# Disruption of the cytokine signaling checkpoint CIS enhances serial-killing and anti-tumor activity of CAR-engineered γδ T cells

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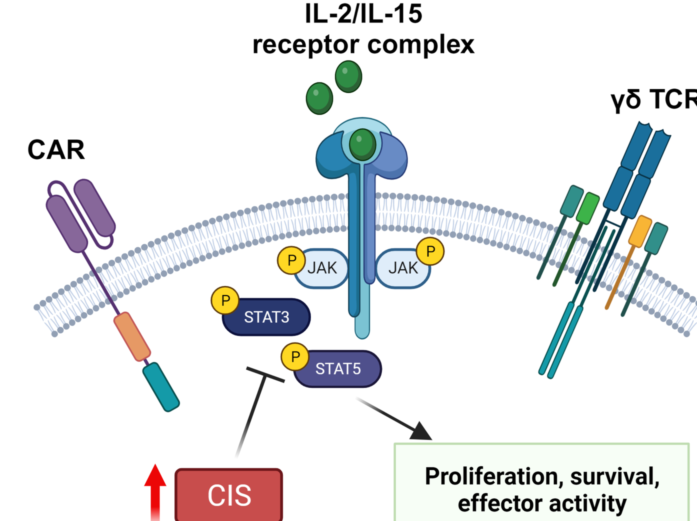
## INTRODUCTION

γδ T cells have emerged as a promising allogeneic cell-based immunotherapy platform for the treatment of cancer. Armed with a unique combination of innate and adaptive immunity to target malignant cells, the infiltration and presence of γδ T cells in various solid tumors is significantly correlated with survival benefit. Additional modifications to engineer γδ T cells to express CARs have shown enhanced tumoricidal activity and encouraging clinical efficacy[1,2]. Opportunities to understand the mechanisms that drive these potent anti-tumor responses in γδ T cells are integral to further augmentation of their effector functions. Cytokine inducible SH2-containing protein (CIS) is a key negative regulator of IL-2/15 and TCR signaling. CIS disruption in αβ T cells and NK cells promotes increased persistence and anti-tumor activity[3,4], but the role of CIS in γδ T cells is not well understood. Here, we report, for the first time, a strategy that couples targeted disruption of the CIS loci within allogeneic CAR γδ T cells for enhanced anti-tumor potential.




## CIS up-regulation by cytokine/TCR/CAR stimulation

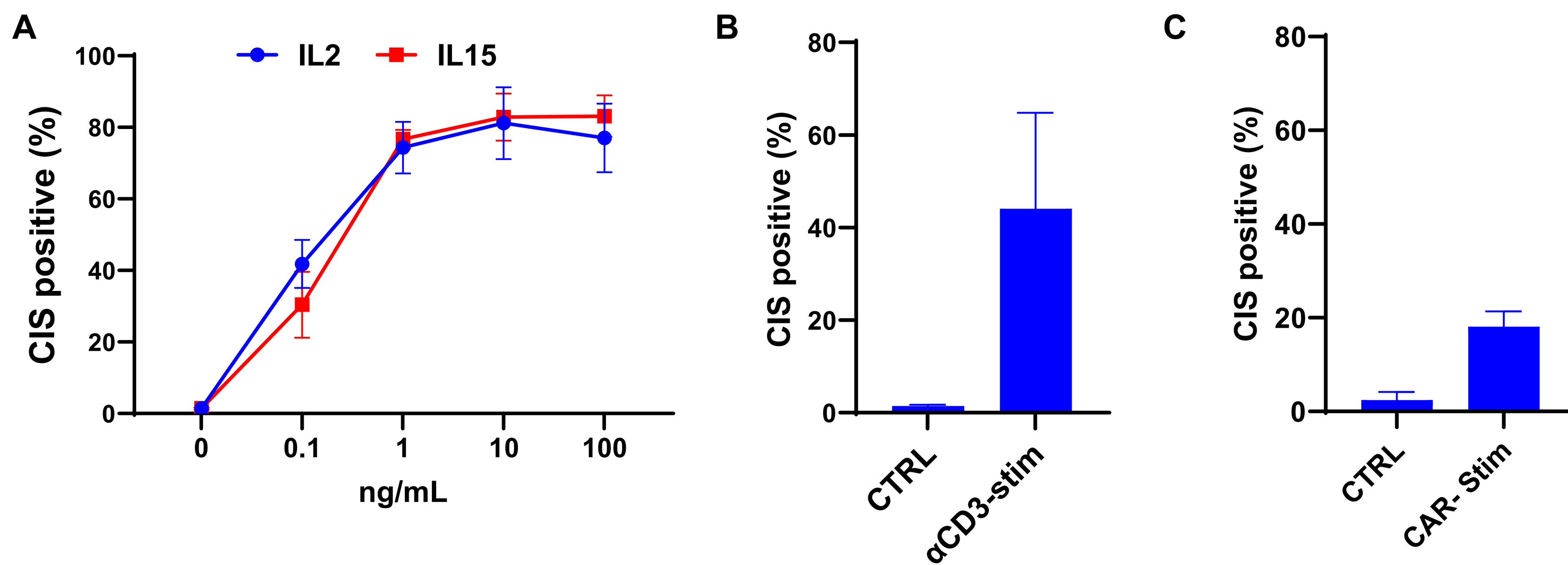



**Figure 1. (A)** CIS up-regulation by IL2/IL15 stimulation. Vδ1 T cells were cultured overnight in cytokine free medium, treated with IL2 or IL15 at the indicated concentrations for five hours, then CIS expression was analyzed using flow cytometry. **(B)** CIS up-regulation through TCR stimulation. Cytokine starved Vδ1 cells were stimulated with plate bound CD3 antibody (OKT3) for five hours, then CIS expression was analyzed using flow cytometry. **(C)** Cytokine starved CAR Vδ1 cells were stimulated by tumor antigen for five hours and CIS expression was analyzed using flow cytometry.

## Efficient Generation of *CISH* gene knockout CAR Vδ1 T cells

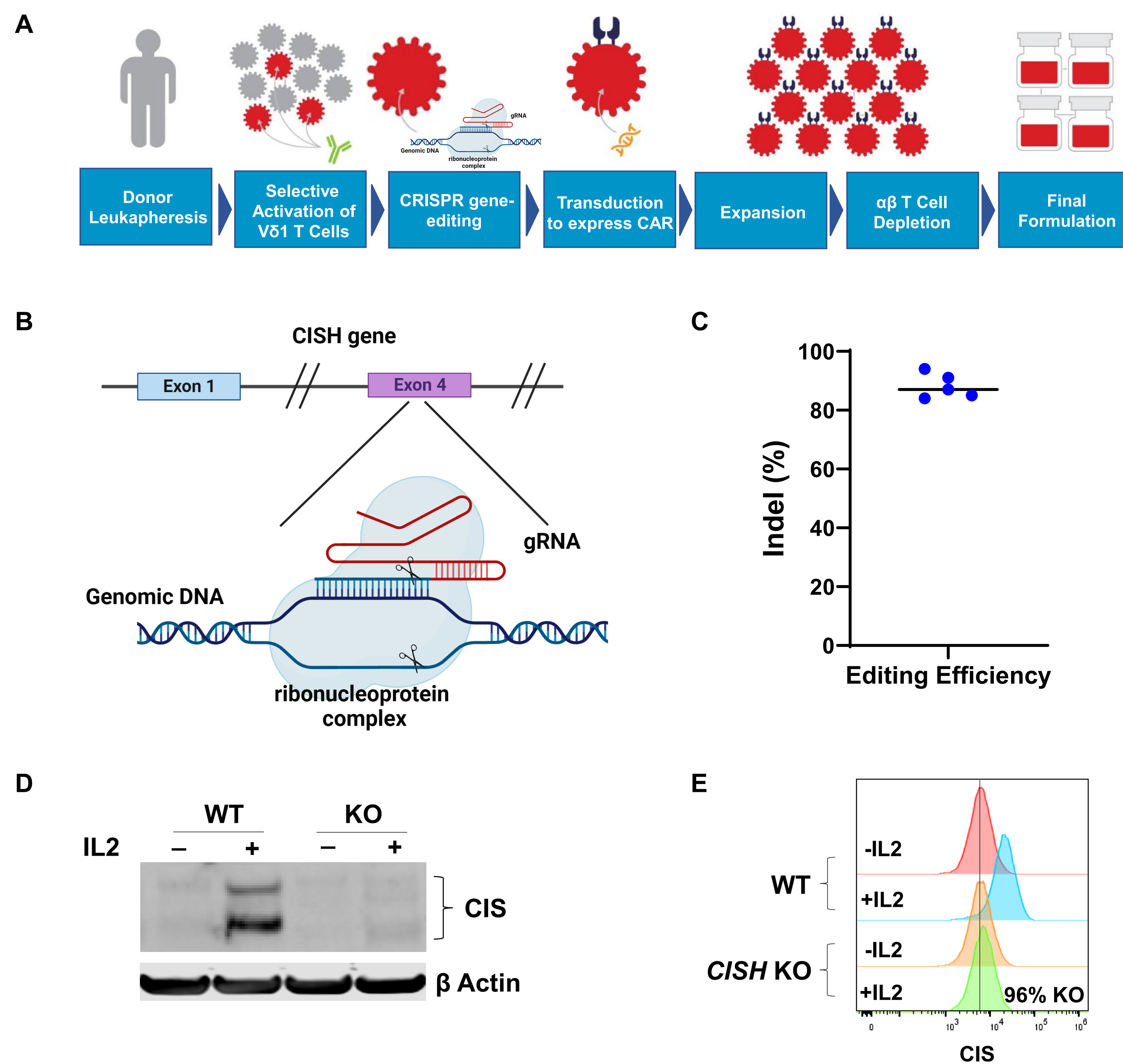



**Figure 2. (A)** Flow chart highlighting the key steps in generation of *CISH* gene knockout CAR Vδ1 T cells. **(B)** Scheme of CRISPR mediated *CISH* KO using guide RNAs (gRNA) located in exon 4 of the *CISH* gene. **(C)** Indel frequencies of the *CISH* gene knockout in Vδ1 cells were analyzed by Inference of CRISPR Edits (ICE) analysis tool. **(D)** WT or *CISH* gene edited CAR transduced Vδ1 T cells were incubated overnight in cytokine free medium and then stimulated with or without IL2 for five hours, and CIS expression was analyzed by immunoblotting. β actin was used as loading control. **(E)** Representative flow cytometry plot of CIS expression on WT or CISH knockout CAR Vδ1 T cells stimulated with or without IL2.

## Robust CAR expression and a less differentiated T cell memory phenotype with minimal activation/exhaustion-associated markers in CISH KO Vδ1 T cells

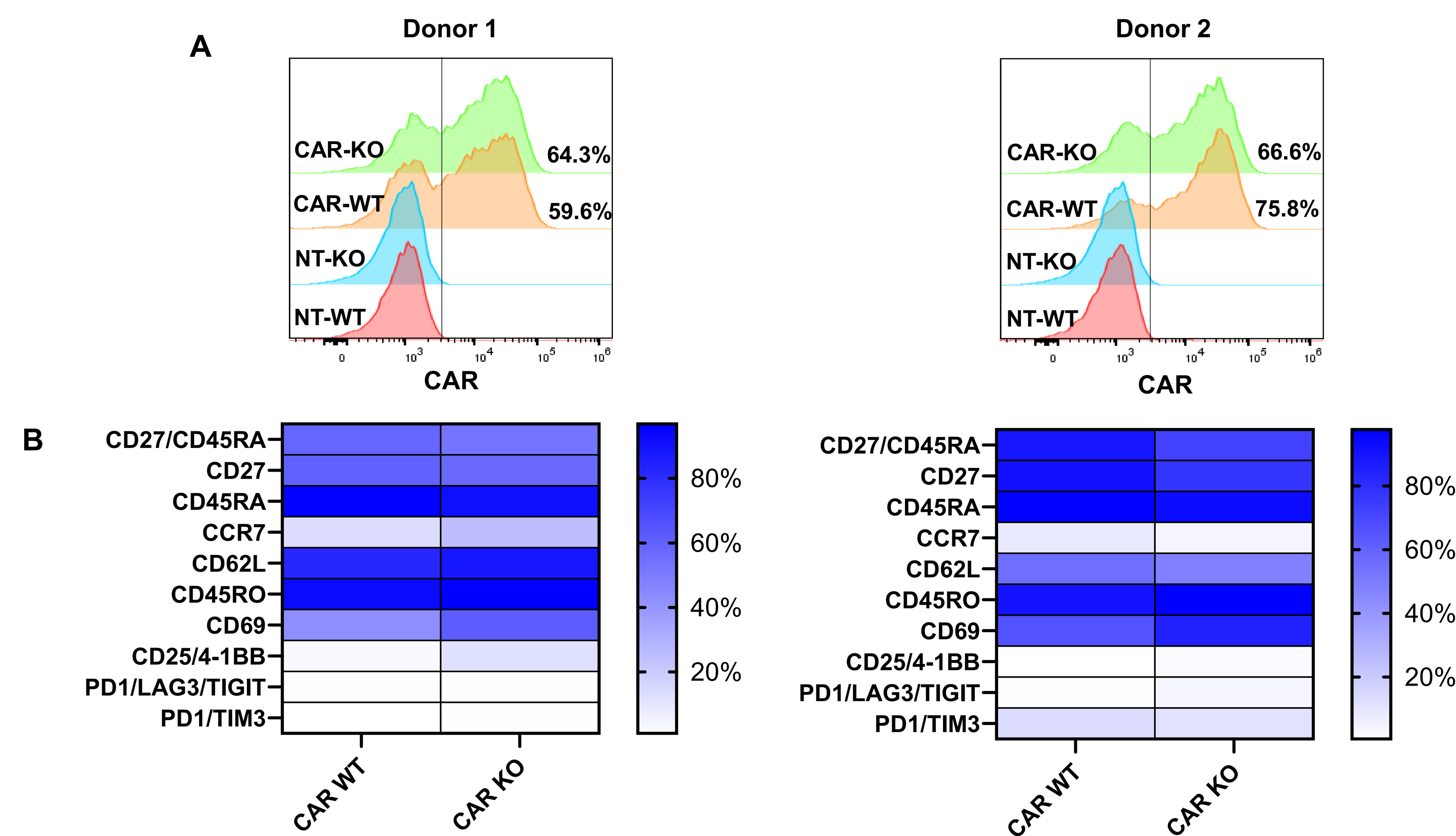



**Figure 3. (A)** Representative flow cytometry plot of anti-B7-H6 CAR expression on WT or CISH knockout CAR and non-transduced (NT) Vδ1 T cells from two different donors. **(B)** Heatmap showing percentages of T cell memory, activation, and exhaustion associated markers expressed by WT or *CISH* KO CAR Vδ1 T cells.

## CISH KO in Vδ1 T cells promoted increased expression of genes and gene-enrichment sets associated with T cell effector function

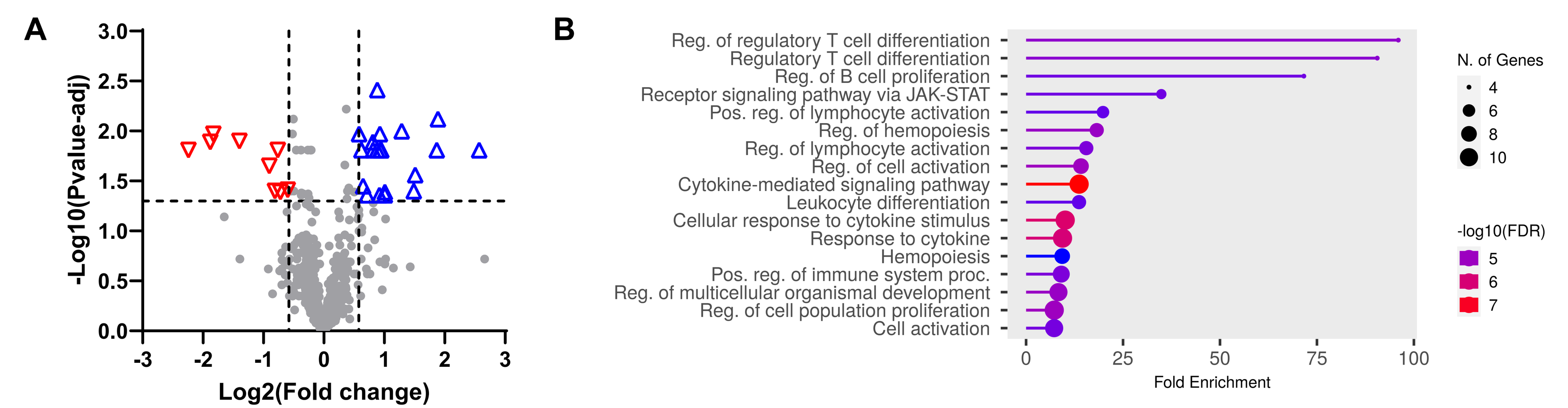



**Figure 4. (A)** Volcano plot of differentially expressed genes (DEGs) between WT and *CISH* KO CAR Vδ1 T cells. Blue triangles △ represent DEGs upregulated and red triangles ▽ represent DEGs downregulated in *CISH* KO vs WT conditions. Gene expression were quantitated using the Nanostring nCounter® CAR T Cell Characterization panel. **(B)** Gene Ontology analysis was performed using ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/) to identify the biological pathways associated with DEGs that were upregulated in the *CISH* KO CAR Vδ1 T cells.

## Enhanced phosphorylation of STAT3/5 and proliferation in *CISH* KO Vδ1 cells

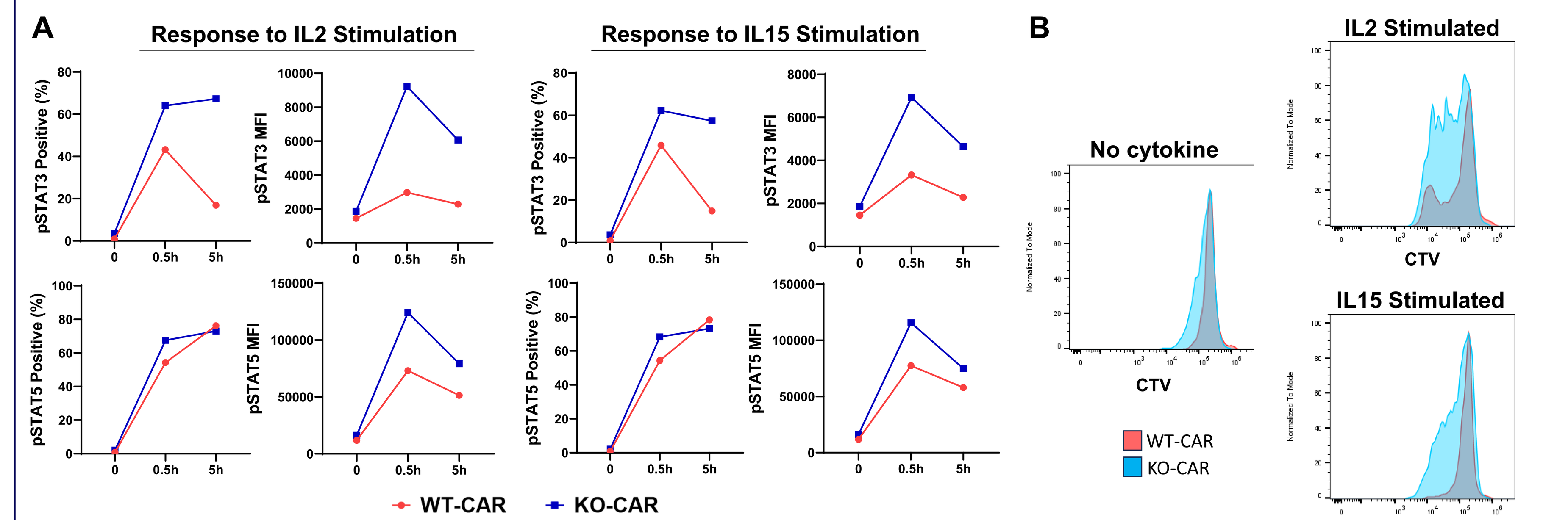



**Figure 5. (A)** Enhanced phosphorylation of STAT3/STAT5 signaling in *CISH* KO Vδ1 T cells. WT or *CISH* KO CAR Vδ1 T cells were cultured overnight in cytokine free medium, then treated with IL2 or IL15 at the indicated times, and percentage of pSTAT3 and pSTAT5 expression (left) or mean fluorescence intensity (MFI, right) was analyzed using flow cytometry. **(B)** Improved proliferation of *CISH* KO compared to WT CAR Vδ1 T cells. WT or *CISH* KO CAR Vδ1 T cells were labelled with cell trace violet (CTV) and cultured for seven days in cytokine free or low levels of IL2/IL15. Representative FACS plots are shown.

## *CISH* KO CAR Vδ1 T cells demonstrated enhanced serial killing and persistence against tumor cell lines with diverse antigen expression levels

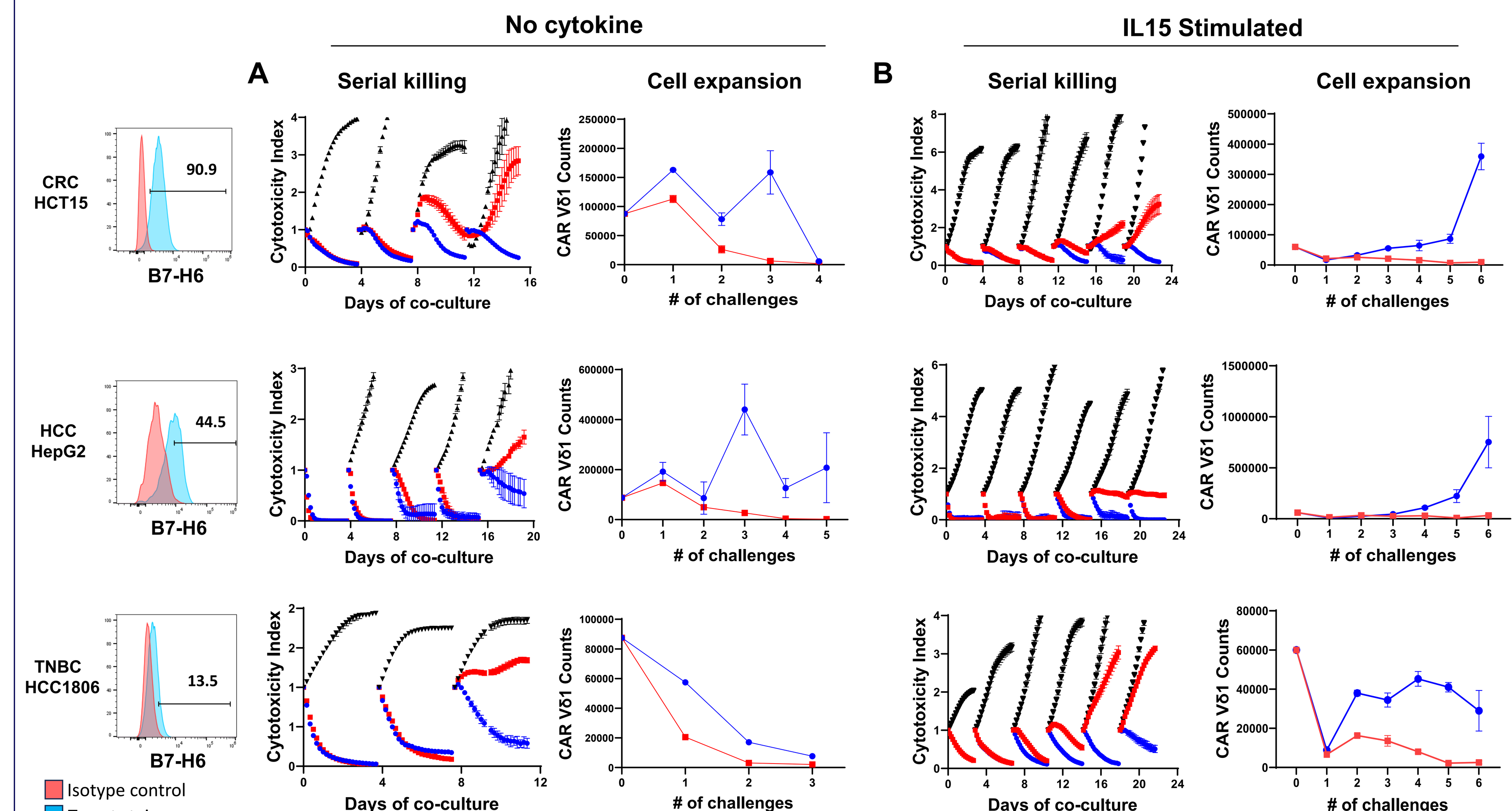



**Figure 6.** Cytotoxic potentials of WT CAR Vδ1 T cells (■ red square) and *CISH* KO CAR Vδ1 T cells (● blue circle) were evaluated against tumor cell lines (▼ black triangle) with diverse antigen expression levels: HCT15 (high), HepG2 (medium) and HCC1806 (low) in the repetitive stimulation Incucyte Immune Cell Killing Assay in cytokine free medium **(A)** or IL15 **(B)**. CAR Vδ1 T cells were cocultured with target cell line for three to four days, then half of the cells were transferred into a new vessel with freshly plated target cells for an additional three to four days. The Cytotoxicity Index was calculated by dividing the total NIR object area (μm²/well) of all time points by the value at time of tumor challenge. Leftover cells in the previous plates were collected and analyzed by flow cytometry to monitor the CAR positive Vδ1 T cell expansion.

## Demonstration of One Step Knock-out Knock-in (KOKI) of CAR through *CISH* locus in Vδ1 T cells

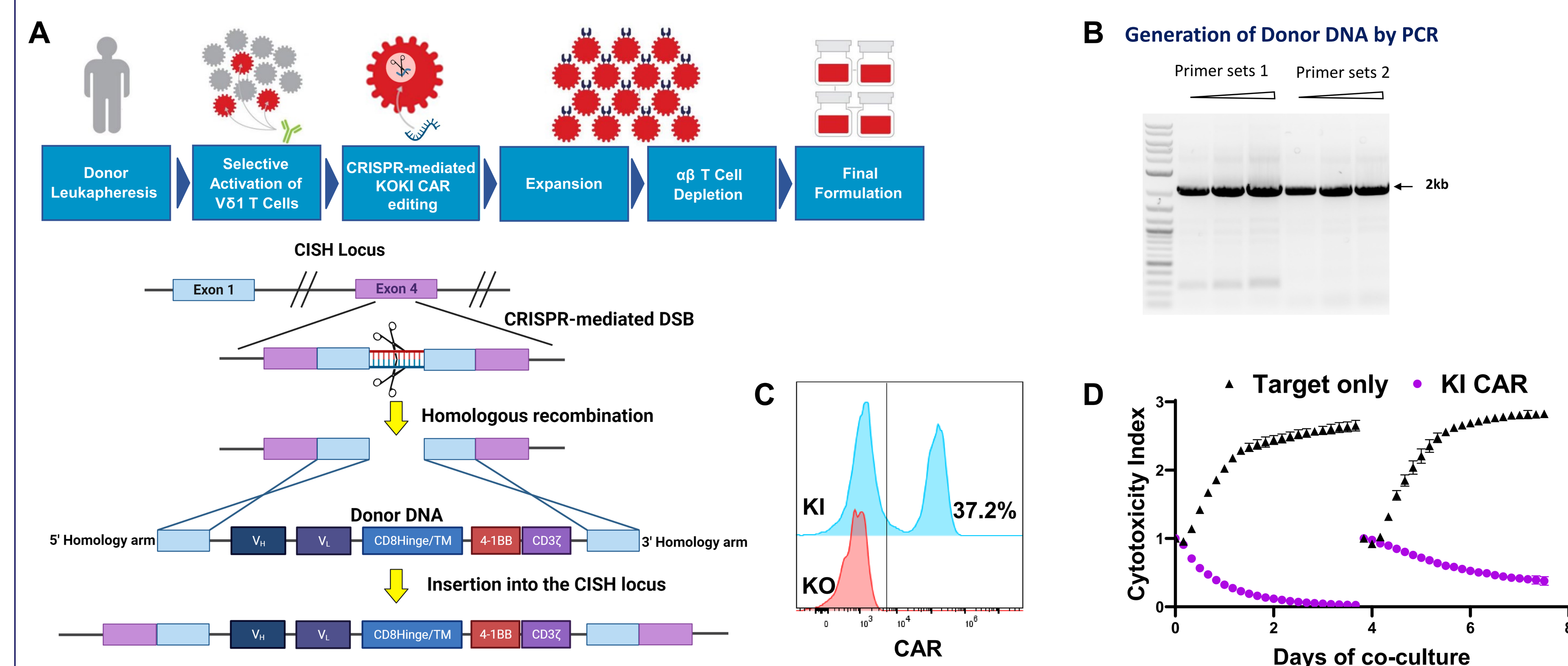



**Figure 7. (A)** Flow chart describing the process of generation of knockout knock-in CAR through the *CISH* locus in Vδ1 T cells. **(B)** Use of PCR primer sets on the homologous arms to generate donor DNA for electroporation. **(C)** Representative flow cytometry plot of CAR expression on *CISH* knockout CAR knock-in Vδ1 T cells. **(D)** Cytotoxic potential of *CISH* knockout knock-in CAR Vδ1 T cells (purple circles) were evaluated against B7-H6 positive tumor cell line HCT-15 in a repetitive stimulation Incucyte Immune Cell Killing Assay. The Cytotoxicity Index was calculated by dividing the total NIR object area (μm²/well) of all time points by the value at time of tumor challenge.

## Summary & Conclusions

- CRISPR-gene editing can be successfully applied to CAR Vδ1 T cells with a high KO efficiency
- *CISH* KO in CAR Vδ1 T cells enhanced pSTAT3/5 signaling, increased proliferation, and promoted robust *in vitro* anti-tumor activity
- Transcripts associated with T cell effector activity were upregulated in *CISH* KO CAR in Vδ1 T cells
- One step knock-out knock-in experiments supports PoC for non-viral vector CAR insertion and expression in Vδ1 T cells
- These approaches provide opportunities to explore novel gene-editing strategies to further enhance allogeneic CAR γδ T cells as a promising platform for cancer immunotherapy.

### References

1. A Phase 1 Safety and Efficacy Study of ADI-001 Anti-CD20 CAR engineered Allogeneic Gamma Delta T Cells in Adults with B Cell Malignancies. NCT04735471.
2. Nishimoto KP et al. Allogeneic CD20-targeted γδ T cells exhibit innate and adaptive antitumor activities in preclinical B-cell lymphoma models. Clin Transl Immunology. 2022 Feb 2;11(2):e1373.
3. Zhu H et al. Metabolic Reprograming via Deletion of CISH in Human iPSC-Derived NK Cells Promotes In Vivo Persistence and Enhances Anti-tumor Activity. Cell Stem Cell. 2020 Aug 6;27(2):224-237.
4. Palmer DC et al. Cish actively silences TCR signaling in CD8+ T cells to maintain tumor tolerance. J Exp Med. 2015 Nov 16;212(12):2095-113.