up to four loci simultaneously while delivering several additional payloads to increase the efficacy and persistence of CAR T-cells. We take it a step further and use a curated combination of genome engineering technologies including TALE base editors to increase the efficiency of multiplexed gene editing while protecting genomic integrity. By carefully choosing a range of gene and cell engineering approaches, we can develop CAR T-cells focused on unmet medical needs with a high level of efficiency for gene editing and targeted-integration. We also show that this high efficiency in TALEN®-mediated gene-edition does not lead to increased gene integrations at undesired loci. Moreover, we show that multiplexed engineering does not compromise CAR T-cell function, which in turn can be enhanced and display improved anti-tumor activity. Thus, multiplexed engineering at superior efficiency rates while preserving genomic integrity has the potential to generate highly functional CAR T-cells to advance in the fight against solid tumors

1 Background

TALEN® overview



TALEN® mediated edits

2 Triple TALEN® transfection is hightly efficient

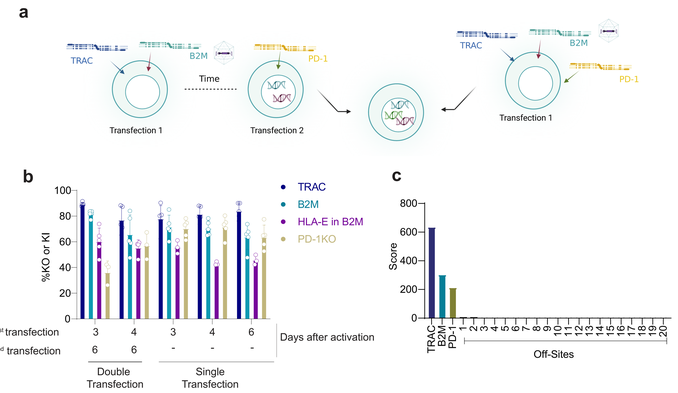


Figure 1. Triple TALEN® transfection can efficiently edit the genome without creating additional off-sites. a. Diagram depicting the two strategies used to deliver 3 TALEN® mediated edits to cells. **b.** Rates of knock-out and knock-in efficiency after 2 indepentent transfections or a single transfection to deliver 3 TALEN® and one repair matrix measured by flow cytometry. **c.** Genome-wide off-target analysis upon simulatenous transfection with 3 TALEN®.

3 Sequential editing attenuates TALEN® cross-talk

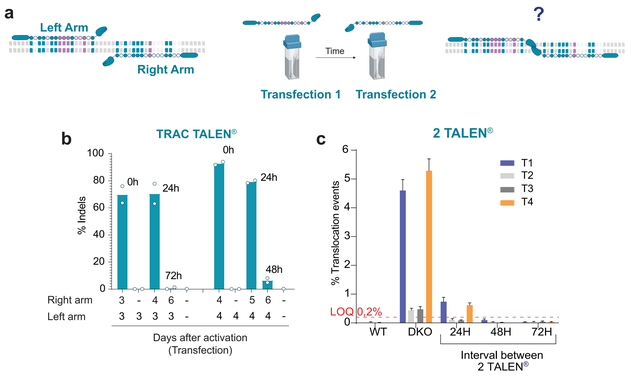


Figure 2. Sequential transfections reduce undesired genomic events. a. Diagram depicting the strategies used to evaluate cross-talk between TALEN® arms. **b.** Rates of gene editing of the *TRAC* locus when both arms are separated at different times. **c.** Identification of translocations when both arms of 2 independent TALEN® are transfected at different time points. Note: For this experiment *TRAC* and *CD52* TALEN® were used. DKO: Double knock-out

4 Compatible TALEN® pairs to optimize the process

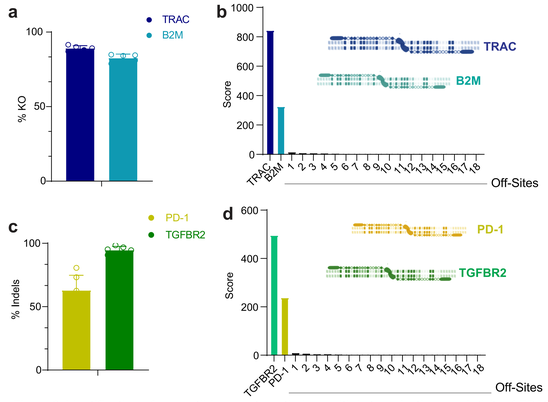


Figure 3. Identification of compatible arms to optimize multiple transfections. a. Activity of *TRAC* and *B2M* TALEN® co-transfected 3 days after activation. **b.** Genome-wide off-target identification upon *TRAC* and *B2M* TALEN® co-transfection 3 days after T-cell activation . **b.** Activity of TGFBR2 and PD-1 TALEN® co-tranfected and measured at the genomic level. **c.** Genome-wide off-target identification upon TGFBR2 and PD-1 TALEN® co-transfection.

5 TALEN® allows the generation of CAR T-cells with 4 KO and 2 KI

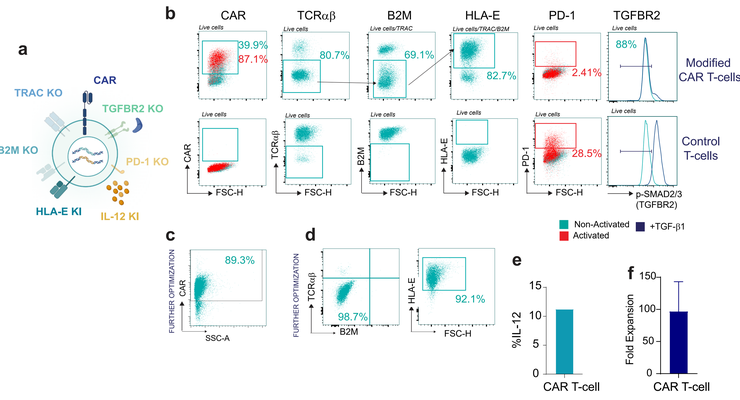


Figure 4 Efficient multiplex engineering. a. Diagram depicting the final CAR T-cell with multiple edits. **b.** Flow cytometry plots showing the knock-out and knock-in efficiency at the end of the process with the edits indicated. Cells were activated with PMA/Ionomycin to evaluate PD-1 KO (red). **c, d** CAR expression and TRAC/B2M KO coupled HLA-E KI after further optimization in the context of complex engineering. Note: data on figure c and d were obtained in different experiments. **e.** Rates of IL-12 integration measured by ddPCR. **f.** Fold cell expansion calculated by dividing the number of cells obtained at the end of the production by the number of cells seeded in culture vessels after the last electroporation.

6 Combining TALE-based approaches for complex engineering

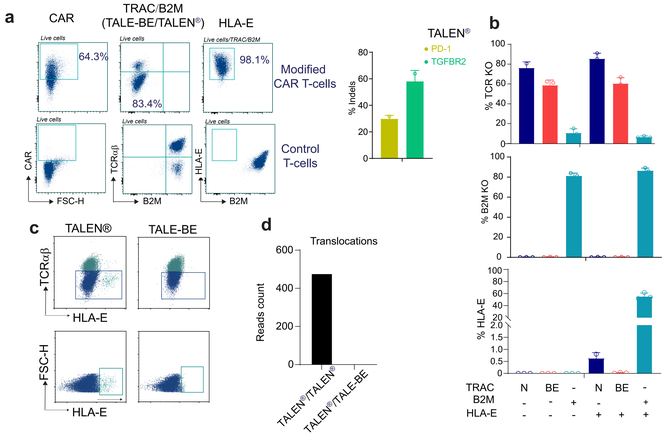


Figure 5. Combination of TALE-BE and TALEN® allows multiplex editing and minimizes genomic risks. a. Flow cytometry showing editing efficiencies with base editor after optimization. **b**, **c** Bargraph and flow cytometry respectively showing potential non-homology arm mediated trappping of HLA-E repair matrix into the TRAC locus and integration in B2M as control (N: nuclease; BE: base editor). **d.** Translocation analysis between 2 TALEN® and 1 TALEN® and 1 TALE-BE.

7 TALEN® multi-edited cells display high activity

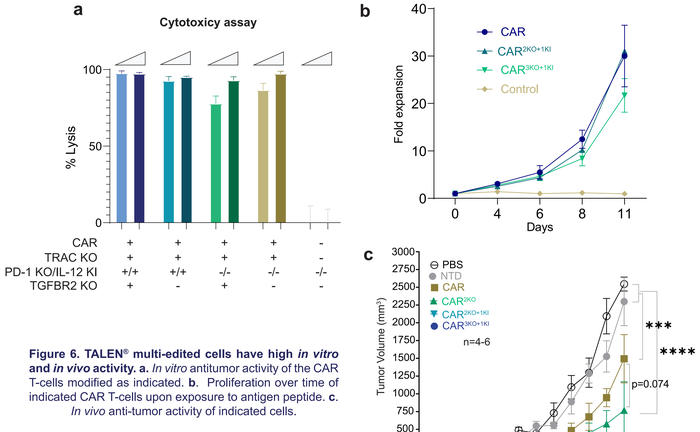
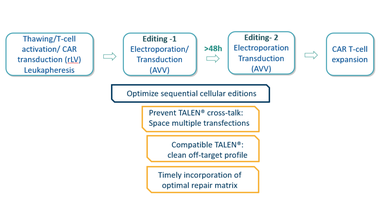


Figure 6. TALEN® multi-edited cells have high *in vitro* and *in vivo* activity. a. *In vitro* antitumor activity of the CAR T-cells modified as indicated. **b.** Proliferation over time of indicated CAR T-cells upon exposure to antigen peptide. **c.** *In vivo* anti-tumor activity of indicated cells.

8 Conclusion



TALEN® technology is suited to generate multiplexed engineered cells with high level of efficiency

Sequential incorporation of edit events minimizes potential genomic risks

TALE-BE can efficiently be incorporated into multiplex cell engineering yielding high levels of editing and favoring genomic safety.