Preclinical Discovery and Characterization of Allogeneic Anti-PSMA γδ CAR-T Therapy for Prostate Cancer

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BACKGROUND

Prostate-specific membrane antigen (PSMA) is a transmembrane glycosylated homodimer overexpressed in >80% of prostate cancers. PSMA expression is increased in advanced stages of the disease, making it an attractive therapeutic target. Clinically, autologous anti-PSMA αβ CAR T cells have shown initial efficacy coupled with significant CRS-like dose-limiting toxicities[1]. Compared to αβ T cells and other innate cells, γδ T cells are associated with multifunctional innate and adaptive targeting and differentiated biodistribution into tumor-associated tissues. Additionally, γδ CAR T cells demonstrate enhanced tumoricidal activity and activation-induced cytokine profiles that may decrease toxicities associated with CRS.

METHODS

We used phage panning to identify 91 unique anti-PSMA scFv sequences, of which a subset were reformatted into CARs in VH-VL and VL-VH orientations and screened in Jurkat Lucia NFAT cells to assess CAR expression and activation in the context of target cell-based stimulation. We transduced functional CARs into Vδ1 T cells activated from healthy donor PBMCs. We performed *in vitro* cell-based cytotoxicity assays and phenotypic assessments of CAR Vδ1 T cells using flow cytometry. Preclinical potency was also assessed in NSG mice bearing subcutaneous PSMA-expressing xenografts. Here we show the discovery and preclinical characterization of γδ T cells modified from a set of novel scFv-based CARs targeting PSMA for prostate cancer. We also engineered armored anti-PSMA CAR Vδ1 T cells expressing a TGFβ dominant-negative receptor (dnTGFβRII) and assessed the functional advantage of armoring (here referred to as a "bolt-on") in cell-based assays and a PCa xenograft model.

Phage panning was used to identify novel anti-PSMA binders with varying affinities

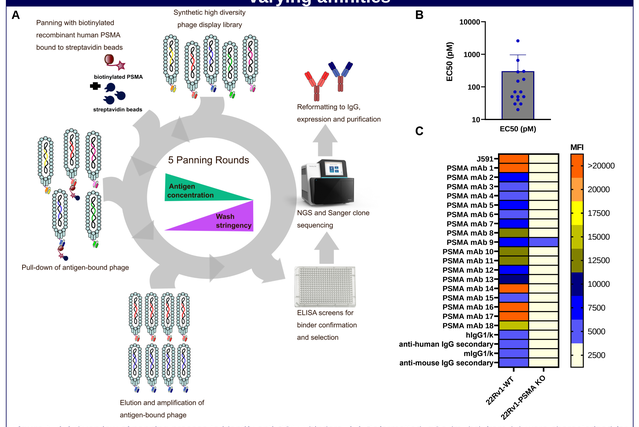

Figure 1. (A) Overview of panning process to identify anti-PSMA binders. **(B)** Reformatted IgGs (mAbs) from (A) were characterized for binding properties on recombinant protein using ELISA. Range of EC50 values are shown for all mAbs tested **(C)** mAbs were additionally tested for binding to PSMA-expressing 22Rv1-WT cells. Specificity of mAbs were also assessed using the 22Rv1-PSMA KO cell line.

Anti-PSMA CAR-expressing Jurkat cells show target-specific activation and low tonicity

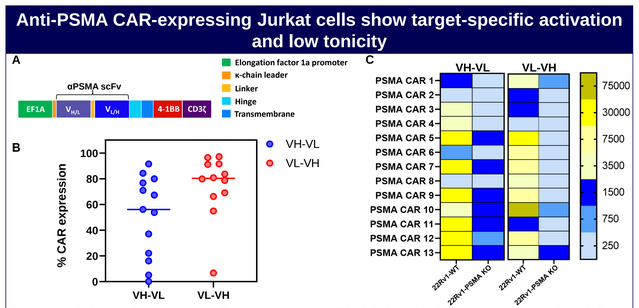

Figure 2. (A) Schematic showing elements of second generation anti-PSMA CARs **(B)** CAR expression in Jurkat Lucia™ NFAT cells transduced with VH-VL (blue) or VL-VH (red) CARs. Each dot represents an independent scFv. **(C)** Jurkat Lucia™ NFAT cells were transduced with gammaretrovirus to express CARs in VH-VL and VL-VH orientations, then co-cultured with 22Rv1-WT cells and 22Rv1-PSMA KO cells to assess antigen-specific activation. Supernatants from co-cultures were incubated with luciferase and resultant luminescence was plotted as NFAT activity in heatmap format, with each row representing an independent scFv.

Anti-PSMA CAR Vδ1 T cells expand robustly and demonstrate potent cytotoxicity and proliferation against prostate cancer cell lines

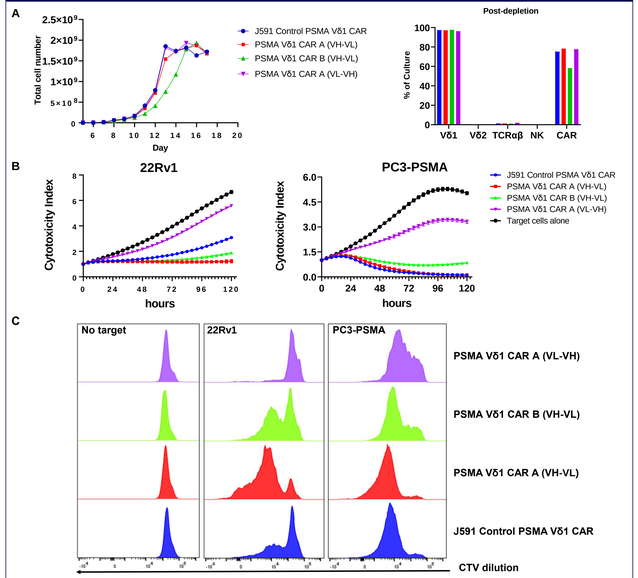

Figure 3. (A) Scatter plot (left) showing expansion of 4 anti-PSMA Vδ1 CARs including the J591 control over 18 days in research-optimized process in shake flasks. Bar graphs (right) display cell populations and CAR percentages in post- αβ T cell-depleted cultures **(B)** 120 hr cytotoxicity assay demonstrates in vitro potency at 1:1 E:T ratio of PSMA-targeting Vδ1 CAR-T when co-cultured PSMA-expressing target cell lines, 22Rv1 (left) and PC3 cells engineered to express PSMA (right). Assay was set up in the Incucyte® SX5 Live-Cell Analysis Instrument with Nuclight-NIR transformed target cells. **(C)** Proliferative potential of CellTrace Violet labeled anti-PSMA Vδ1 CAR T cells following target antigen exposure in a 7-day co-culture period, using 22Rv1-WT or PC3-PSMA cells. Aliquots of cells harvested on Day 7 were analyzed by flow cytometry to assess cellular proliferation by dye dilution, and compared to unstimulated controls.

Anti-PSMA CAR Vδ1 T cells significantly inhibit *in vivo* tumor growth in heterogeneous 22Rv1 xenograft model

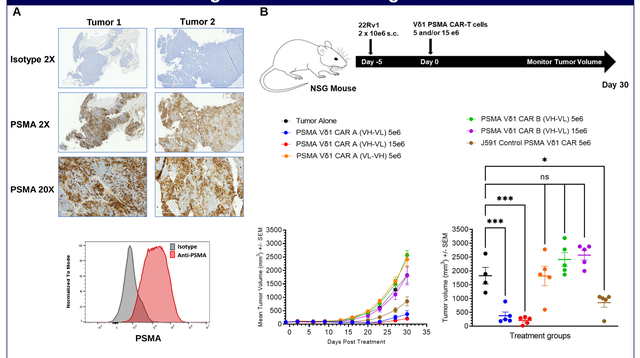

Figure 4. (A) IHC staining of FFPE tumor sections from NSG mice (upper panel) reveals heterogeneity in expression of PSMA in 22Rv1 cells, a cell line known to express moderate levels of PSMA (lower panel). 22Rv1 cells were stained with either isotype antibody (grey) or anti-PSMA antibody (red) and analyzed by flow cytometry **(B)** Demonstration of in vivo potency in a 22Rv1 PCa xenograft model with PSMA-targeting Vδ1 CAR-T cells. Schematic outlines the study design (top panel). Graphs detail tumor volumes determined for the entire study duration (bottom, left panel) as well as statistical comparison of treatment groups relative to the untreated tumor alone control at study termination (bottom, right panel).

"Bolt-on" engineered anti-PSMA CAR Vδ1 T cells express dnTGFβRII with corresponding reduction in pSMAD 2/3 activity

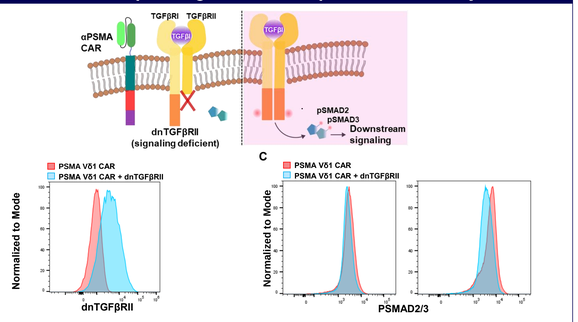

Figure 5. (A) Schematic showing anti-PSMA Vδ1 CAR with dominant negative TGF-β receptor II (dnTGFβRII) "bolt on" (left, unshaded), which can still bind TGFβ1 but cannot signal like the native receptor (right, shaded pink) **(B)** Expression of dnTGFβRII is increased in "bolt-on" engineered CAR Vδ1 T cells (blue) in comparison to the naked CAR (red). **(C)** Evaluation of TGFβ1 signaling (pSMAD 2/3) from Vδ1 cells containing dnTGFβRII CAR constructs in the presence or absence of TGFβ1

"Bolt-on" expressing anti-PSMA CAR Vδ1 T cells demonstrate potent *in vitro* cytotoxicity against prostate cancer cell lines and retain enhanced cytotoxic potential after rechallenge

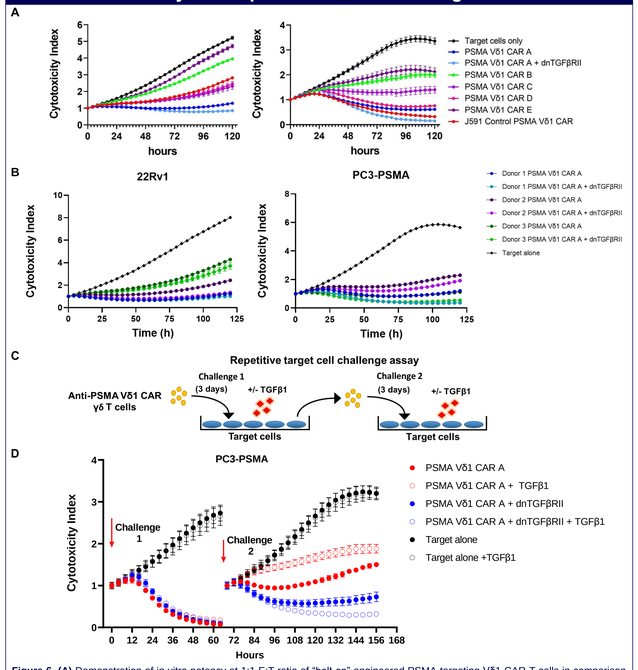

Figure 6. (A) Demonstration of in vitro potency at 1:1 E:T ratio of "bolt-on" engineered PSMA-targeting Vδ1 CAR-T cells in comparison to its naked CAR and other CAR constructs against 22Rv1 (left) and PC3-PSMA (right) at submaximal 1:1 E:T ratio. **(B)** Demonstration of in vitro potency at submaximal 1:1 E:T ratio of "bolt-on" engineered PSMA-targeting CAR Vδ1 T cells in 3 donors compared to corresponding naked CARs **(C)** Schematic describing *in vitro* target re-challenge assay to assess cytotoxic ability of "bolt-on" engineered anti-PSMA CAR Vδ1 T cells in the presence or absence of TGFβ1 **(C)** anti-PSMA CAR Vδ1 T cells, with or without the "bolt-on" were co-cultured with PC3-PSMA target cells at an E:T ratio of 3:1 in the presence or absence of TGFβ1 for 3 days before re-challenging with fresh PC3-PSMA target cells in the presence or absence of TGFβ1. Cytotoxicity indices are plotted over time.

Transcriptional and proteomic profiles of anti-PSMA CAR Vδ1 T cells support functional enhancement provided by "bolt-on"

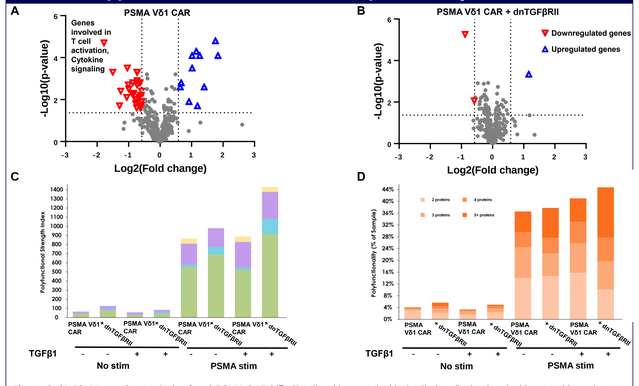

Figure 8. (A-B) Nanostring analysis of anti-PSMA CAR Vδ1 T cells without and with the "bolt-on" stimulated with recombinant human PSMA protein in the presence and absence of TGFβ1 reveals downregulation of genes associated with T cell activation and cytokine signaling for CAR without a "bolt-on" **(C,D)** Isolight single cell secretome assay showing "bolt-on" engineered anti-PSMA CAR Vδ1 T cells demonstrate higher polyfunctional strength index (C) and higher % polyfunctionality (D) compared to their naked CAR counterparts when stimulated with recombinant human PSMA protein in the presence of TGFβ1

"Bolt-on" expressing anti-PSMA CAR Vδ1 T cells demonstrate enhanced tumor control *in vivo* in PC3-PIP xenograft model

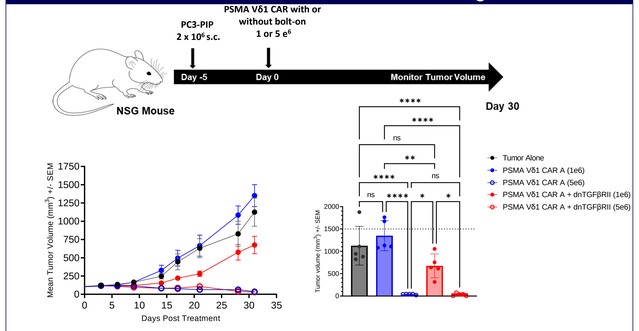

Figure 9. Demonstration of in vivo potency in a PC3-PIP PCa xenograft model with PSMA-targeting Vδ1 CAR-T cells with or without the dnTGFβRII "bolt-on", tested at two doses, including a submaximal dose (1e6). Schematic outlines the study design (top panel). Graphs detail tumor volumes determined for the entire study duration (bottom, left panel) as well as statistical comparison of treatment groups relative to the untreated tumor alone control and each other at study termination (bottom, right panel)

SUMMARY & CONCLUSIONS

- Vδ1 T cells modified to express *de novo* PSMA CARs were successfully generated and characterized.
- The resulting Vδ1 CAR T cells expressed a predominant naïve-like memory phenotype and were associated with potent in vitro cytotoxicity, production of proinflammatory cytokines, and proliferation against PSMA+ tumor cell lines.
- Potent tumor growth inhibition was observed in heterogeneous and uniform PCa tumor xenograft models.
- A functional advantage with "bolt-on" armoring (dnTGFβRII) was demonstrated for the anti-PSMA CAR Vδ1 T cells both *in vitro* and *in vivo*
- In summary, these preclinical data support further development of an armored allogeneic γδ CAR T cell therapy for prostate cancer

References

1. Narayan, V., Barber-Rotenberg, J.S., Jung, IY. et al. PSMA-targeting TGFβ-insensitive armored CAR T cells in metastatic castration-resistant prostate cancer: a phase 1 trial. Nat Med 28, 724–734 (2022). https://doi.org/10.1038/s41591-022-01726-1