Preclinical Discovery And Evaluation of Allogeneic “off-the-shelf” γδ CAR T Cells Targeting B7-H6+ Tumors

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BACKGROUND

B7-Homolog 6 (B7-H6) is a B7 family member and the natural ligand for NK cell-activation receptor, NKp30. B7-H6 is expressed on multiple tumor types but has limited expression in normal tissues. Given this tumor specificity, B7-H6 represents an attractive target for CAR T therapy. CAR T cell therapy is associated with high clinical response rates in hematologic malignancies, but opportunities for improved efficacy in solid tumors remain. γδ T cells, whose solid tumor infiltration has demonstrated a significant correlation with survival, combine innate and adaptive mechanisms to recognize and kill tumors while complimenting the CAR-based targeting. Here, we evaluated the antitumor activity of γδ T cells modified with *de novo* scFv-based CARs targeting B7-H6, potentially applicable against multiple cancer indications for which natural tissue tropism of γδ T cells may offer advantages.

METHODS

Phage-display libraries were used to identify scFvs against B7-H6. To confirm activation upon target engagement, scFvs formatted into CARs were evaluated in a Jurkat-Lucia™ NFAT reporter cell line. Donor-derived PBMCs were used to activate, expand, and engineer Vδ1 T cells to express CARs. Vδ1 CAR T cells were assessed for phenotype and *in vitro* activity using flow cytometry and cell-based assays. Tumor xenograft models were further used to evaluate and assess B7-H6 CAR candidates for *in vivo* efficacy. Here we describe an archetypical discovery subset from a larger set of 154 hits that were generated.

Phage Display and B7-H6 scFv CAR construct design

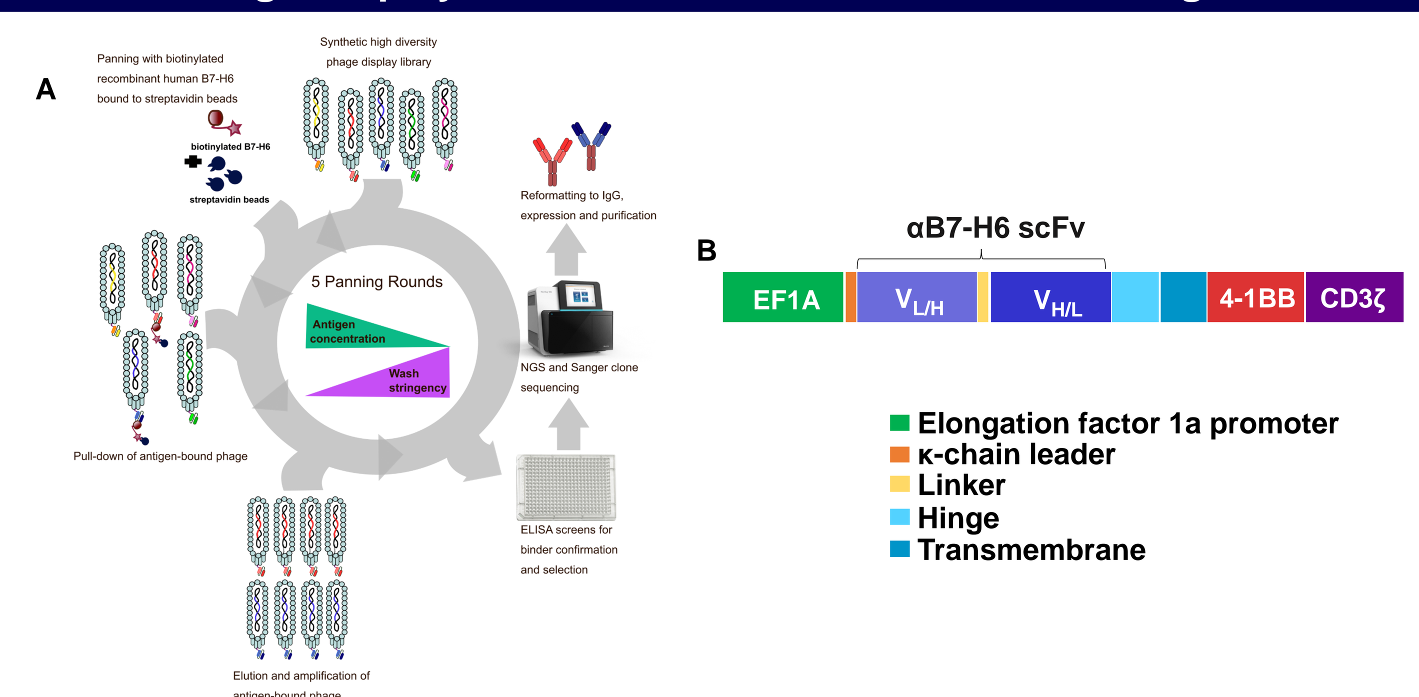

Figure 1. **(A)** Phage display panning scheme used to identify binders against B7-H6. Synthetic phage display libraries containing fully human antibody sequences were used to pan against recombinant human B7-H6 (rhuB7-H6). Selected scFvs were sequenced and reformatted into IgG antibodies (Abs) for further binding assessments. **(B)** Candidate anti-B7-H6 scFv sequences were formatted into a second-generation CAR containing 4-1BB and CD3ζ costimulatory domains.

Candidate scFvs derived from phage display panning are specific against B7-H6

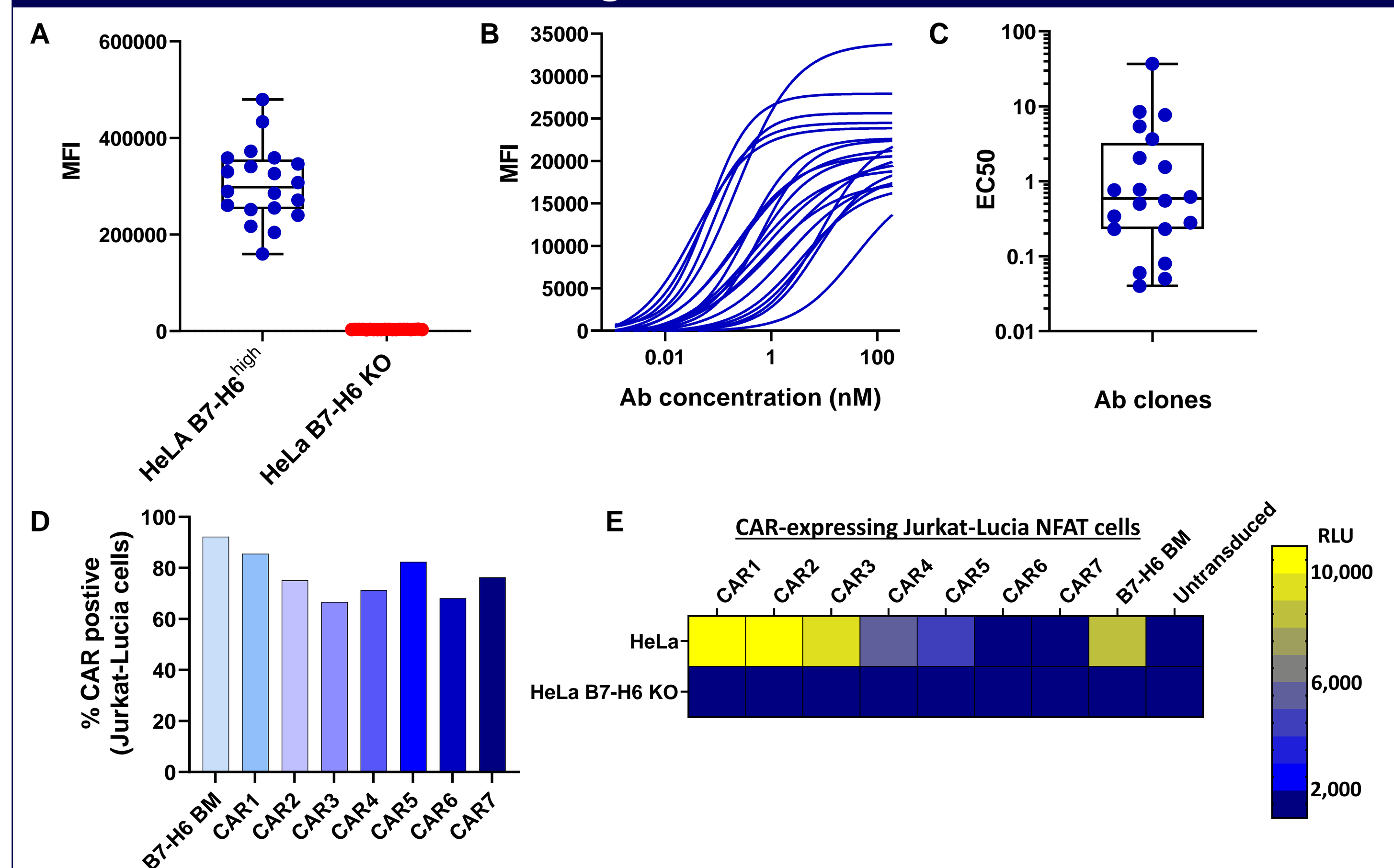

Figure 2. **(A)** Selected anti-B7-H6 scFv clones derived from phage display panning were reformatted into human IgG Abs and tested using flow cytometry against HeLa cells with B7-H6 knock-out (KO) and B7-H6 KO HeLa cells re-engineered to express B7-H6 (HeLa B7-H6^{high}). Circles represent the MFI from individual Ab clones. **(B, C)** Anti-B7-H6 IgG Ab clones were evaluated against rhuB7-H6 conjugated to MagPlex-C Microspheres. The Luminex FLEXMAP 3D® instrument was used to obtain the MFI at various Ab concentrations. EC50 values (circles) from the curves were determined for each Ab clone. **(D)** Candidate anti-B7-H6 scFvs were formatted into CARs (CAR1-7), and gammaretroviral vector encoding each CAR were transduced into Jurkat-Lucia™ NFAT cells. % CAR was detected with biotinylated rhuB7-H6 using flow cytometry. **(E)** CAR transgene activity in Jurkat-Lucia™ NFAT cells were evaluated in a co-culture assay with HeLa or HeLa B7-H6 KO cells. Supernatants were collected after 18-hrs to detect Lucia Luciferase activity. B7-H6 benchmark CAR (B7-H6 BM) represents an anti-B7-H6 scFv previously validated from the literature.

Generation of B7-H6 CAR Vδ1 T cells show robust expansion from donor PBMCs

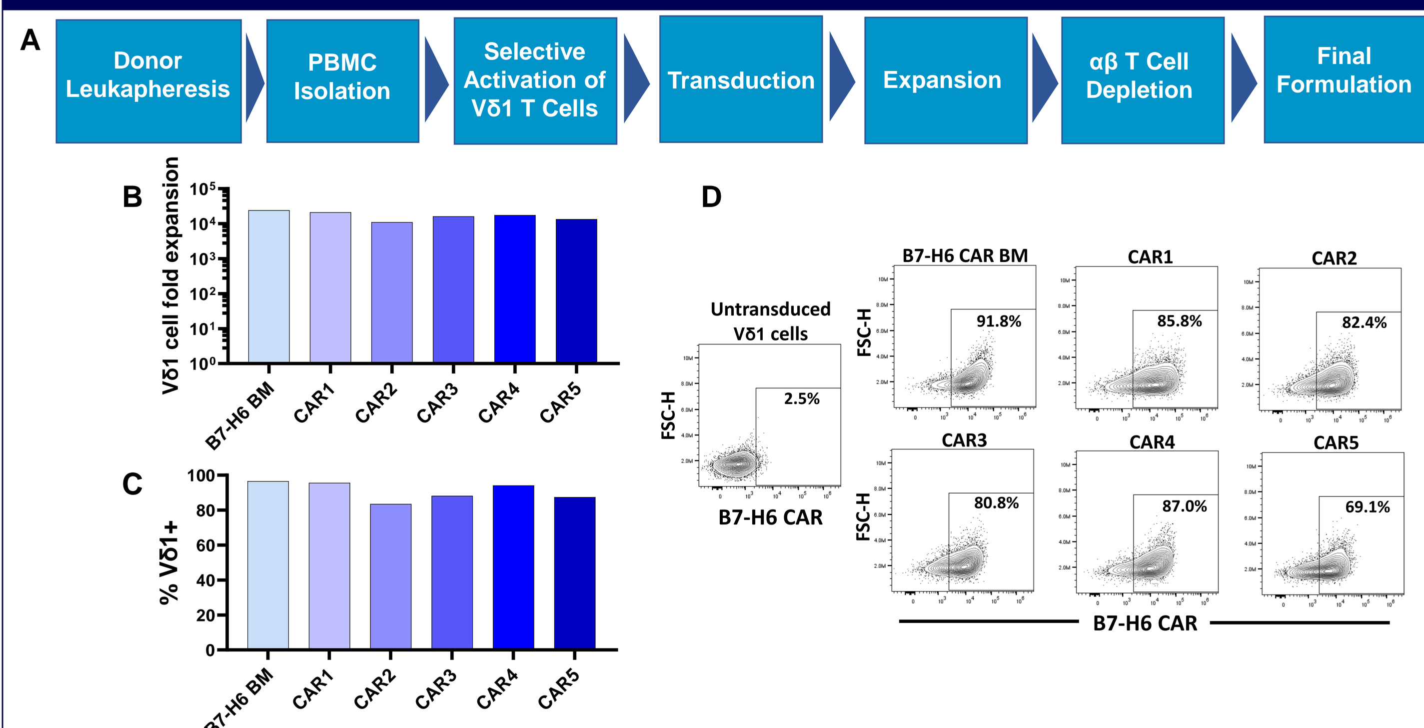

Figure 3. Selective activation and expansion of Vδ1 T cells using agonistic mAb from donor-derived PBMCs. **(A)** Flow chart highlighting the key steps in the generation of allogeneic B7-H6 CAR Vδ1 T cells. **(B)** B7-H6 CAR Vδ1 T cell generation process resulted in a substantial fold-expansion of Vδ1 T cells from the different candidate CARs (CAR1-5). **(C)** % of Vδ1 T cells expressing the B7-H6 CAR from the generation process as measured by flow cytometry. **(D)** Representative flow cytometric contour plots showing % Vδ1 T cells expressing the B7-H6 CARs.

B7-H6 CAR Vδ1 T cells exhibit potent cytotoxicity against multiple B7-H6+ tumor cell lines across a range of antigen densities

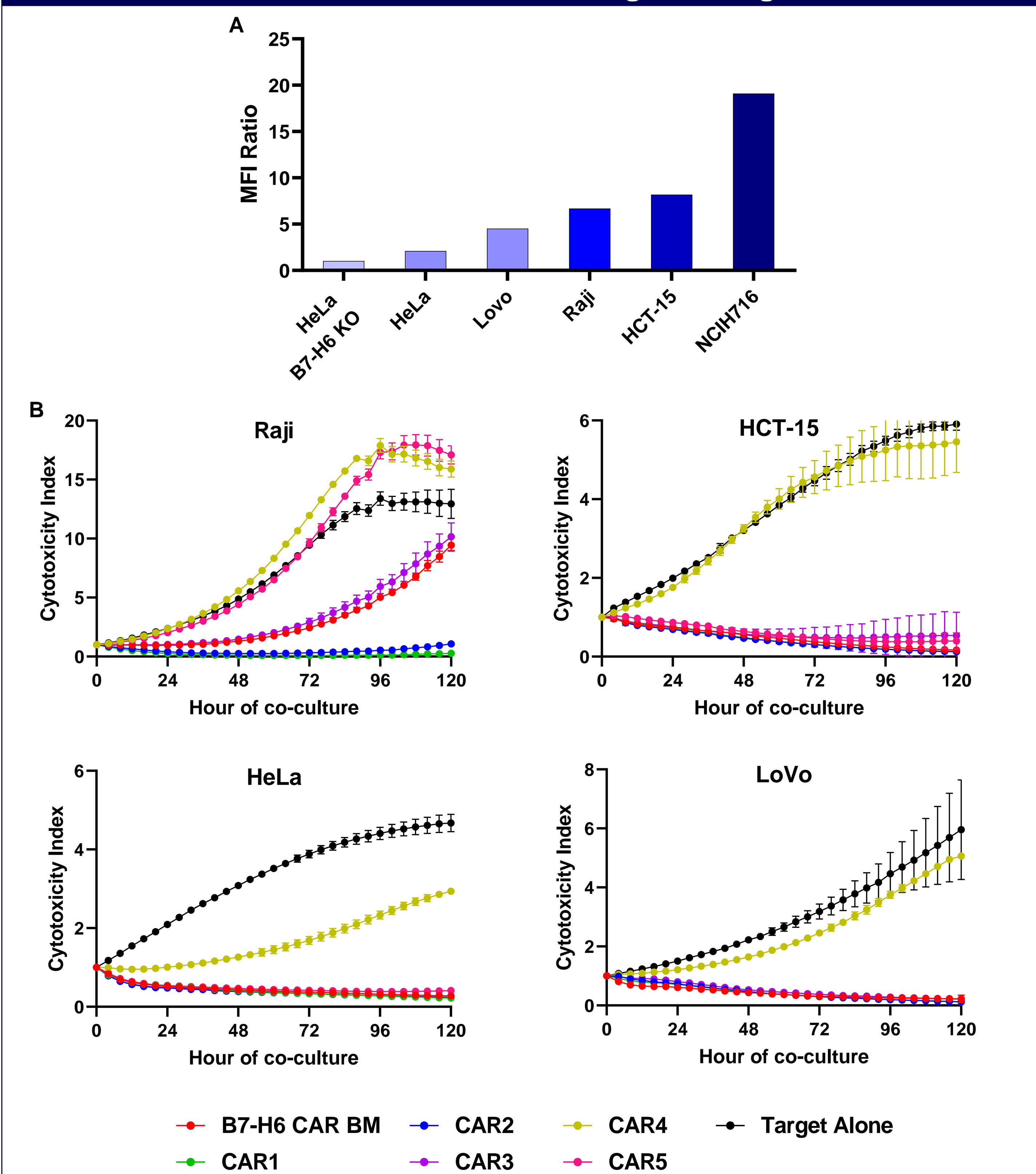

Figure 4. **(A)** Relative B7-H6 surface expression levels on tumor cell line panel as determined by flow cytometry. Relative B7-H6 expression was determined by B7-H6 geoMFI / isotype control geoMFI **(B)** Cytotoxic potentials of B7-H6 CAR Vδ1 T cells (CAR1-5) were evaluated against B7-H6+ tumor cell lines in a 120-hour Incucyte Immune Cell Killing Assay, in which T cells were co-cultured with NucNIR-expressing target cells at an E:T ratio of 5:1. The Cytotoxicity Index was calculated by dividing the total NIR object area (mm²/well) of all time points by the value at time = 0. B7-H6 benchmark (BM) CAR represents an anti-B7-H6 scFv previously validated from the literature.

B7-H6 CAR Vδ1 T cells exhibit a naive-like T cell memory phenotype, robust proliferation, and polyfunctional cytokine activity

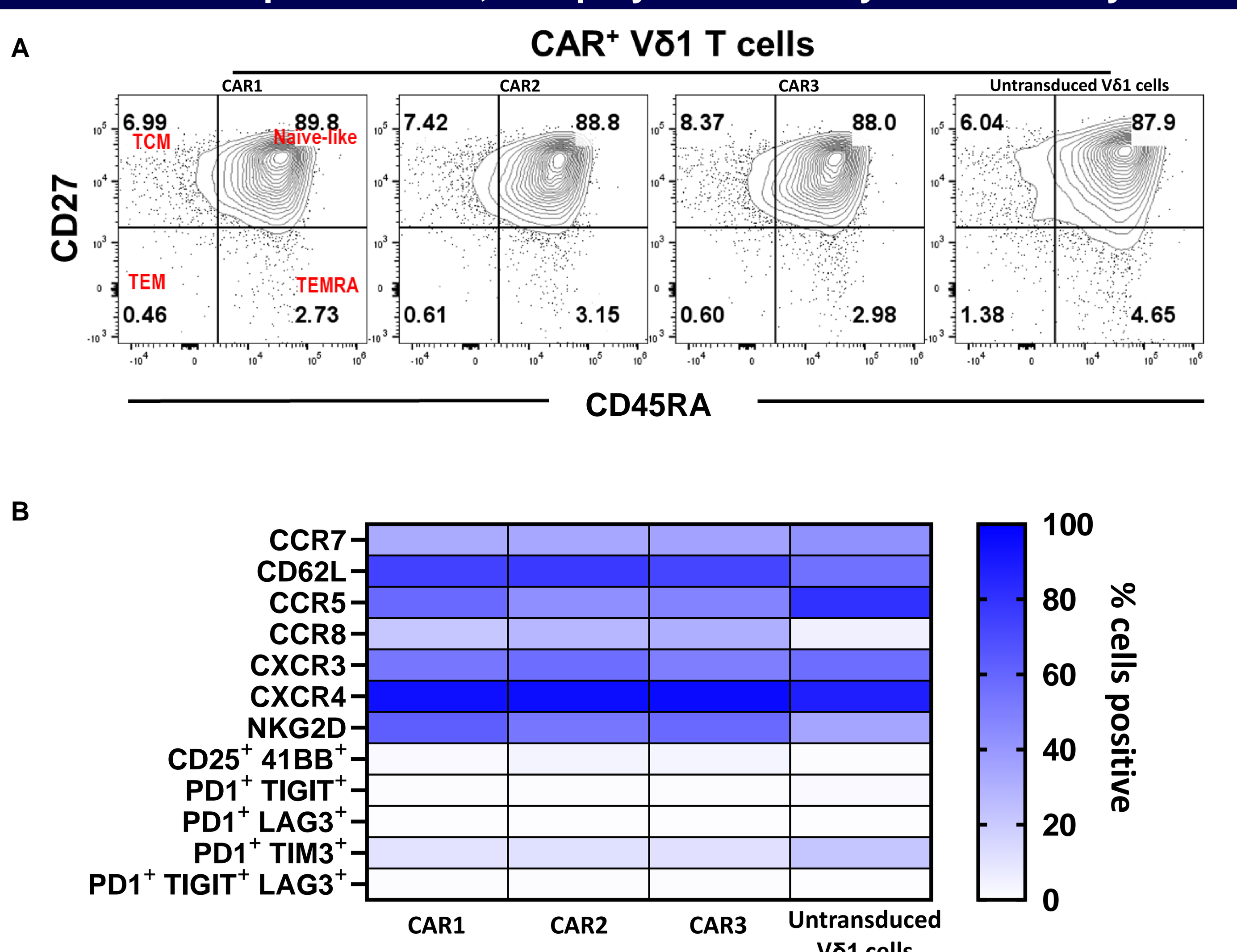

Figure 5. **(A)** Flow cytometric expression profile showing a predominant naïve-like T cell memory phenotype of the candidate B7-H6 CAR+ Vδ1 T cells (CAR1-3) **(B)** Heatmap showing percentages of B7-H6 CAR+ Vδ1 T cells that express multiple chemokine receptors, activation markers, and cells that co-express PD-1 and another co-inhibitory receptor. **(C)** Proliferative potential of B7-H6 CAR Vδ1 T cells labeled with Cell Trace Violet (CTV) following target antigen exposure with B7-H6+ cell lines in a 7-day culture period. 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). **(D)** B7-H6 CAR Vδ1 T cells were stimulated with rhuB7-H6 for 18 hrs prior to loading CAR T cells onto the IsoCode chip for single-cell multiplex cytokine analysis. Polyfunctional strength index (PSI), is defined by the percentage of polyfunctional cells with the intensity of the cytokines measured *(left)*. Percent polyfunctionality is the % of cells secreting 2 or more cytokines *(right)*. Gray bars correspond to analytes that were not significantly secreted by the sample.

B7-H6 CAR Vδ1 T cells maintain cytotoxicity against HCT-15 cells in the presence of soluble B7-H6

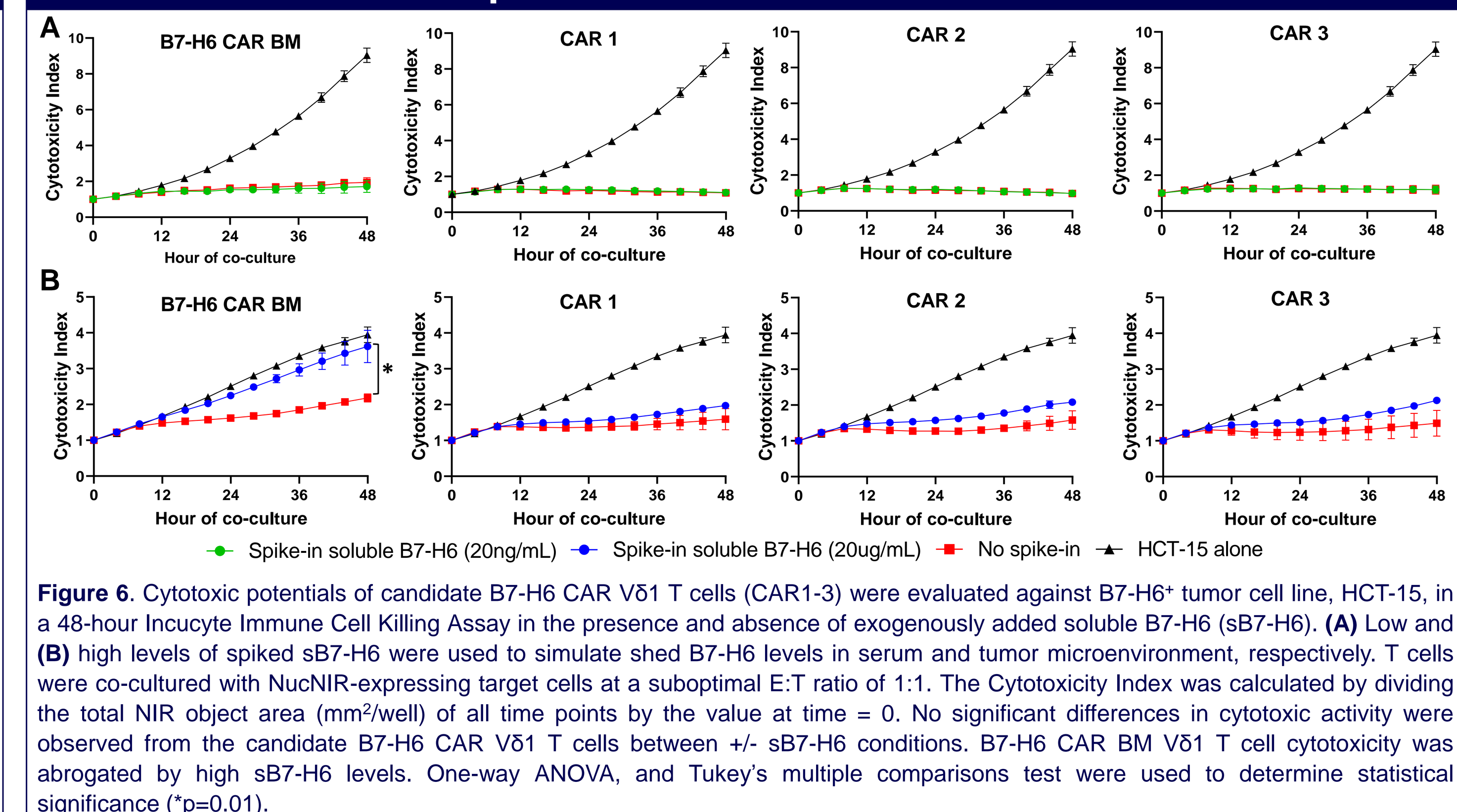

Figure 6. Cytotoxic potentials of candidate B7-H6 CAR Vδ1 T cells (CAR1-3) were evaluated against B7-H6+ tumor cell line, HCT-15, in a 48-hour Incucyte Immune Cell Killing Assay in the presence and absence of exogenously added soluble B7-H6 (sB7-H6). **(A)** Low and **(B)** high levels of spiked sB7-H6 were used to simulate shed B7-H6 levels in serum and tumor microenvironment, respectively. T cells were co-cultured with NucNIR-expressing target cells at a suboptimal E:T ratio of 1:1. The Cytotoxicity Index was calculated by dividing the total NIR object area (mm²/well) of all time points by the value at time = 0. No significant differences in cytotoxic activity were observed from the candidate B7-H6 CAR Vδ1 T cells between +/- sB7-H6 conditions. B7-H6 CAR BM Vδ1 T cell cytotoxicity was abrogated by high sB7-H6 levels. One-way ANOVA, and Tukey's multiple comparisons test were used to determine statistical significance (*p=0.01).

B7-H6 CAR Vδ1 T cells significantly inhibit *in vivo* tumor growth in HCT-15 colorectal carcinoma xenografts

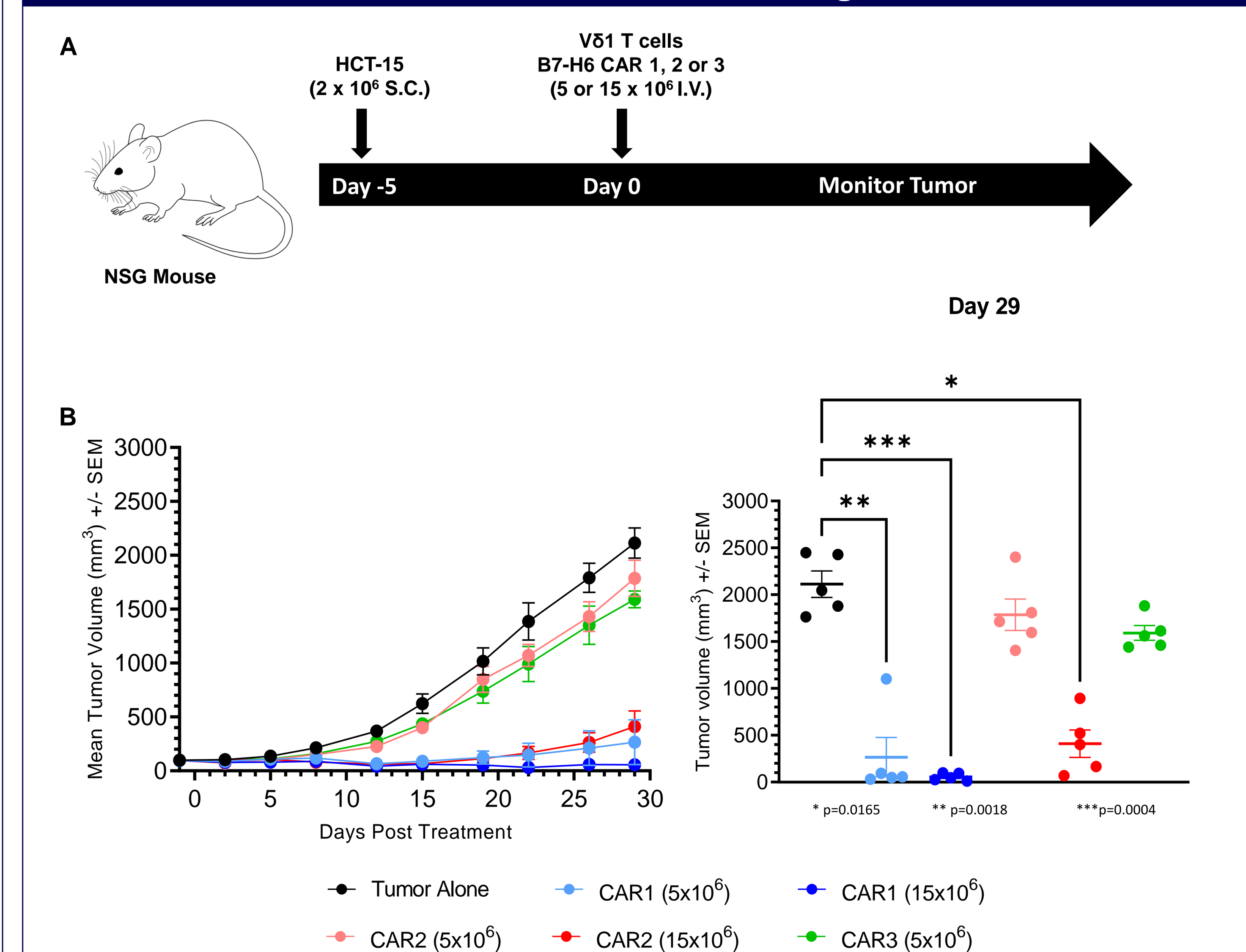

Figure 7. *In vivo* efficacy of a single dose of candidate B7-H6 CAR Vδ1 T cells (CAR1-3) in HCT-15 tumor-bearing NSG mice. **(A)** Study schematic. **(B)** Tumor growth kinetics (*left*) and tumor volumes on Day 29 (*right*). Data shown as mean ± SEM for 5 mice/group. Kruskal-Wallis with Dunn's post hoc was used to assess statistical significance among the groups at the last time point when all control mice were alive.

CONCLUSIONS

- Phage-display panning successfully yielded a diverse range of *de* novo binders specific against B7-H6.
- Selected B7-H6 scFv CARs were associated with increased NFAT activity post-activation and minimal antigen-independent tonic signaling when transduced into Jurkat-Lucia NFAT cells.
- PBMC-derived B7-H6 CAR Vδ1 T cells were successfully activated, expanded, and genetically engineered using established processes.
- Candidate B7-H6 CAR Vδ1 T cells demonstrated a predominant naïve-like phenotype with low levels of exhaustion-associated markers.
- Candidate B7-H6 CAR Vδ1 T cells inhibited *in vitro* tumor cell growth against various B7-H6+ cell lines, demonstrated robust proliferation, and polyfunctional cytokine responses.
- *In vivo* efficacy was observed against tumor xenografts in NSG mice with candidate B7-H6 CAR Vδ1 γδ T cells.
- In summary, we present the initial preclinical discovery and generation of allogeneic γδ CAR T cells targeting B7-H6 with potential applications across numerous cancer indications.