

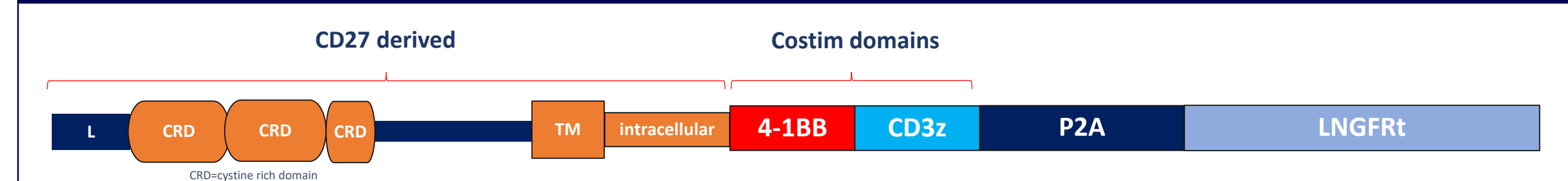
## INTRODUCTION

CD70, a member of the TNF receptor ligand family, represents a compelling target for the development of CAR T cell therapies due to its high expression in multiple solid and hematological malignancies. CAR T cells have shown remarkable clinical benefit in hematological malignancies, but efficacy in solid tumors has highlighted key challenges. Among the emerging strategies to improve clinical responses is the use of alternative cytotoxic effector cells with multifunctional tumoricidal activity.  $\gamma\delta$  T cells combine innate and adaptive immunity to recognize and kill malignant cells. In addition, the infiltration of  $\gamma\delta$  T cells into various cancer types, including those expressing CD70, significantly correlates with survival. Strategies for targeting CD70 have explored scFvs or engineering its natural receptor (CD27) as the antigen-recognition moiety of a CAR. A recent study demonstrated superior preclinical antitumor activity using the CD27- compared to scFv-based CAR, suggesting a functional advantage is associated with the CD27 natural receptor CAR approach<sup>1</sup>. Here we report on the functional characterization and manufacturability of  $\gamma\delta$  T cells expressing a CD27-based CAR for targeting a set of CD70<sup>+</sup> cancers.

## METHODS

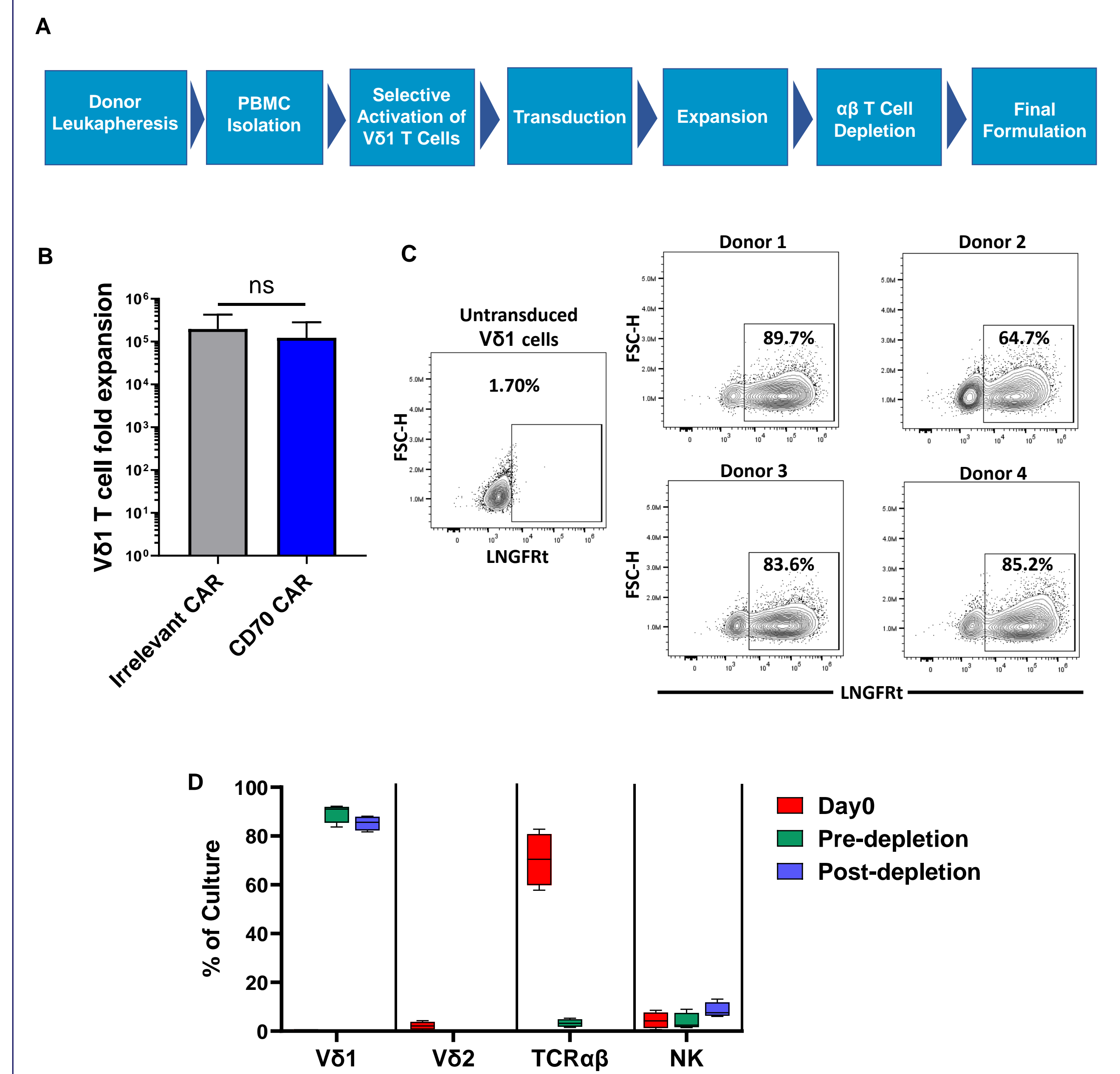
Donor PBMCs were used to activate, expand, and engineer cytotoxic V $\delta$ 1 T cells to express CD27-containing CAR (CD70 CAR). *In vitro* phenotype and antitumor functionality of V $\delta$ 1 CAR T cells were determined using flow cytometry and cell-based cytotoxicity assays against a panel of cell lines having a wide range of CD70 expression. Human tumor xenograft models in immunodeficient mice were used to evaluate *in vivo* efficacy after a single dose of CD70 CAR V $\delta$ 1 T cells. In addition, CD70 CAR V $\delta$ 1 T cells were armored with a dominant-negative receptor (dnTGF $\beta$ RII) “bolt-on”, and *in vitro* functional assays were used to determine their resistance to the immunosuppressive effects of TGF- $\beta$ .

## CD27-containing CAR construct design (CD70 CAR)



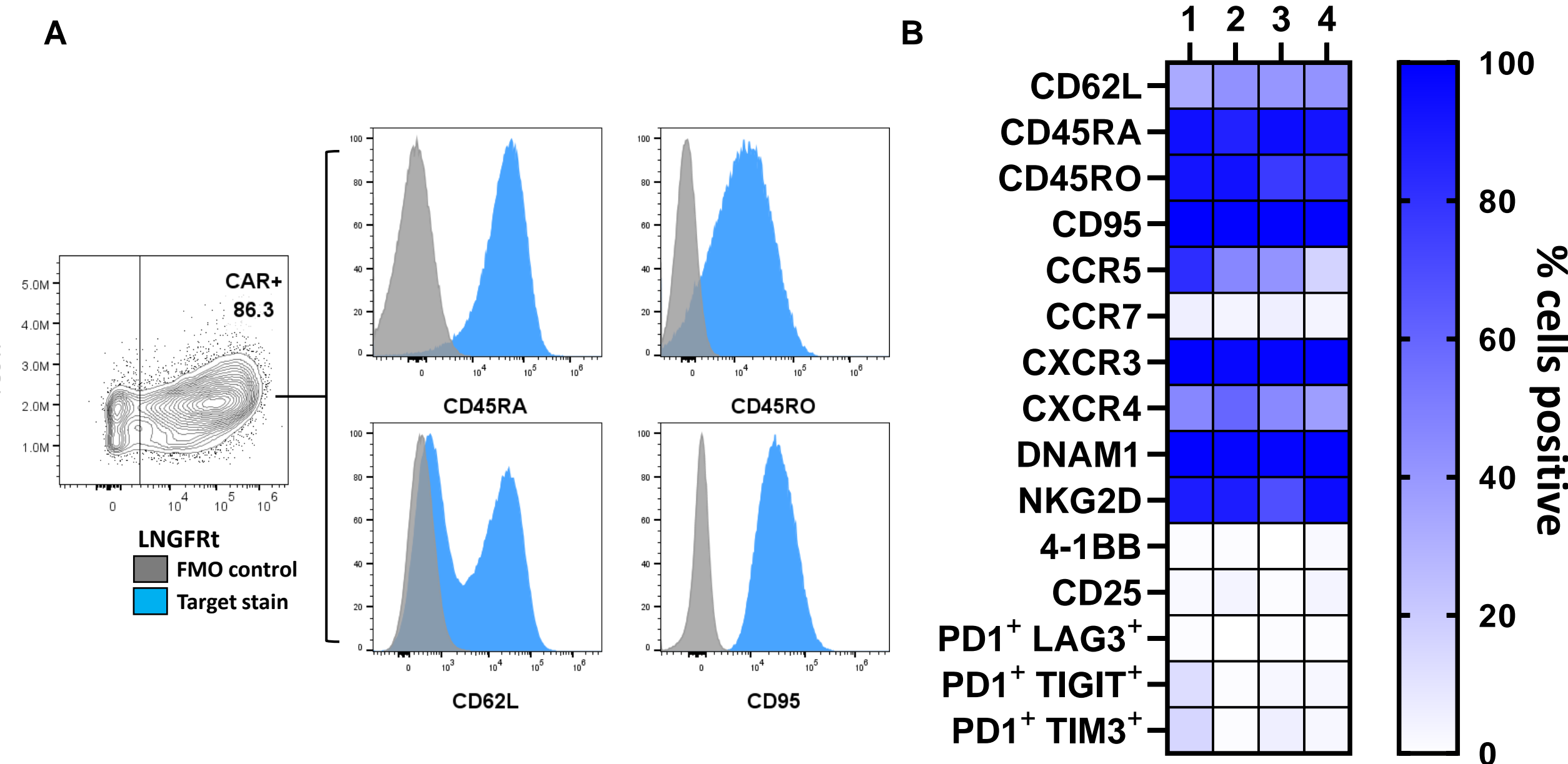
**Figure 1.** Schematic diagram of the CD70 CAR construct. The CD70 CAR consists of the CD27-derived receptor fused to costimulatory domains, 4-1BB and CD3z. The CD70 CAR contains a truncated low affinity nerve growth factor receptor (LINGFRt), separated by P2A, for research purposes only for the determination of CAR transduction efficiency in preclinical experiments.

## Generation of allogeneic CD70 CAR V $\delta$ 1 T cells



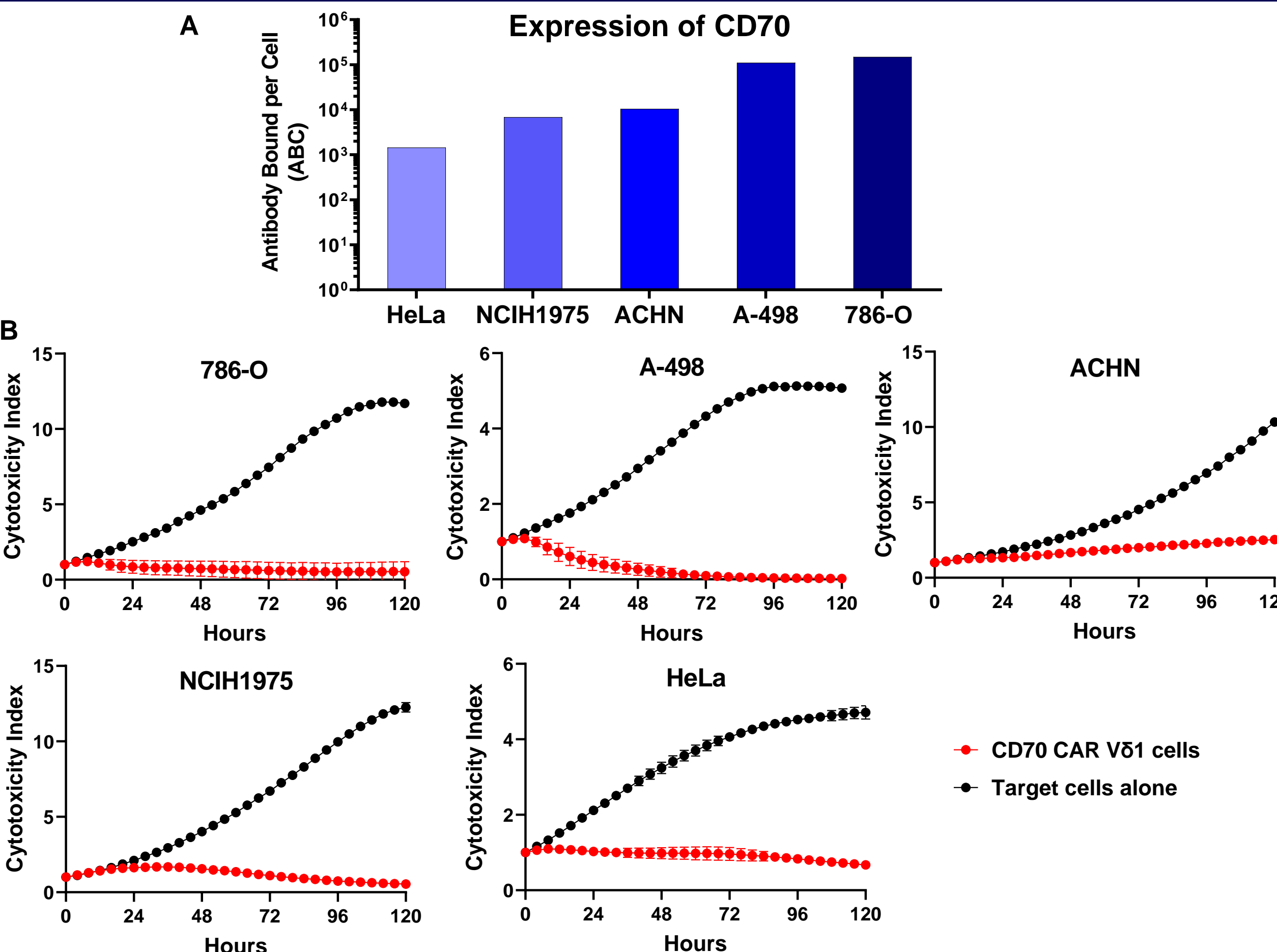
**Figure 2.** Selective activation and expansion of V $\delta$ 1 T cells using agonistic mAb from healthy donor-derived PBMCs. (A) Flow chart highlighting the key steps in the generation of allogeneic CD70 CAR V $\delta$ 1 T cells. (B) CD70 CAR V $\delta$ 1 T cell generation process resulted in a substantial fold-expansion of V $\delta$ 1 T cells with no effect of fratricide when compared to control irrelevant CAR expansions. Paired t-test was used to assess statistical significance. (C) Contour plots displaying the transduction efficiency (% LINGFRt expression) of the CD70 CAR V $\delta$ 1 T cells derived from 4 different donors as measured by flow cytometry. (D) % cell composition throughout the expansion of CD70 CAR V $\delta$ 1 T cell products derived from 4 different donors analyzed using flow cytometry.

## T cell memory phenotype, NKR, chemokine receptor, and activation/exhaustion expression profile in CD70 CAR V $\delta$ 1 T cells



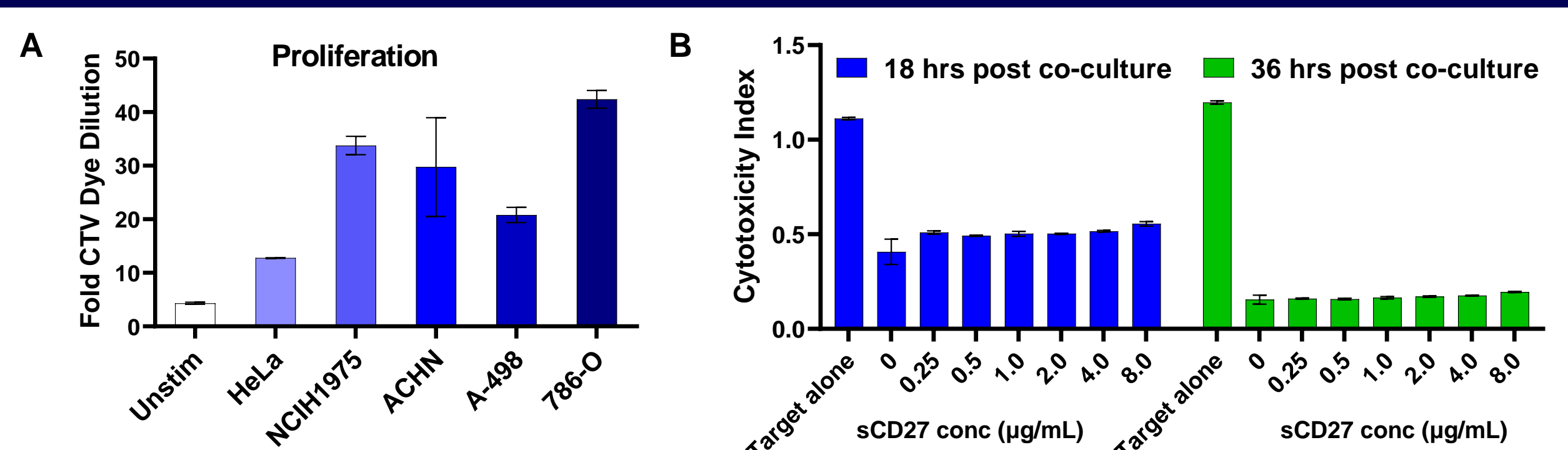
**Figure 3.** (A) CD70 CAR<sup>+</sup> V $\delta$ 1 T cells exhibit a naïve-like T cell memory phenotype assessed by flow cytometry. (B) Heatmap showing percentages from CD70 CAR<sup>+</sup> V $\delta$ 1 T cells (4 different donors) of T cell memory markers, multiple chemokine receptors, NKRs, activation markers, and the co-expression of PD1 and another co-inhibitory receptor.

## CD70 CAR V $\delta$ 1 T cells exhibit potent cytotoxic activity against various CD70<sup>+</sup> solid tumor cell lines



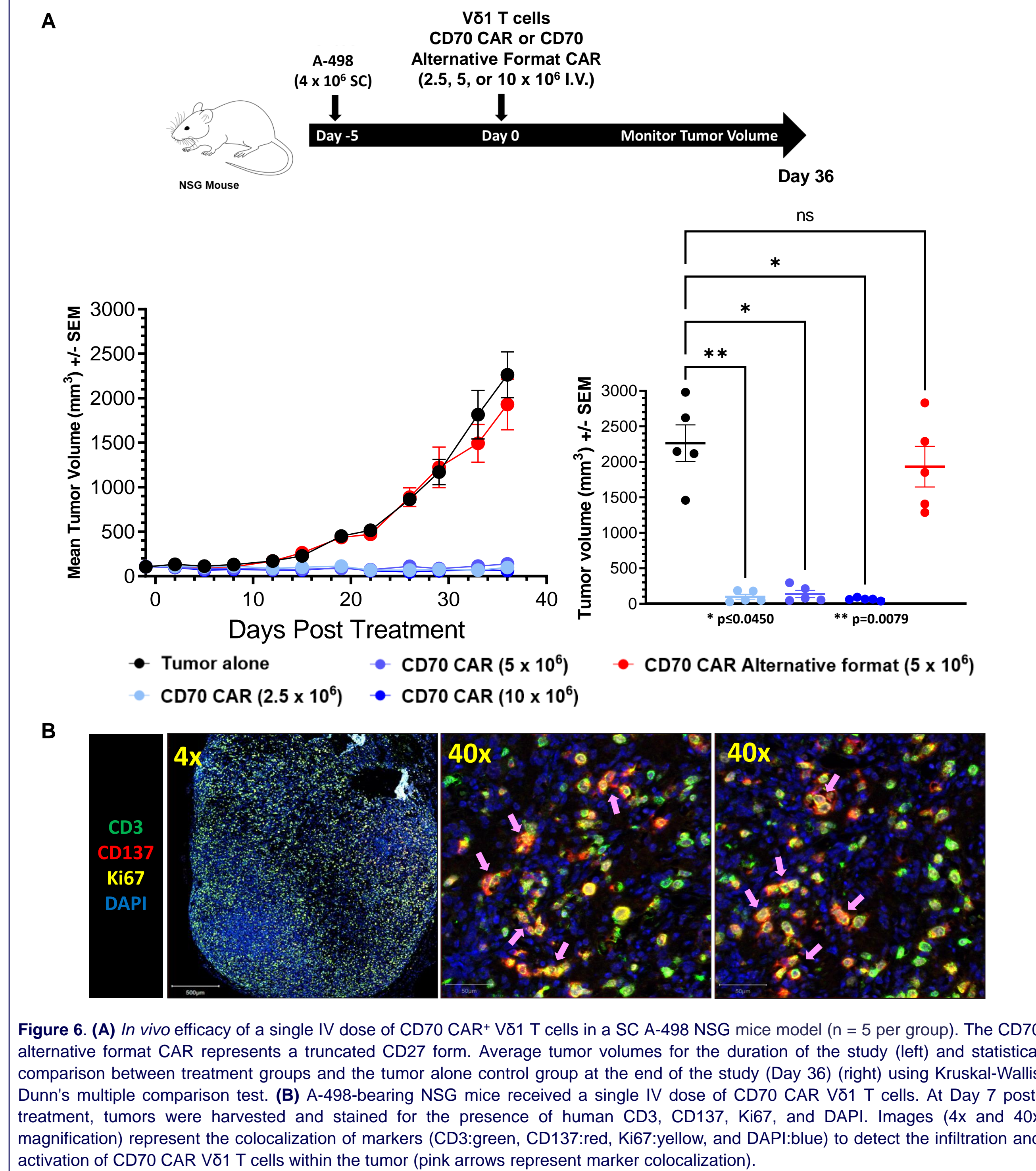
**Figure 4.** (A) CD70 quantitative expression on tumor cell line panel determined by flow cytometry using BD Quantibrite™ Beads. (B) Cytotoxic potentials of CD70 CAR V $\delta$ 1 T cells (red circle) were evaluated against CD70<sup>+</sup> tumor cell lines in a 120-hour Incucyte Immune Cell Killing Assay, in which T cells were co-cultured with NuclIR-expressing target cells at an E:T ratio of 2:1. The Cytotoxicity Index was calculated by dividing the total NIR object area (mm<sup>2</sup>/well) of all time points by the value at time = 0. Data depicted are the average of two different donors.

## CD70 CAR V $\delta$ 1 T cells proliferate and maintain cytotoxic activity in the presence of soluble CD27



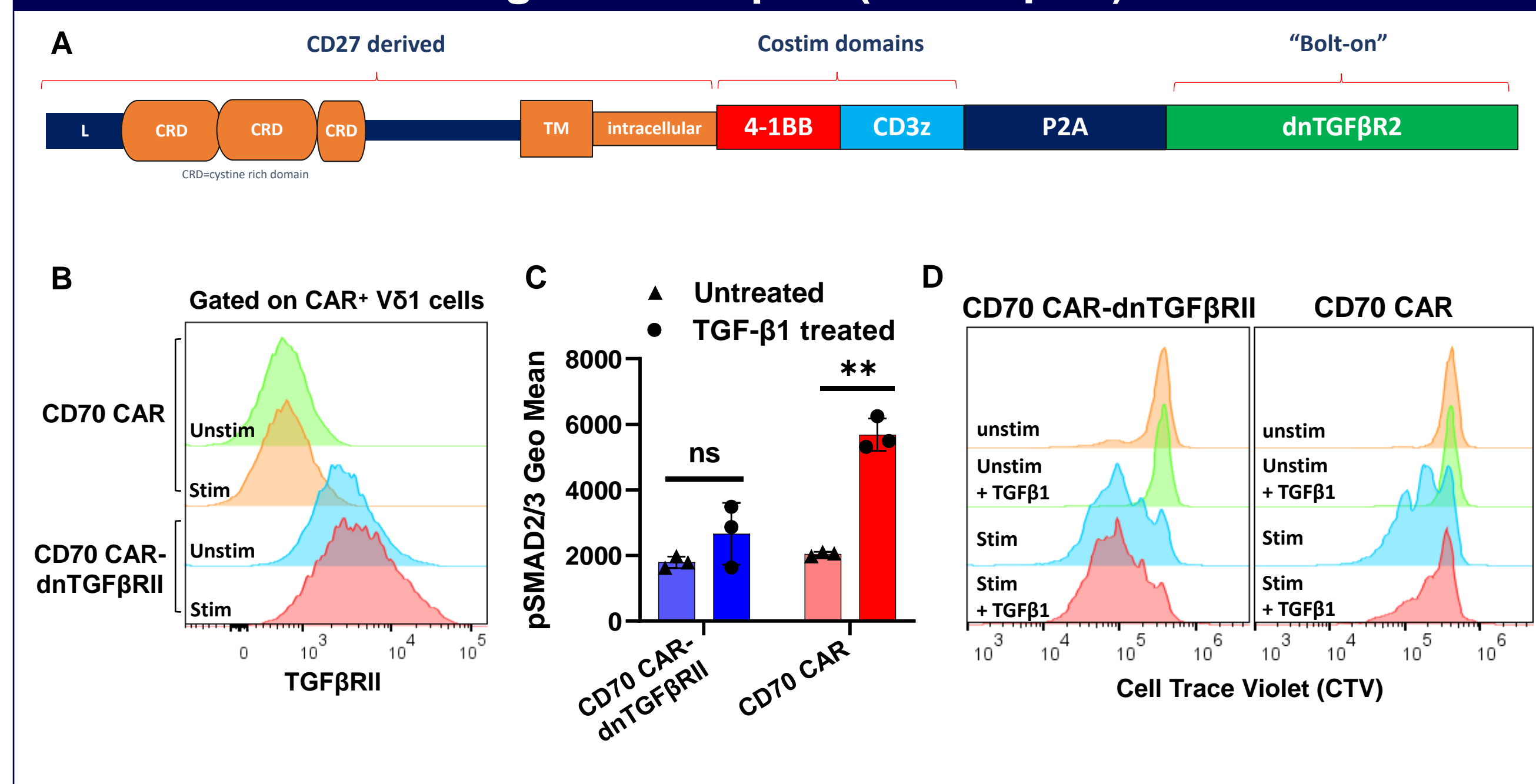
**Figure 5.** (A) Proliferative potential of Cell Trace Violet (CTV) labeled CD70 CAR V $\delta$ 1 T cells in a 7-day co-culture assay with CD70<sup>+</sup> tumor cell lines. Flow cytometry was used to determine the fold change of CTV dye dilution (CTV Geometric mean at day 0 / CTV Geometric mean at day 7). (B) CD70 CAR V $\delta$ 1 T cells were co-cultured with A-498 cells in the presence and absence of exogenously added soluble CD27 (sCD27) and cell killing was measured using an Incucyte Cell Killing Assay. No differences were observed in Cytotoxicity Index between +/- sCD27 added conditions.

## CD70 CAR V $\delta$ 1 T cells significantly inhibit *in vivo* tumor growth in renal cell carcinoma xenografts



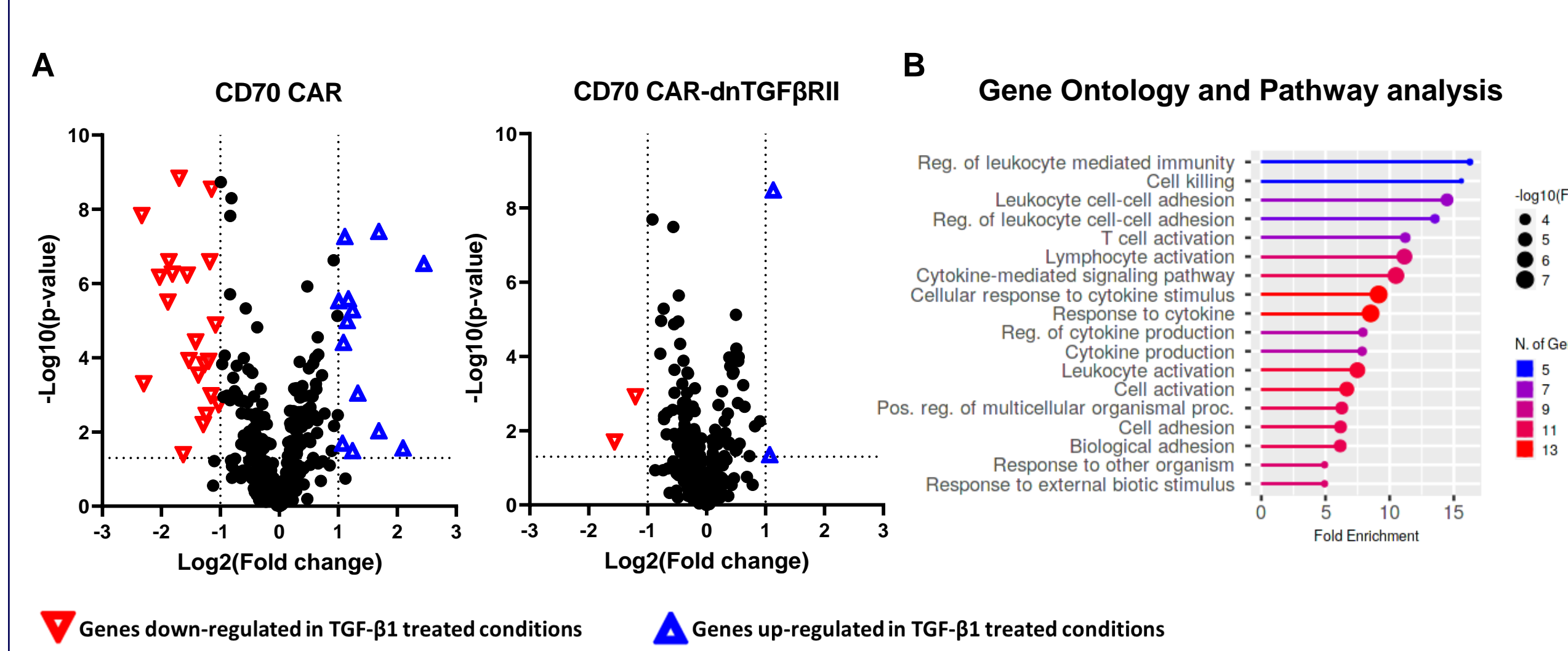
**Figure 6.** (A) *In vivo* efficacy of a single IV dose of CD70 CAR<sup>+</sup> V $\delta$ 1 T cells in a SC A-498 NSG mice model (n = 5 per group). The CD70 alternative format CAR represents a truncated CD27 form. Average tumor volumes for the duration of the study (left) and statistical comparison between treatment groups and the tumor alone control group at the end of the study (Day 36) (right) using Kruskal-Wallis Dunn's multiple comparison test. (B) A-498-bearing NSG mice received a single IV dose of CD70 CAR V $\delta$ 1 T cells. At Day 7 post-treatment, tumors were harvested and stained for the presence of human CD3, CD137, Ki67, and DAPI. Images (4x and 40x magnification) represent the colocalization of markers (CD3:green, CD137:red, Ki67:yellow, and DAPI:blue) to detect the infiltration and activation of CD70 CAR V $\delta$ 1 T cells within the tumor (pink arrows represent marker colocalization).

## Armoring against immunosuppressive effects of TGF- $\beta$ with a dominant-negative receptor (dnTGF $\beta$ RII) “bolt-on”



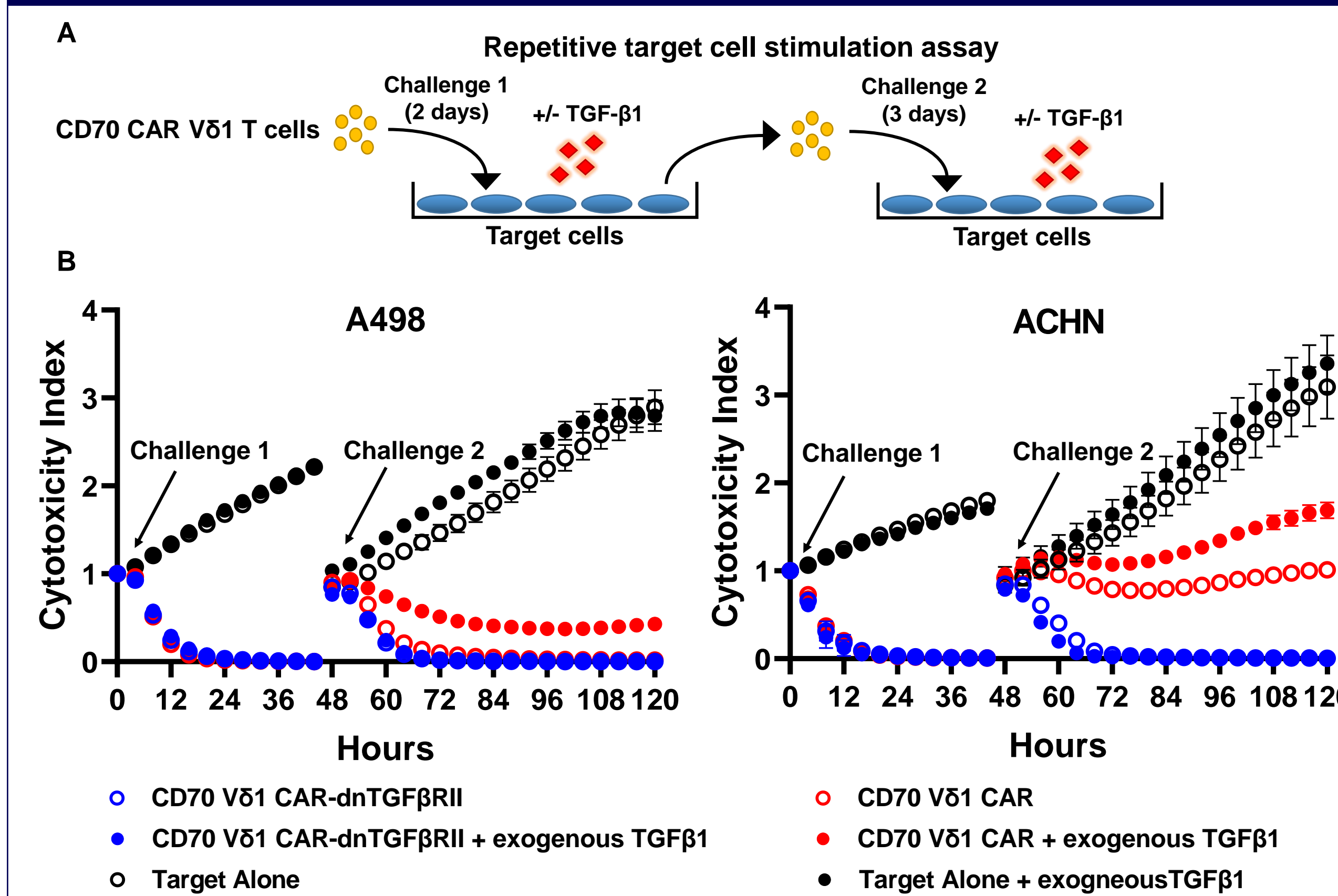
**Figure 7.** (A) Schematic diagram of the CD70 CAR with dnTGF $\beta$ RII “bolt-on”. (B) Detection of TGF $\beta$ RII surface expression in CD70 CAR V $\delta$ 1 T cells with or without “bolt-on” using flow cytometry. TGF $\beta$ RII expression was evaluated pre- and post-stimulation with immobilized rhuCD70 (5 $\mu$ g/mL) after an 18-hr incubation. (C) CD70 CAR V $\delta$ 1 T cells with the “bolt-on” showed lower intracellular staining levels of pSMAD2/3 (Geo Mean) in the presence of TGF- $\beta$ 1 (20ng/mL) compared to CD70 CAR V $\delta$ 1 T cells without “bolt-on”, confirming the functionality of the dnTGF $\beta$ RII. Statistical analysis was performed using paired t-test \*\* p<0.005. (D) CTV labeled CD70 CAR V $\delta$ 1 T cells with or without “bolt-on” were stimulated with CD70<sup>+</sup> A-498 tumor cells for 7 days in the presence and absence of TGF- $\beta$ 1 (20ng/mL). Increased cell proliferation is indicated by the dilution of the CTV dye (histogram plots) compared to unstimulated controls. CD70 CAR-dnTGF $\beta$ RII V $\delta$ 1 T cells become resistant to the effects of TGF- $\beta$ 1 compared to CD70 CAR V $\delta$ 1 T cells without “bolt-on”. The data is a representative of 3 different donors.

## CD70 CAR-dnTGF $\beta$ RII V $\delta$ 1 T cells are armored against TGF- $\beta$ 1 mediated alterations to activation expression profile



**Figure 8.** (A) Volcano plots of differentially expressed genes (DEGs) from CD70 CAR +/- dnTGF $\beta$ RII V $\delta$ 1 T cells activated with rhuCD70 (5 $\mu$ g/mL) in the presence or absence of TGF- $\beta$ 1 (20ng/mL) for 24 hrs from 2 different donors. Blue triangles represent DEGs upregulated in TGF- $\beta$ 1 treated vs untreated conditions. Red triangles represent DEGs downregulated in TGF- $\beta$ 1 treated vs untreated conditions. Gene expression was quantitated using the Nanostring nCounter® CAR T Cell Characterization panel. (B) Gene Ontology analysis was performed using ShinyGO 0.76.1 (<http://bioinformatics.sdstate.edu/go/>) to identify the biological pathways associated with DEGs that were downregulated in the presence of TGF- $\beta$ 1 from CD70 CAR V $\delta$ 1 T cells without the dnTGF $\beta$ RII “bolt-on”.

## CD70 CAR-dnTGF $\beta$ RII V $\delta$ 1 T cells have enhanced functional persistence against renal cell carcinoma cell lines



**Figure 9.** (A) Diagram of the *in vitro* repetitive stimulation assay to measure the cytolytic activity of CD70 CAR V $\delta$ 1 T cells co-cultured with target cell lines in the presence and absence of TGF- $\beta$ 1 (20ng/mL). CAR V $\delta$ 1 T cells were stimulated with target cell line for 48 hours, then transferred into a new vessel with freshly plated target cells for an additional 72 hours. (B) Cytotoxic potentials of CD70 CAR V $\delta$ 1 T cells (red circles) and CD70 CAR-dnTGF $\beta$ RII V $\delta$ 1 T cells (blue circles) were evaluated against CD70<sup>+</sup> tumor cell lines A-498 and ACHN in the repetitive stimulation Incucyte Immune Cell Killing Assay. The Cytotoxicity Index was calculated by dividing the total NIR object area (mm<sup>2</sup>/well) of all time points by the value at time of tumor challenge (challenge 1 or challenge 2 time points).

## SUMMARY & CONCLUSIONS

- V $\delta$ 1 T cells modified to express CD70 CAR were successfully generated and expanded, demonstrating product expansion without indications of fratricide.
- The resulting CD70 CAR V $\delta$ 1 T cells expressed a predominant naïve-like memory phenotype and were associated with potent *in vitro* cytotoxicity, and proliferation against multiple CD70<sup>+</sup> tumor cell lines.
- To assess the potential impacts of soluble CD27 (sCD27) competition on cytotoxicity, exogenously added sCD27 did not significantly impact anti-tumor activity.
- Highly potent tumor growth inhibition was observed with CD70 CAR V $\delta$ 1 T cells against tumor xenografts in immunodeficient mice with evidence of T cell infiltration and activation within the tumor bulk.
- Armoring CD70 CAR V $\delta$ 1 T cells with the dnTGF $\beta$ RII “bolt-on” maintained activity in the presence of TGF- $\beta$ .
- In summary, these preclinical data support further development of an armored allogeneic  $\gamma\delta$  CAR T cell therapy utilizing the CD27 natural receptor CAR format for targeting CD70<sup>+</sup> cancers.

## References

1. Sauer T, Parikh K, Sharma S, Omer B, Sedlov D, Chen Q, Angenendt L, Schliemann C, Schmitt M, Müller-Tidow C, Gottschalk S, Rooney CM. CD70-specific CAR T cells have potent activity against acute myeloid leukemia without HSC toxicity. *Blood*. 2021 Jul 29;138(4):318-330.