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TITLE: Interrogating the Safety and Efficacy of a Novel STEAP1 Chimeric Antigen Receptor T-Cell Therapy in Prostate Cancer

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14. ABSTRACT In this reporting period, we completed studies profiling the response of the prostate cancer tumor immune microenvironment to collagen binding domain (CBD)-IL-12 fusion cytokine therapy in conjunction with STEAP1 CAR T cell therapy. CBD-IL-12 induced a significant increase in mature antigen-presenting macrophages with M1 polarization and an expansion of type 1 conventional dendritic cells. These data indicate that CBD-IL-12 can help revert the hostile immunosuppressive tumor microenvironment to a pro-inflammatory state that enhances the activity of adoptively transferred STEAP1 CAR T cells. Further, we previously showed that CBD-IL-12 can restore a deficit in antigen processing and presentation associated with STEAP1 antigen loss arising from STEAP1 CAR T cell therapy. These findings taken together provide a strong rationale for combination therapy with CBD-IL-12 and STEAP1 CAR T cell therapy in advanced prostate cancer.					
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INTRODUCTION

Background: Chimeric antigen receptor (CAR) T cell immunotherapy combines specificity, potency, and memory and is poised to revolutionize the treatment of solid tumors including prostate cancer. However, the heterogeneous nature of target antigen expression in advanced prostate cancer suggests that no single CAR T cell therapy may be able to achieve cure. Further, CAR T cell therapies under clinical investigation for prostate cancer have not addressed the immune-suppressive tumor microenvironment that renders the disease resistant to immunotherapy or have not been investigated in immune-competent model systems prior to first-in-human trials.

The work proposed here focuses on a novel CAR T cell therapy that was developed from the ground up by our group to selectively recognize and kill prostate cancer cells that express six transmembrane epithelial antigen of the prostate 1 (STEAP1). STEAP1 is a cell surface antigen whose expression is generally tied to androgen receptor signaling and thus is found on most advanced prostate cancer. In substantive preliminary work, we have demonstrated that STEAP1 CAR T cells exhibit sensitivity, specificity, and potency in the killing of prostate cancer cells and inhibition of tumor growth. We have now generated a murinized STEAP1 CAR construct and human STEAP1 knock-in mice which will enable us to define the antitumor efficacy and off-tumor toxicity of STEAP1 CAR T cell therapy in preclinical models that recapitulate critical barriers found in human prostate cancer.

KEYWORDS

Prostate cancer, STEAP1, chimeric antigen receptor, adoptive T cell therapy, immunotherapy

ACCOMPLISHMENTS

To summarize the research accomplishments to date, the tasks described in the proposed Statement of Work are itemized here with a brief update for each task.

SA 1: Determine the safety and activity of STEAP1 CAR T cells in immune-competent hSTEAP1-KI mice (months 1-12)

Task 1: *Characterize STEAP1 expression in hSTEAP1-KI mice (months 1-4)* Completed in the prior reporting period.

Task 2: *Define the antitumor activity and off-tumor toxicity of STEAP1 CAR T cells in hSTEAP1-KI mice (months 5-12)* Completed in the prior reporting period.

SA 2: Investigate combinatorial strategies to augment STEAP1 CAR T cell activity against PC (months 1-36)

Task 1: *Evaluate the antitumor efficacy of STEAP1 CAR T cell therapy when combined with CBD-IL-12, anti-PD-1, or both (months 10-30)* In progress. To further characterize antitumor responses in human STEAP1 knock-in (hSTEAP1-KI) mice bearing disseminated syngeneic RM9 tumors, we isolated lung tumors from mice treated with untransduced T cells, STEAP1 CAR T cells, untransduced T cells + CBD-IL-12, or STEAP1 CAR T cells + CBD-IL-12 at a compassionate endpoint (relapse) based on tumor burden. For the STEAP1 CAR T cells + CBD-IL-12, we also collected lung tumors at day 10 of treatment when we anticipated maximal tumor response (nadir). This allowed us to compare the tumor immune microenvironment at nadir and at relapse. Lung tumors were viably dissociated to single cells and analyzed using a multiparametric flow cytometry panel (**Appendix, Supplementary Figure 18**) to characterize distinct immune cell subsets. We found that, after STEAP1 CAR T cells + CBD-IL-12 treatment, there was a significant increase in CD11b+Ly6C-/F4/80+MHC-II+ macrophages (**Appendix, Figure 8c, 8d, Supplementary Figure 19a**) with increased induced nitric oxide synthase (iNOS) indicative of pro-inflammatory M1 macrophages and increased CD11b+XCR1+ conventional type 1 dendritic cells (**Appendix, Supplementary Figure 19b**). We also observed a concomitant reduction in conventional type 2 dendritic cells. Another key finding was an increase in Ly6G+ tumor-

associated neutrophils with STEAP1 CAR T cell therapy which was diminished in conditions with CBD-IL-12 therapy (**Appendix, Figure 8f, 8g**). These data indicate that CBD-IL-12 can favorably remodel the tumor immune microenvironment to a pro-inflammatory state which may contribute to enhanced tumor control.

We have also evaluated the combination of CBD-IL-12 and anti-PD-1 therapy in subcutaneous syngeneic RM9 tumors established in C57Bl/6 mice (**Figure 1**) and Myc-CaP tumors established in FVB mice (**Figure 2**). While these tumors demonstrate no response to anti-PD-1 therapy alone, CBD-IL-12 induces tumor growth inhibition. The combination of CBD-IL-12 and anti-PD-1 treatment results in significant tumor regression with evidence of synergy in the RM9 model. In contrast, Myc-CaP tumors did not demonstrate an additive or synergistic response to combined CBD-IL-12 and anti-PD-1 therapy. Thus, potential benefit from combining these two agents may be tumor model-specific.

Task 2: *Perform multiplex CRISPR/Cas9 genome editing combined with single-cell DNA amplicon sequencing of indels to identify genetic interactions that suppress CAR T cell function (months 1-36) In progress.* The Paddison laboratory has generated a series of viral constructs enabling expression of STEAP1 CAR and expression of guide RNAs (gRNAs) for CRISPR/Cas9 genome engineering. These include control constructs with gRNAs targeting NR4A1, NR4A2, and NR4A3.

While the initial proposal indicated the use of multiplexed gRNAs to evaluate cooperative genetic interactions that restrain CAR T cell activity, we will no longer have access to the Mission Bio Tapestry platform upon moving our laboratory to UCLA. Therefore we will take the alternative approach of more standard CRISPR/Cas9 genetic screening using a diverse gRNA library introduced at low multiplicity-of-infection (MOI = 0.3) to maintain at least 50-100x library representation. Genome-edited STEAP1 CAR T cells will then be applied to an optimized *in vitro* serial tumor rechallenge assay (**Figure 3**) to select for CAR T cells with sustained survival/cytotoxicity over time. gRNAs will be sequenced from the initially transduced pool of STEAP1 CAR T cells and after selection in the serial tumor rechallenge assay to evaluate for statistical enrichment.

Given the success of our CBD-IL-12 studies, we believe that co-administration of pro-inflammatory cytokines with STEAP1 CAR T cells is a highly promising strategy to enhance CAR T cell activity and favorably remodel the tumor microenvironment of prostate cancer. However, IL-12 has previously been associated with toxicities in human clinical trials. In contrast, interleukin-18 (IL-18) has been found to be safe but not particularly effective. Published preclinical studies have indicated that IL-18 arming of CAR T cells enhances antitumor activity (Hu *et al*, Cell Reports 2017; Avanzi *et al*, Cell reports 2018; Jaspers *et al*, JCI 2023) and preliminary data from a phase I IL-18 armored CD19 CAR

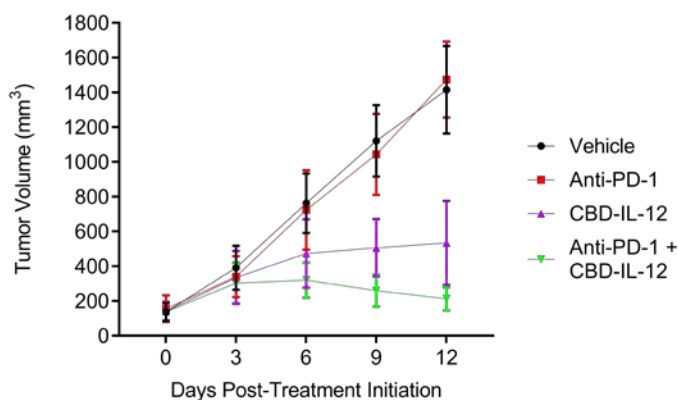


Figure 1. RM9 tumors demonstrate a synergistic response to CBD-IL-12 and anti-PD-1 therapy. Volumes over time of subcutaneous RM9 tumors established in C57Bl/6 mice after treatment with vehicle, anti-PD-1 (clone 29F.1A12) 200 ug ip every 5 days, CBD-IL-12 25 ug iv every 5 days, or both anti-PD-1 and CBD-IL-12. n > 5 mice per group.

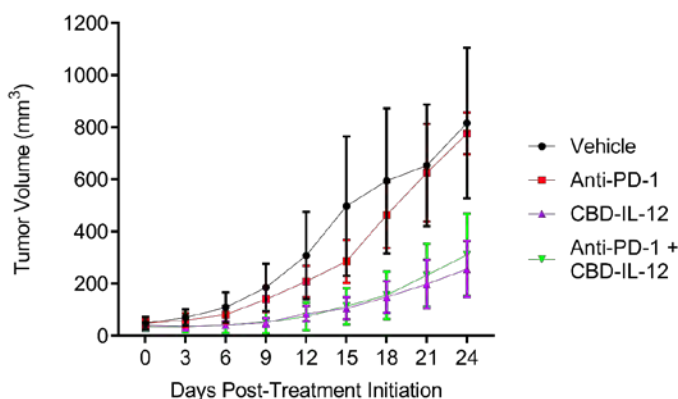


Figure 2. Myc-CaP tumors do not demonstrate an additive or synergistic response to CBD-IL-12 and anti-PD-1 therapy. Volumes over time of subcutaneous Myc-CaP tumors established in FVB mice after treatment with vehicle, anti-PD-1 (clone 29F.1A12) 200 ug ip every 5 days, CBD-IL-12 25 ug iv every 5 days, or both anti-PD-1 and CBD-IL-12. n > 5 mice per group.

T cell therapy trial for patients with relapsed/refractory non-Hodgkin's lymphoma has indicated safety and promising activity (Svoboda *et al*, Blood [Supplement 1] 2022). Thus, we developed constructs to express IL-18 or IL-18 and STEAP1 CAR from T cells. We conducted co-culture assays to evaluate antigen-specific target cell killing using the RM9 cells line across effector:target ratios (**Figure 4**). Our preliminary results indicate that arming STEAP1 CAR T cells for secretion of IL-18 significantly enhances antigen-dependent cytotoxicity of target cells. We are now preparing to evaluate IL-18 armored STEAP1 CAR T cell therapy in the disseminated RM9 tumor model established in hSTEAP1-KI mice to investigate safety and efficacy.

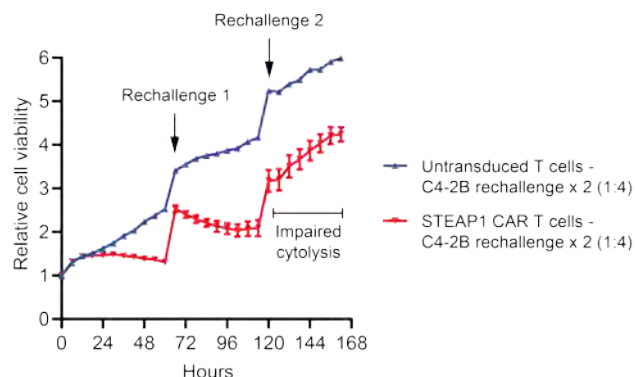


Figure 3. Serial CAR T tumor rechallenge assay. Plot showing relative cell viability of C4-2B tumor cells over time upon co-culture with untransduced or STEAP1 CAR T cells at an effector:ratio of 1:4 with rechallenge of tumor cells where indicated.

SA 3: Determine the effects of antiandrogen therapies on STEAP1 CAR T cell activity (months 1-36)

Task 1: Compare the functional characteristics of STEAP1 CAR T cell products from intact, surgically castrate, chemically castrate, or enzalutamide-treated mice (months 1-4) Not yet started. These studies will be initiated after the laboratory relocates.

Task 2: Characterize the antitumor efficacy of STEAP1 CAR T cell therapy in the context of global perturbation of androgen levels and AR signaling (months 13-21) Not yet started. These studies will be initiated after the laboratory relocates.

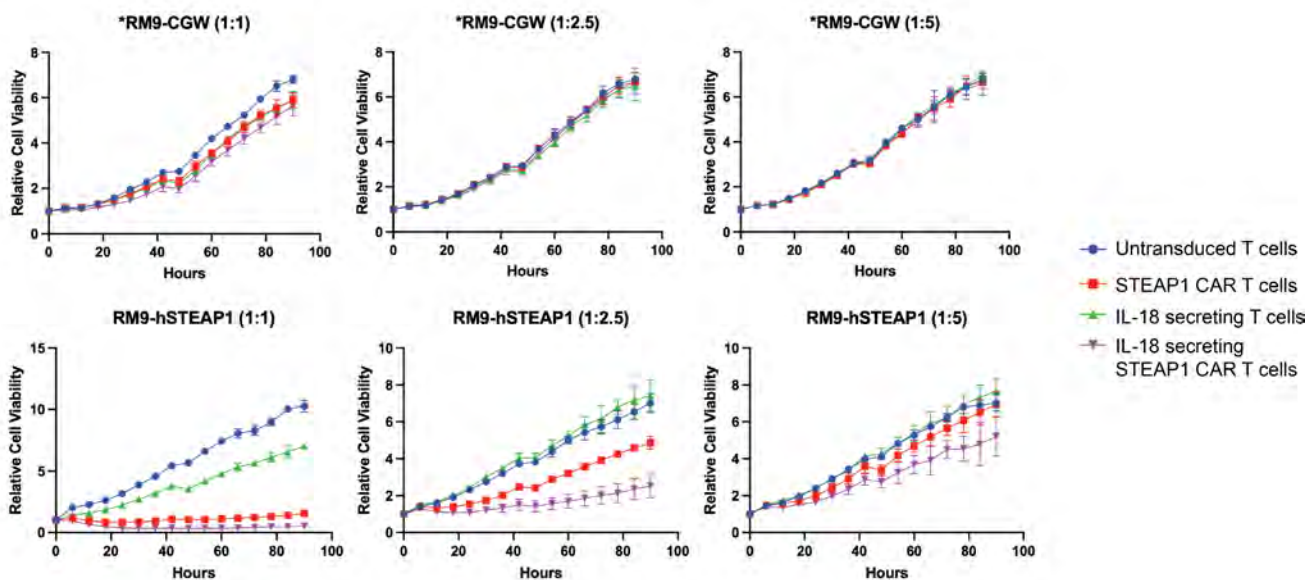


Figure 4. Armoring STEAP1 CAR T cells to secrete IL-18 enhances antigen-dependent cytotoxicity. Plots showing relative cell viability of RM9-CGW (STEAP1-negative control) or RM9-hSTEAP1 (STEAP1-positive) target cells over time upon co-culture with untransduced T cells, STEAP1 CAR T cells, IL-18 secreting T cells, or IL-18 armored STEAP1 CAR T cells across effector:target ratios of 1:1, 1:2.5, and 1:5.

Key Research Accomplishments:

- Demonstrated the enhanced antitumor efficacy of combined STEAP1 CAR T cell therapy and CBD-IL-12 therapy with characterization of changes in the tumor immune microenvironment.

- Showed that CBD-IL-12 and anti-PD-1 therapy exhibit synergistic antitumor effects in preclinical prostate cancer models.
- Preliminary evidence that IL-18 armoring of STEAP1 CAR T cells enhances antigen-specific cytotoxicity *in vitro*.
- Publication of our initial findings in the journal *Nature Communications*.

Opportunities for Training and Professional Development:

This reporting period included my fifth year as an independent investigator in the Prostate Cancer Program in the Human Biology Division at Fred Hutch. I have met regularly with my junior faculty mentoring committee consisting of Drs. Pete Nelson, Valeri Vasioukhin, and Bob Eisenman.

My professional development includes multiple areas of service to the Fred Hutchinson Cancer Center as a member of the Cancer Consortium Scientific Review Committee, the Faculty Diversity and Inclusion Committee, and the Proteomics Faculty Advisory Committee. I am also actively involved with the Prostate Cancer Foundation and the Pacific Northwest Prostate Cancer SPORE of which I am a co-investigator on Project 2.

I remain a co-investigator on the UCLA SPORE in Prostate Cancer Project 3 with Drs. Owen Witte, Stephen Forman, and Saul Priceman. Dr. Priceman and I are in communication through teleconference calls to discuss the progress of our shared research projects related to CAR T cell therapy for prostate cancer through the UCLA SPORE and Department of Defense Prostate Cancer Research Program.

I have been recruited to UCLA as an Associate Professor-in-Residence in the Division of Hematology/Oncology, Department of Medicine and the Institute for Urologic Oncology, Department of Urology at the David Geffen School of Medicine at UCLA. I started my new position on July 1, 2023.

Dissemination of Results:

The findings of our work have been published in the journal *Nature Communications* in April 2023.

Plan for the Next Reporting Period:

In the next reporting period, we will conduct further studies of IL-18 armored STEAP1 CAR T cells as well as CRISPR/Cas9 studies to evaluate for genetic interactions that may suppress STEAP1 CAR T cell function. We will also initiate studies evaluating the role of antiandrogen therapies (surgical castration or pharmacologic inhibition) in modulating STEAP1 CAR T cell activity.

IMPACT

Based on the promise of our preclinical findings, we are currently translating STEAP1 CAR T cell therapy to a phase I clinical study for men with metastatic castration-resistant prostate cancer. We have partnered with the Biopharmaceutical Development Program (BDP) at Frederick National Laboratory for Cancer Research through the NCI Experimental Therapeutics (NExT) Program. Further, the STEAP1 CAR T cell therapy has been licensed to a startup company called PromiCell Therapeutics, Inc. that has raised significant capital to support clinical development. The BDP has generated GMP vector, completed process development, and is starting GMP process development. We have received approval from the Clinical Trials Advisory Committee at the Fred Hutchinson Cancer Center Immunotherapy Integrated Research Program to proceed to a clinical trial

and have completed a full draft of the clinical protocol. Drafting for an Investigation New Drug submission is currently underway with an anticipated submission in November 2023.

In the meantime, our goal is to continue preclinical studies investigating approaches to enhance the efficacy of STEAP1 CAR T cell therapy in parallel with clinical development to allow future iterative optimization.

CHANGES/PROBLEMS

I have been recruited from Fred Hutchinson Cancer Center to UCLA. My lab will be relocating during the summer and early fall of 2023. This may consequently cause a delay in scientific progress.

PRODUCTS

Manuscript:

Bhatia V*, Kamat NV*, Pariva TE*, Wu L, Tsao A, Sasaki K, Sun H, Javier G, Nutt S, Coleman I, Hitchcock L, Zhang A, Rudoy D, Gulati R, Patel RA, Roudier MP, True LD, Srivastava S, Morrissey CM, Haffner MC, Nelson PS, Priceman SJ, Ishihara J[#], Lee JK[#]. Targeting advanced prostate cancer with STEAP1 chimeric antigen receptor T cell and tumor-localized IL-12 immunotherapy. *Nat Commun.* 2023 Apr 11;14(1):2041. doi: 10.1038/s41467-023-37874-2. PMID: 37041154

Patent:

Filed February 11, 2022
U.S. Provisional Patent Application No. 63/309,389
Chimeric Antigen Receptors Binding STEAP1
Inventors: John K. Lee and Tiffany Pariva

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	<i>John K. Lee</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-6570-2180
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Lee has performed data analysis and overseen the conduct of the study.</i>
Funding Support:	

Name:	<i>Vipul Bhatia</i>
Project Role:	<i>Post-Doctoral Research Fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>4</i>

Contribution to Project:	<i>Dr. Bhatia has been responsible for CAR T cell production, cell viability assays, animal studies, tissue processing, histology, immunohistochemistry,</i>
Funding Support:	

Name:	<i>Li-Ting Wu</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>6</i>
Contribution to Project:	<i>Ms. Li has assisted with animal studies, tissue processing, cell viability assays, and histology.</i>
Funding Support:	

Name:	<i>Patrick Paddison</i>
Project Role:	<i>Co-Investigator (Fred Hutch)</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Paddison has overseen the generation of constructs related to multiplex CRISPR/Cas9 genome editing of CAR T cells.</i>
Funding Support:	

Name:	<i>Bethany Stackhouse</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Ms. Stackhouse has been performing molecular cloning to generate and test constructs to enable the co-expression of multiple guide RNAs to facilitate multiplex CRISPR/Cas9 genome editing of CAR T cells.</i>
Funding Support:	

Name:	<i>Jun Ishihara</i>
Project Role:	<i>Subaward PD/PI (Imperial College London)</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Ishihara has overseen the production and quality control testing of CBD-IL-12.</i>
Funding Support:	

Name:	<i>Koichi Sasaki</i>
-------	----------------------

Project Role:	<i>Post-Doctoral Research Fellow (Imperial College London)</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	<i>Dr. Sasaki has affinity purified recombinant CBD-IL-12 from the transfection of mammalian cells, performed quality control, and shipped batches to the Lee laboratory for the conduct of in vivo experiments.</i>
Funding Support:	

SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

APPENDICES

Bhatia V*, Kamat NV*, Pariva TE*, Wu L, Tsao A, Sasaki K, Sun H, Javier G, Nutt S, Coleman I, Hitchcock L, Zhang A, Rudoy D, Gulati R, Patel RA, Roudier MP, True LD, Srivastava S, Morrissey CM, Haffner MC, Nelson PS, Priceman SJ, Ishihara J[#], Lee JK[#]. Targeting advanced prostate cancer with STEAP1 chimeric antigen receptor T cell and tumor-localized IL-12 immunotherapy. *Nat Commun.* 2023 Apr 11;14(1):2041. doi: 10.1038/s41467-023-37874-2. PMID: 37041154

Targeting advanced prostate cancer with STEAP1 chimeric antigen receptor T cell and tumor-localized IL-12 immunotherapy

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 Check for updates

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Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is a cell surface antigen for therapeutic targeting in prostate cancer. Here, we report broad expression of STEAP1 relative to prostate-specific membrane antigen (PSMA) in lethal metastatic prostate cancers and the development of a STEAP1-directed chimeric antigen receptor (CAR) T cell therapy. STEAP1 CAR T cells demonstrate reactivity in low antigen density, antitumor activity across metastatic prostate cancer models, and safety in a human STEAP1 knock-in mouse model. STEAP1 antigen escape is a recurrent mechanism of treatment resistance and is associated with diminished tumor antigen processing and presentation. The application of tumor-localized interleukin-12 (IL-12) therapy in the form of a collagen binding domain (CBD)-IL-12 fusion protein combined with STEAP1 CAR T cell therapy enhances antitumor efficacy by remodeling the immunologically cold tumor microenvironment of prostate cancer and combating STEAP1 antigen escape through the engagement of host immunity and epitope spreading.

Metastatic prostate cancer represents an incurable disease responsible for over 33,000 deaths per year in the United States¹. Prostate cancer is critically reliant on androgen receptor (AR) signaling and thus the suppression of gonadal androgen production through surgical or chemical castration (androgen deprivation therapy) has been a mainstay of treatment for advanced disease. However, metastatic prostate cancer inevitably develops resistance to androgen deprivation therapy

and enters a stage called metastatic castration-resistant prostate cancer (mCRPC). mCRPC is currently incurable and is considered the end-stage of the disease and is associated with a median overall survival of three years². In the past decade, multiple therapies including an inhibitor of extragonadal androgen synthesis (abiraterone acetate)³, second-generation AR antagonists (enzalutamide)⁴, radioactive isotope (radium-223)⁵, and a prostate-specific membrane antigen

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(PSMA)-specific radioligand therapy (lutetium Lu 177 vipivotide tetraxetan)⁶ have been approved for mCRPC. Each of these agents extends survival on average by several months but long-term remissions are rare.

Strategies to reprogram the immune system to combat prostate cancer first gained traction with the clinical approval of the dendritic cell vaccine sipuleucel-T for asymptomatic mCRPC⁷. More recently, several types of immunotherapies including immune checkpoint inhibitors, a DNA cancer vaccine, antibody-drug conjugates (ADC), T cell engaging bispecific antibodies (T-BsAb), and chimeric antigen receptor (CAR) T cell therapies have been under active clinical investigation^{8,9}. CARs are synthetic receptors that leverage the potency, expansion, and memory of T cells and can be engineered against virtually any tumor-associated cell surface antigen. The adoptive transfer of CAR T cells has rapidly become an established treatment for hematologic malignancies with exceptional response rates leading to six clinical approvals in the last five years¹⁰. In contrast, CAR T cell therapies targeting solid tumors have lagged due to additional challenges related to the lack of bona fide tumor-specific antigens, inhospitable tumor microenvironments, and poor trafficking, persistence, and expansion of CAR T cells¹¹.

Despite the challenges observed in driving effective immune responses toward solid tumors, recent early phase clinical trials investigating CAR T cell therapies targeting PSMA in mCRPC have reported safety and evidence of significant biochemical and radiographic responses^{12,13}. These preliminary results serve to embolden efforts to develop and optimize new CAR T cell therapies for prostate cancer. While PSMA is the preeminent target for therapeutic and diagnostic development in prostate cancer, recent work indicates that PSMA expression may be quite heterogeneous in mCRPC¹⁴. Tumor antigen heterogeneity, especially in the context of single antigen-targeted CAR T cell therapies for solid tumors like prostate cancer, is an important barrier to therapeutic efficacy¹⁵. Thus, identifying cell surface antigens with broad and relatively homogeneous expression in prostate cancer is imperative. In addition, very few if any tumor-associated antigens demonstrate tumor-restricted expression—most also exhibit low level expression in normal tissues that could represent liabilities for CAR T cell therapies due to on-target off-tumor toxicities which can lead to devastating consequences including death¹⁶.

We previously performed integrated transcriptomic and cell surface proteomic profiling of human prostate adenocarcinoma cell lines and identified six transmembrane epithelial antigen of the prostate 1 (STEAP1) as one of the most highly enriched cell surface antigens¹⁷. STEAP1 was first described over two decades ago¹⁸ and was recognized as being highly expressed in prostate cancer. STEAP1 is strongly expressed in >80% of mCRPC with bone or lymph node involvement¹⁹, 62% of Ewing sarcoma²⁰, and multiple other cancer types²¹. STEAP1 belongs to the STEAP family of metalloendopeptidases that can form homotrimers or heterotrimers with other STEAP proteins²². STEAP1 has an established functional role in promoting cancer cell proliferation, invasion, and epithelial-to-mesenchymal transition^{18,23–26}. Furthermore, STEAP1 demonstrates limited expression in normal tissue²⁷ which makes it a highly compelling target for cancer therapy.

Multiple immunotherapeutic agents have been developed to target STEAP1 yet none are clinically approved. The ADC vandortuzumab vedotin (DSTP3086S) consisting of a humanized anti-STEAP1 IgG1 antibody linked to monomethyl auristatin E was found to have an acceptable safety profile in a phase I clinical trial in mCRPC but few objective tumor responses were observed²⁸. A T-BsAb incorporating two anti-STEAP1 fragment-antigen binding (Fab) domains, an anti-CD3 single chain variable fragment (scFv), and a fragment crystallizable (Fc) domain engineered to lack effector function called AMG 509 is currently being evaluated in a phase I clinical trial (NCT04221542) in mCRPC²⁹. A symmetric dual bivalent T-BsAb called BC261 was also recently reported to demonstrate potent antitumor activity across

multiple preclinical models of prostate cancer and Ewing sarcoma³⁰. In addition, a human leukocyte antigen (HLA) class I-restricted T cell receptor (TCR) specific for a STEAP1 peptide has been shown to inhibit local and metastatic Ewing sarcoma growth in a preclinical xenograft model after adoptive transfer of transgenic T cells³¹.

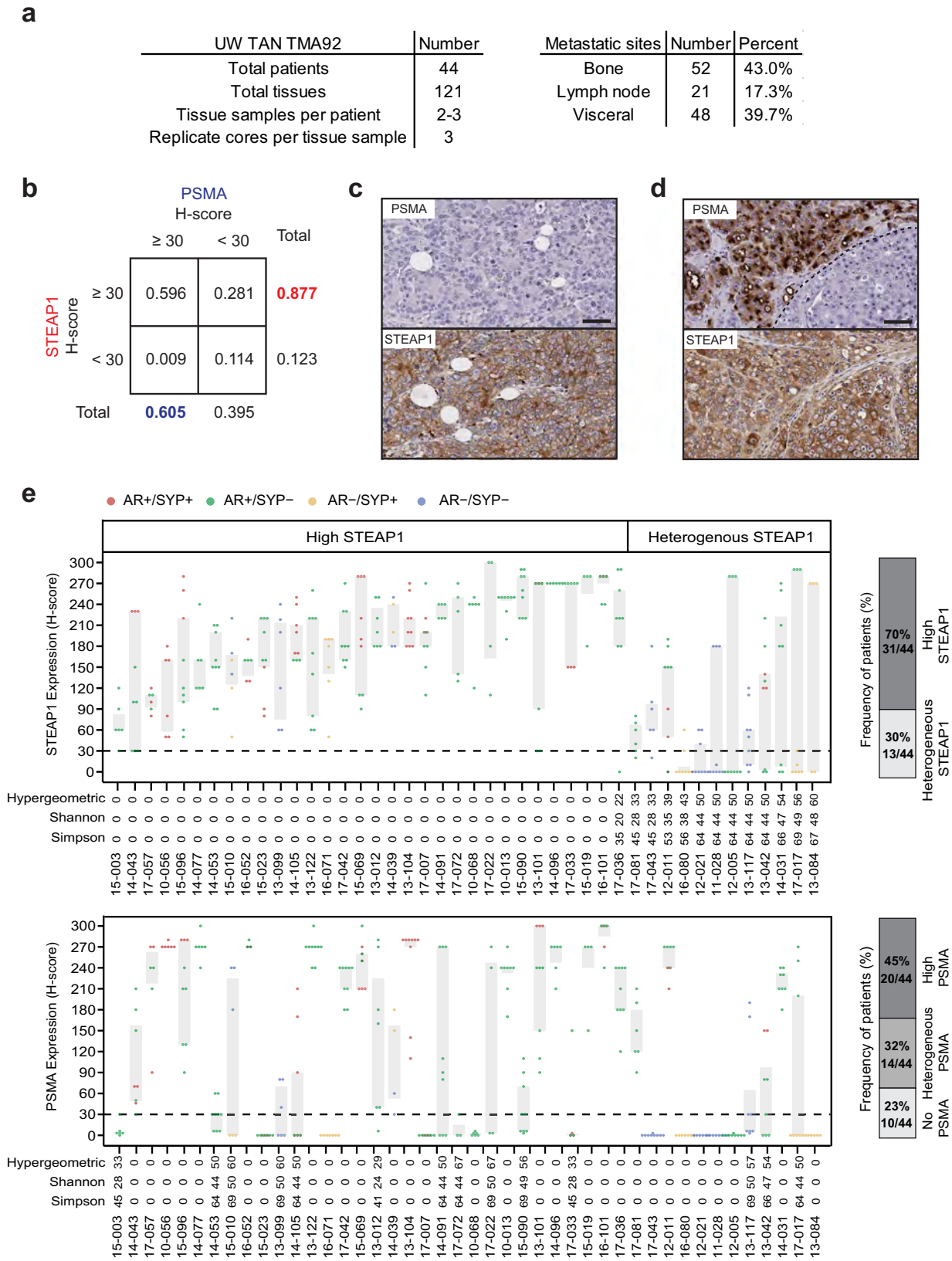
In this study, we perform comparative analysis of the relative expression of STEAP1 and PSMA in lethal mCRPC to investigate the utility of targeting STEAP1 in the current era of PSMA theranostics. We engineer and screen second-generation STEAP1 CARs for antigen-specific T cell activation and target cell cytotoxicity to yield a lead candidate for further characterization. We determine the functional epitope specificity of STEAP1 CAR T cells and profile the expansion and immunophenotype of STEAP1 CAR T cell products from multiple donors. We then establish the potency and preliminary safety of STEAP1 CAR T cell therapy in relevant preclinical models of prostate cancer but observe recurrent loss of STEAP1 antigen expression as a mechanism of treatment resistance. To overcome this issue, we evaluate the concomitant administration of CBD-IL-12 which remodels the immunosuppressive tumor microenvironment of prostate cancer and engages endogenous immunity to broaden antitumor responses. Collectively, these studies provide strong rationale for the clinical translation of STEAP1 CAR T cell therapy to men with mCRPC and guide strategies to overcome potential mechanisms of therapeutic resistance.

Results

STEAP1 is broadly expressed in treatment-refractory mCRPC tissues

We first set out to determine the pattern and extent of STEAP1 expression relative to PSMA in advanced metastatic prostate cancer. We performed immunohistochemical (IHC) staining on a duplicate set of tissue microarrays consisting of 121 metastatic tumors (each with up to three cores represented) collected from 44 men with lethal mCRPC patients collected by rapid autopsy between the years 2010 and 2017 through the University of Washington Tumor Acquisition Necropsy Program³² (Fig. 1a). Plasma membrane staining for STEAP1 and PSMA in each tissue was scored by a research pathologist and semiquantitative H-scores were determined based on the staining intensity (Supplementary Fig. 1a) multiplied by the percentage of cancer cells staining at each intensity (Supplementary Fig. 1b). By implementing a minimal staining threshold with an H-score cut-off of 30, we found that 87.7% of evaluable matched mCRPC tissues (100 of 114) demonstrated staining for STEAP1 compared to only 60.5% (69 of 114) for PSMA (Fig. 1b). In addition, 28.1% of mCRPC tissues (32 of 114) showed STEAP1 but not PSMA staining (Fig. 1b, c) whereas only 0.9% (one of 114) exhibited PSMA but not STEAP1 staining. Based on these results, we used a linear mixed statistical model to determine that the odds of non-zero (H-score >0) staining was 22-fold (95% CI 6–173) higher for STEAP1 than for PSMA and the odds of an H-score ≥30 are 84-fold (95% CI 30–317) higher for STEAP1 than for PSMA. The mean STEAP1 H-score in bone (193; 95% CI 171 to 215) was significantly higher than in lymph node metastases (difference −48; 95% CI −21 to −76; $p < 0.001$) and significantly higher than in visceral metastases (difference −59; 95% CI −42 to −77; $p < 0.001$). There was no significant difference between the mean STEAP1 H-score in lymph node metastasis compared to visceral metastases (difference 11; 95% CI −16 to 39; $p = 0.4$) (Supplementary Fig. 1c). We also observed several cases with heterogeneous expression of PSMA within cores (Fig. 1d) which is consistent with a recent report of intratumoral PSMA heterogeneity in mCRPC biopsies¹⁴.

Patient-level analysis using a mean H-score threshold of ≥30 and McNemar's test revealed that 95% of evaluable patients (42 of 44) had tumors with STEAP1 expression while 68% (30 of 44) were positive for PSMA (Supplementary Fig. 1d). To study the patterns of inter-patient and intra-patient heterogeneity associated with STEAP1 and PSMA expression, we used STEAP1 and PSMA H-scores to evaluate the hypergeometric, Simpson, and Shannon diversity scores. We observed



two patterns of STEAP1 expression (Fig. 1e) with 68% (30/44) of patients showing STEAP1 expression across all metastatic sites (high STEAP1) and 32% (14/44) patients showing all metastatic sites with and without STEAP1 expression (heterogenous STEAP1). No patients were identified in which all metastatic tissues lacked STEAP1 expression. A similar analysis for PSMA expression in the same cohort revealed 45%

(20/44) patients with high PSMA expression, 32% (14/44) with heterogeneous PSMA expression, and 23% (10/44) with no PSMA expression. Based on molecular subclassification of mCRPC tissues using AR and the neuroendocrine marker synaptophysin (SYP) expression assessed by IHC, most patients with high or heterogeneous STEAP1 and PSMA expression had AR-positive prostate cancer (AR+/SYP- or

Fig. 1 | Comparative analysis of STEAP1 and PSMA in lethal, metastatic castration-resistant prostate cancer (mCRPC). **a** Characteristics of the mCRPC tissues represented on University of Washington Tissue Acquisition Necropsy Tissue Microarray 92 (UW TAN TMA92). **b** Contingency table showing the frequency of mCRPC tissues with STEAP1 or PSMA IHC staining above or below an H-score threshold of 30. Micrographs of select mCRPC tissues after STEAP1 and PSMA IHC staining to highlight the **(c)** absence of PSMA but presence of STEAP1 expression and **(d)** intratumoral heterogeneity of PSMA expression but not STEAP1. Scale bars = 50 μ m. For panels **(c, d)** $n = 332$ mCRPC cores were immunostained for STEAP1 and PSMA. **e** Dot and box plot showing the distribution of STEAP1 (top) and

PSMA (bottom) H-scores in 44 patients from the UW TAN TMA92 cohort. Each dot represents a tumor specimen/core ($n = 319$ cores for PSMA and 333 cores for STEAP1) and the color indicates the molecular subtype: AR+/SYP+ (red), AR+/SYP- (green), AR-/SYP+ (yellow) and AR-/SYP- (purple). Gray rectangles show inter-quartile ranges spanning the 25th to the 75th percentiles of PSMA H-scores from each patient. Bar plots (on the right) summarize the frequencies of patients classified based on STEAP1 and PSMA expression as no expression (all cores with H-score ≤ 30 , light grey), heterogeneous expression (at least one core with H-score ≤ 30 and H-score > 30 , mid grey) and high expression (all cores with H-scores > 30 , dark grey). Source data are provided in the Source Data file.

AR+/SYP+) while those with no PSMA expression had AR-null prostate cancer (AR-/SYP+ or AR-/SYP-). We identified a positive correlation between the expression of STEAP1 and AR ($p < 0.001$) by a fitted linear mixed model with random effect in cases represented on the tissue microarray (Supplementary Fig. 2a, b) which was expected given that *STEAP1* is an androgen-regulated gene^{33,34}. In contrast, a negative trend was appreciated between the expression of STEAP1 and SYP (Supplementary Fig. 2c). These findings suggest that, like PSMA³⁵, STEAP1 expression may be lost with neuroendocrine transdifferentiation of prostate cancer.

Development of a potent, antigen-specific STEAP1 CAR

Given the widespread expression of STEAP1 in late-stage mCRPC and its reported functional role in cancer progression^{27,36,37}, we next started to engineer a lentiviral STEAP1-specific second-generation CAR. We used the pCCL-c-MNDU3-X lentiviral backbone³⁸ which has been widely used for hematopoietic stem cell gene therapy³⁹ and CAR expression in T cells driven by the internal MNDU3 promoter has been shown to be higher than that achieved with an EFS promoter⁴⁰. A 4-1BB costimulatory domain was favored due to its association with T cell memory formation and prolonged persistence⁴¹ and a CD28 transmembrane domain was introduced as this has been shown to reduce the antigen threshold for second-generation 4-1BB CAR T cell activation⁴². We incorporated the fully humanized scFv derived from vandortuzumab vedotin, an ADC targeting STEAP1 whose development was discontinued after a phase I clinical trial²⁸. This scFv is a humanized variant of the murine monoclonal antibody (mAb 120.545) originally developed by Agensys, Inc. that demonstrates 1 nM affinity in cell-based binding assays⁴³. To potentially tune CAR activity, we implemented three different hinge/spacer lengths including short (IgG4 hinge), medium (IgG4 hinge-CH3), and long (IgG4 hinge-CH2-CH3). The long spacer was engineered with previously described 4/2-NQ mutations⁴⁴ in the CH2 domain to prevent Fc-gamma receptor binding and activation-induced cell death that occurs with the adoptive transfer of long spacer CAR T cells into immunodeficient mice. The three candidate CARs were cloned into the lentiviral vector (Fig. 2a) that also co-expresses truncated epidermal growth factor receptor (EGFRt) as a transduction marker. Lentiviruses were generated and used to transduce human CD4 and CD8 T cells enriched from human donor peripheral blood mononuclear cells (PBMCs) collected from pheresis. Expanded CD4 and CD8 CAR T cells were immunophenotyped (Supplementary Fig. 3a) and reconstituted into cell products of a defined composition with a normal CD4/CD8 ratio to evaluate their functional activities.

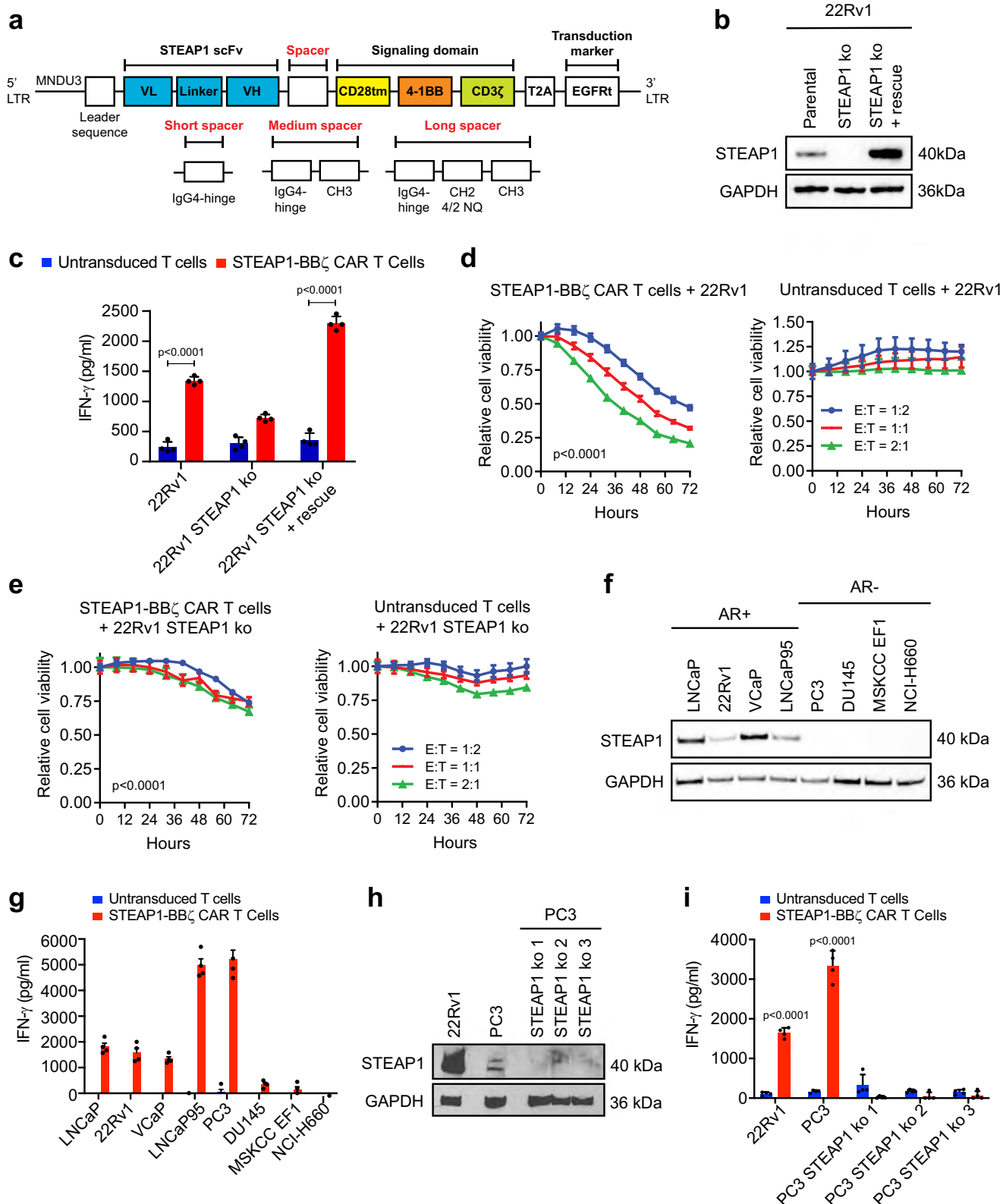
To control for STEAP1 expression in an isogenic manner, we focused on the 22Rv1 human prostate cancer cell line that demonstrates native STEAP1 expression and performed STEAP1 knockout (ko) by CRISPR/Cas9 genome editing. We then generated a STEAP1 rescue line from the 22Rv1 STEAP1 ko by transduction with a STEAP1 expressing lentivirus (Fig. 2b). These lines were then used to screen the three short, medium, and long spacer STEAP1 CAR T cells in co-culture assays with a readout of interferon-gamma (IFN- γ) release as an indicator of T cell activation. Only the long spacer STEAP1 CAR T cells (hereafter called STEAP1-BB ζ CAR T cells) demonstrated the

anticipated antigen-specific pattern of IFN- γ release, while the short and medium spacer STEAP1 CAR T cells did not (Fig. 2c, Supplementary Fig. 3b, c). Further, STEAP1-BB ζ CAR T cells showed substantial dose-dependent cytotoxicity of 22Rv1 cells compared to untransduced T cells (Fig. 2d) and demonstrated relative sparing of 22Rv1 STEAP1 ko cells (Fig. 2e). Similar studies were then performed in the DU145 human prostate cancer cell line that lacks native STEAP1 expression but was engineered to express STEAP1 (DU145 STEAP1) by lentiviral transduction (Supplementary Fig. 4a). In this setting, STEAP1-BB ζ CAR T cell activation was only observed in co-cultures with DU145 STEAP1 cells and not the parental DU145 cells (Supplementary Fig. 4b). Cytolytic activity was only appreciated with STEAP1-BB ζ CAR T cells and not untransduced T cells in co-cultures with DU145 STEAP1 cells (Supplementary Fig. 4c).

We subsequently analyzed a larger panel of human prostate cancer cell lines to characterize their native STEAP1 expression by immunoblot analysis. The cell lines with known AR expression/activity (LNCaP, 22Rv1, VCaP, and LNCaP95) showed varying levels of STEAP1 expression while the AR-null cell lines (PC3, DU145, MSKCC EF1, and NCI-H660) did not appear to express detectable levels of STEAP1 (Fig. 2f). We proceeded to perform co-cultures of STEAP1-BB ζ CAR T with these lines to further validate their antigen-specific activation based on IFN- γ release (Fig. 2g). However, we observed a discordant finding in that the PC3 line, which showed no apparent STEAP1 protein expression (Fig. 2f), induced substantial activation of STEAP1-BB ζ CAR T cells. Prior literature suggested that STEAP1 is expressed in the PC3 cell line at low levels⁴⁵. Indeed, prolonged immunoblot exposure revealed a band suggesting the presence of very low expression of STEAP1 (Fig. 2h). To confirm whether the STEAP1-BB ζ CAR T cell activation was due to this minor STEAP1 expression in PC3 cells, we generated three PC3 STEAP1 ko sublines (Fig. 2h) and again performed co-cultures with STEAP1-BB ζ CAR T cells. STEAP1 ko in the PC3 line led to the abrogation of STEAP1-BB ζ CAR T cell activation (Fig. 2i), further validating specificity and providing evidence of the sensitivity of STEAP1-BB ζ CAR T cells to low antigen density conditions.

Lack of cross-reactivity of STEAP1-BB ζ CAR with mouse Steap1 and human STEAP1B

Consistent with the anti-human specificity of vandortuzumab vedotin, STEAP1-BB ζ CAR T cells did not demonstrate cross reactivity with mouse Steap1 (Supplementary Fig. 4a, d, e). However, we used this as an opportunity to individually reconstitute the three human STEAP1 extracellular domains (ECDs) onto mouse Steap1 (Supplementary Fig. 4f) to determine which ECDs are critical for epitope recognition by STEAP1-BB ζ CAR T cells. Co-culture experiments were performed with STEAP1-BB ζ CAR T cells and DU145 cells engineered to express mouse Steap1 with individual replacement of mouse ECDs with human ECDs. We found that human STEAP1 ECD2 but not ECD1 or ECD3 was associated with STEAP1-BB ζ CAR T cell activation (Supplementary Fig. 4g). Interestingly, the human STEAP1 and mouse Steap1 ECD2 demonstrate 93.9% (31/33 amino acids) homology (Supplementary Fig. 4h), indicating that Q198 and/or I209 of human STEAP1 are critical to productive recognition by STEAP1-BB ζ CAR T cells. Q198 has been shown



to interact with the Fab of 120.545 as part of an interaction hotspot based on a recent structure resolved by cryogenic electron microscopy²².

Of the human STEAP family of proteins, STEAP1B has the greatest homology to STEAP1⁴⁵. Three STEAP1B transcripts have been identified, of which all demonstrate complete conservation of the amino acid sequence of human STEAP1 ECD2 (Supplementary Fig. 5a). The

consensus membrane topology prediction algorithm TOPCONS⁴⁶ predicted ECD2 domain sequences as being extracellular in the three STEAP1B protein isoforms (Supplementary Fig. 5b) albeit with low reliability scores for STEAP1B as compared to hSTEAP1 due to a lack of consensus between the five topology prediction models (OCTOPUS, Philius, PolyPhobius, SCAMPI, and SPOCTOPUS) used by TOPCONS (Supplementary Fig. 5c). Prior analysis using another in silico hidden

Fig. 2 | Screening second-generation 4-1BB chimeric antigen receptors (CARs) to identify a lead for STEAPI CAR T cell therapy. **a** Schematic of the lentiviral STEAPI CAR construct and variation based on short, medium, and long spacers. LTR long terminal repeat, MNDU3 Moloney murine leukemia virus U3 region, scFv single-chain variable fragment, VL variable light chain, VH variable heavy chain, tm transmembrane, EGFRt truncated epidermal growth factor receptor, 4/2 NQ = CH2 domain mutations to prevent binding to Fc-gamma receptors. **b** Immunoblots of STEAPI in 22Rv1 parental cells, STEAPI knockout (ko) cells, and STEAPI ko cells with rescue of STEAPI. **c** IFN- γ enzyme-linked immunosorbent assay (ELISA) results from co-cultures of either untransduced T cells or STEAPI-BB ζ CAR T cells with each of the 22Rv1 sublines at a 1:1 ratio at 24 h ($p < 0.001$). Relative cell viability of **(d)** 22Rv1 and **(e)** 22Rv1 STEAPI ko target cells over time measured by fluorescence live cell imaging upon co-culture with (left) STEAPI-BB ζ CAR T cells ($p < 0.001$) or (right) untransduced T cells at variable effector-to-target (E:T) cell ratios. **f** Immunoblots

demonstrating expression of STEAPI in androgen receptor (AR)-positive human prostate cancer cell lines but not AR-negative prostate cancer cell lines. **g** IFN- γ quantification by ELISA from co-cultures of either untransduced T cells or STEAPI-BB ζ CAR T cells with each of the human prostate cancer cell lines in **(f)** at a 1:1 ratio at 24 h. **h** Immunoblots for STEAPI in 22Rv1, PC3, and PC3 STEAPI ko sublines. **i** IFN- γ quantification by ELISA from co-cultures of either untransduced T cells or STEAPI-BB ζ CAR T cells with each cell line in **(h)** at a 1:1 ratio at 24 h ($p < 0.001$). For panels **(c–e, g and i)** $n = 4$ biological replicates per conditions were used and error bars represent mean with SEM. Panel **(b, f, h)** displays results representative of $n = 3$ biological replicates. GAPDH was used as a protein loading control. For panel **(c)** and **(i)**, two-way ANOVA with Sidak's multiple comparison test was used. For panels **(d)** and **(e)**, two-way ANOVA with Tukey's multiple comparisons test was used. Source data are provided in the Source Data file.

Markov model-based topology prediction tool TMHMM⁴⁷ had also suggested that this sequence could be intracellular rather than extracellular in STEAPIB protein isoforms 1 and 2⁴⁵. However, the crystal structure of STEAPIB has not yet been determined to directly substantiate these predictions. To functionally evaluate whether STEAPI-BB ζ CAR T cells might also be reactive against STEAPIB, we performed co-cultures using DU145 lines engineered to express each of the three isoforms of STEAPIB. We did not identify evidence of STEAPI-BB ζ CAR T cell activation (Supplementary Fig. 5d), suggesting that the STEAPI epitope recognized by STEAPI-BB ζ CAR T cells may not be presented as part of an ectodomain by STEAPIB despite apparent sequence homology.

Characterization of STEAPI-BB ζ CAR T cell products across a series of donors

We next profiled the expansion, transduction efficiency, and immunophenotype of STEAPI-BB ζ CAR T cell products using three independent sets of peripheral blood mononuclear cells (PBMCs) collected from healthy donors. We generally observed a 20- to 40-fold expansion of STEAPI-BB ζ CAR T cells within 11 days of culture (Supplementary Fig. 6a). The percentage of EGFRt⁺ CD8 T cells ranged from 24.3 to 54.2% while the percentage of EGFRt⁺ CD4 T cells was higher and ranged from 60.1 to 74.9% in our STEAPI-BB ζ CAR T cell products (Supplementary Fig. 6b). We examined the expression of the T cell exhaustion markers PD-1 and LAG-3 in the untransduced and STEAPI-BB ζ CAR T cell subsets and observed no significant increase in expression (Supplementary Fig. 6c). This finding suggested low or absent tonic signaling by the STEAPI-BB ζ CAR which was encouraging as constitutive CAR signaling can negatively impact CAR T cell effector function⁴⁸.

Both stem cell memory T cell (Tscm) and central memory T cell (Tcm) phenotypes have been associated with the therapeutic efficacy of CAR T cell therapy as they promote sustained proliferation and persistence in vivo^{49–51}. Immunophenotyping of untransduced and STEAPI-BB ζ CAR T cell subsets demonstrated higher frequencies of Tscm cells compared to the T cell subsets in donor PBMCs from which the cell products were derived (Supplementary Fig. 6d). This effect is likely due to the addition of IL-7 and/or IL-15 to the T cell expansion media as these cytokines have been shown to preserve and enhance Tscm differentiation^{51,52}. Our analysis also revealed an enrichment in Tcm populations particularly in the CD8 STEAPI-BB ζ CAR T cells (Supplementary Fig. 6e).

STEAPI-BB ζ CAR T cells demonstrate substantial antitumor effects in disseminated prostate cancer models with native STEAPI expression established in immunodeficient mice

As an initial screen for in vivo antitumor activity, we established 22Rv1 subcutaneous xenograft tumors in male NOD scid gamma (NSG) mice. When tumors grew to approximately 100 mm³, mice were treated with a single intratumoral injection of either 5×10^6 untransduced

T cells or STEAPI-BB ζ CAR T cells. Intratumoral treatment with STEAPI-BB ζ CAR T cells was associated with significant tumor growth inhibition that was statistically significant by day 18 of treatment (Fig. 3a). Mice were sacrificed on day 25 and residual tumors from mice treated with STEAPI-BB ζ CAR T cells showed large areas of necrotic debris and regions of viable tumor were infiltrated with CD3⁺ STEAPI-BB ζ CAR T cells (Supplementary Fig. 7a). STEAPI expression was conserved in the tumors across treatment groups (Supplementary Fig. 7b).

We transduced 22Rv1 cells with lentivirus to enforce firefly luciferase (fLuc) expression and 10⁶ 22Rv1-fLuc cells were injected into the tail veins of male NSG mice. Metastatic colonization was visualized by live bioluminescence imaging (BLI) after two weeks, at which point mice were treated with a single intravenous injection of either 5×10^6 untransduced T cells or STEAPI-BB ζ CAR T cells (Fig. 3b). Serial BLI revealed rapid disease progression in mice treated with untransduced T cells while those receiving STEAPI-BB ζ CAR T cells demonstrated a significant delay in tumor progression (Fig. 3c, d) and extension of survival (97 days versus 31 days, $p = 0.0018$ by log-rank test, Fig. 3e). There was no significant difference in mouse weights between treatment arms (Supplementary Fig. 7c). IHC staining of tumors at the end of study showed a significant reduction in STEAPI expression (Supplementary Fig. 7d), indicating that antigen escape was a mechanism of resistance. However, this was unlikely a result of transdifferentiation to a variant prostate cancer state as we did not appreciate morphologic changes, loss of AR and PSMA expression⁵³, or gain of SYP expression (Supplementary Fig. 7d).

To investigate the global impact of STEAPI loss in prostate cancer, we performed transcriptome profiling of the isogenic 22Rv1 wildtype (wt), 22Rv1 STEAPI ko, and 22Rv1 STEAPI ko + rescue cell lines we had previously prepared (Fig. 2b). Differential gene expression analysis comparing 22Rv1 STEAPI ko cells with 22Rv1 wt cells identified ~1700 genes significantly downregulated ($FDR \leq 0.05$, fold-change < 2) with STEAPI knockout. Rescue of STEAPI expression in the 22Rv1 STEAPI ko cells revealed that ~600 genes were significantly upregulated ($FDR \leq 0.05$, fold-change > 2) by STEAPI addback (Supplementary Fig. 8a). Gene Set Enrichment Analysis (GSEA) nominated several biological pathways that may be dysregulated by modulation of STEAPI expression. Prominent among these were cell cycle progression and multiple metabolic processes including the Krebs cycle and glycolysis which were negatively enriched by STEAPI knockout and rescued upon addback of STEAPI (Supplementary Fig. 8b). We applied the validated 31-gene cell cycle progression (CCP) signature⁵⁴ to our data which showed a significant downregulation of the CCP signature associated with STEAPI knockout with a score of -0.8 which increased substantially to 0.5 with rescue of STEAPI expression (Supplementary Fig. 8c). These data are consistent with a prior publication indicating that knockdown of STEAPI in the LNCaP prostate cancer cell line impairs cell viability and proliferation while inducing apoptosis³⁷.

We also noted that antigen processing and presentation was one of the most significantly de-enriched KEGG pathways with STEAPI

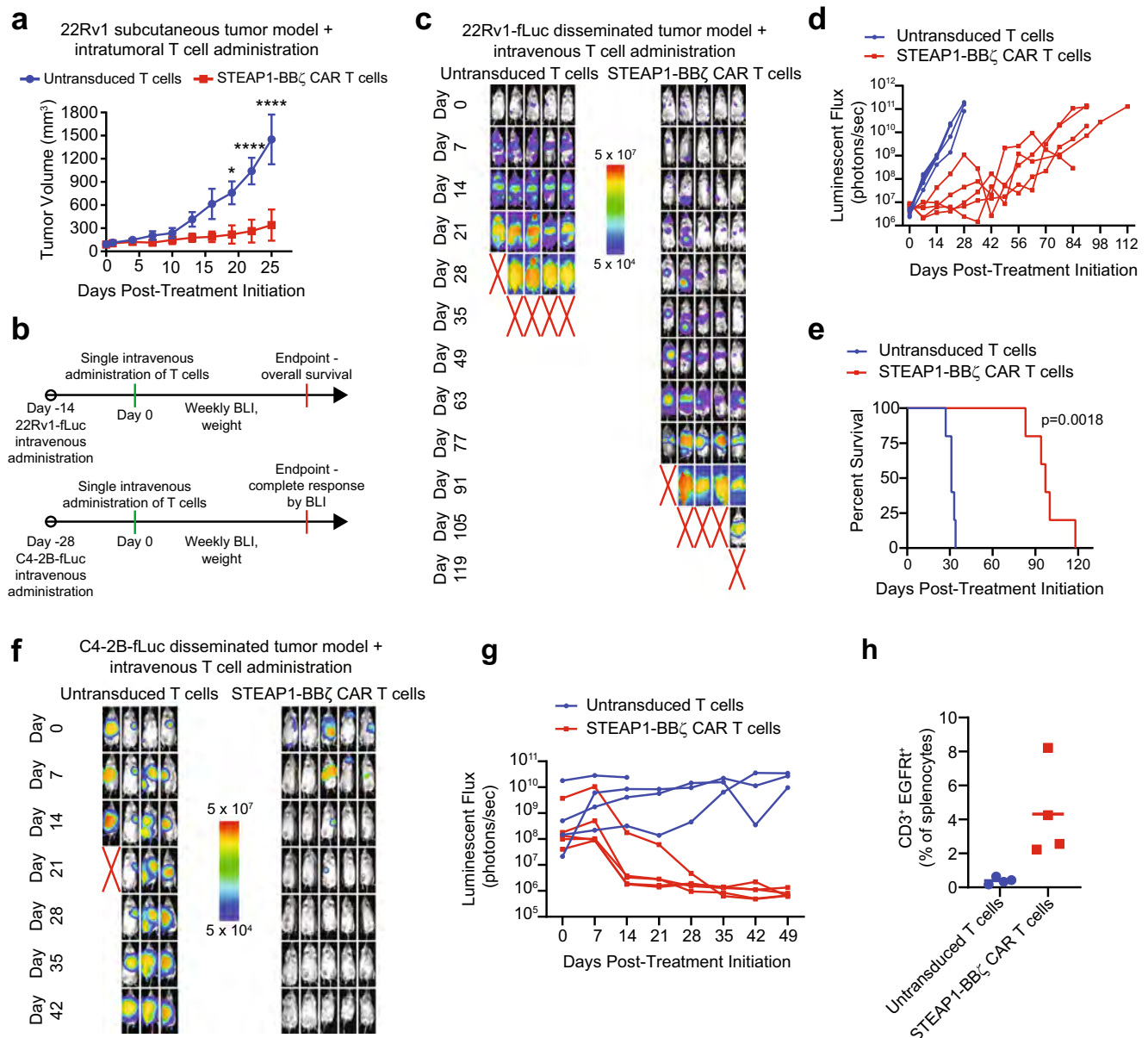


Fig. 3 | In vivo antitumor activity of STEAP1-BB ζ CAR T cell therapy in prostate cancer models with native STEAP1 expression. **a** Volumes of 22Rv1 subcutaneous tumors in NSG mice ($n=4$ for untransduced T cells group and $n=5$ for STEAP1-BB ζ CAR T cells group) over time after a single intratumoral injection of 5×10^6 untransduced T cells or STEAP1-BB ζ CAR T cells at normal CD4/CD8 ratios. $p < 0.0001$ at day 20 and 25. Bars represent mean with SEM. **b** Schematic of tumor challenge experiments for 22Rv1 (top) and C4-2B (bottom) disseminated models. fLuc firefly luciferase, BLI bioluminescence imaging. **c** Serial live bioluminescence imaging (BLI) of NSG mice engrafted with 22Rv1-fLuc metastases and treated with a single intravenous injection of 5×10^6 untransduced T cells or STEAP1-BB ζ CAR T cells at normal CD4/CD8 ratios on day 0. Red X denotes deceased mice. Radiance scale is shown. **d** Plot showing the quantification of total flux over time from live BLI of each mouse in (c). **e** Kaplan–Meier survival curves of mice in (c) with statistical

significance determined by log-rank (Mantel–Cox) test. For panels (c–e) $n=5$ mice per condition were used. **f** Serial live BLI of NSG mice engrafted with C4-2B metastases and treated with a single intravenous injection of 5×10^6 untransduced T cells or STEAP1-BB ζ CAR T cells at normal CD4/CD8 ratios on day 0. Red X denotes deceased mice. Radiance scale is shown. **g** Plot showing the quantification of total flux over time from live BLI of each mouse in (f). For panels (f, g) $n=4$ mice were used in the untransduced T cells group and $n=5$ mice in the STEAP1-BB ζ CAR T cells group. **h** Quantification of CD3⁺EGFR⁺ STEAP1-BB ζ CAR T cells by flow cytometry from splenocytes of mice treated with STEAP1-BB ζ CAR T cells ($n=4$) at the end of experiment on day 49. Bars represent mean. For panel (a), two-way ANOVA with Sidak’s multiple comparison test was used. Source data are provided in the Source Data file.

knockout (Supplementary Fig. 8b). We observed a significant down-regulation of genes including *PSME1* (proteasome activator subunit 1) which is a member of the immunoproteasome complex, *TAP1* (transporter 1, ATP binding cassette subfamily member) which is critical to the major histocompatibility complex (MHC) class I peptide-loading complex, and several MHC class I and II genes such as *MRI* (major histocompatibility complex, class I-related), *HLA-DQ-B1*, and *HLA-DQ-B2* (Supplementary Fig. 8d). We further investigated the tumors

collected from the 22Rv1 disseminated models treated with STEAP1-BB ζ CAR T cell therapy that demonstrated antigen loss (Fig. 3c, Supplementary Fig. 7d) by transcriptome analysis. Differential gene expression analysis and subsequent GSEA comparing 22Rv1 metastatic tumors from mice treated with STEAP1-BB ζ CAR T cell therapy to those treated with untransduced T cells showed negative enrichment of pathways involved in MHC, cytotoxic lymphocytes, and T cell activation (Supplementary Fig. 9a, b). We also specifically evaluated the

expression of MHC class I and II genes and observed their marked downregulation in 22Rv1 tumors treated with STEAP1-BB ζ CAR T cell therapy (Supplementary Fig. 9c). This result was further substantiated by a significant reduction in HLA-A,B,C staining by IHC in these tumors (Supplementary Fig. 9d). The potential implication of these data is that treatment with STEAP1-BB ζ CAR T cell therapy and resultant loss of STEAP1 tumor antigen expression in prostate cancer may result in further immunotherapy resistance through impaired antigen processing and presentation.

We also inoculated male NSG mice with C4-2B-fLuc cells by tail vein injection. C4-2B is a castration-resistant subline of LNCaP⁵⁵ with growth kinetics more in line with typical prostate cancer. Four weeks after injection, metastatic colonization was confirmed by BLI and mice were treated with a single intravenous injection of either 5×10^6 untransduced T cells or STEAP1-BB ζ CAR T cells (Fig. 3b). Serial BLI showed a complete response in all mice who received STEAP1-BB ζ CAR T cells within five weeks of treatment (Fig. 3f, g). We identified a trend of increased weight loss in the untransduced T cell treatment group (Supplementary Fig. 10a) but this was not statistically significant. Necropsy of mice treated with STEAP1-BB ζ CAR T cells showed no macroscopic disease and ex vivo BLI of organs did not reveal any signal (Supplementary Fig. 10b), suggesting that these mice were likely cured. We identified peripheral persistence of STEAP1-BB ζ CAR T cells at the end of the experiment based on the presence of detectable CD3⁺EGFRt⁺ splenocytes (Fig. 3h).

Mouse-in-mouse STEAP1 CAR T cell studies demonstrate anti-tumor therapeutic efficacy

The activation and cytolytic activity of STEAP1-BB ζ CAR T cells observed in the very low STEAP1 antigen density (~1500 molecules/cell) context of the PC3 cell line (Fig. 2g–i, Supplementary Fig. 11a, b) and evidence of in vivo antitumor activity in a disseminated PC3-fLuc tumor model (Supplementary Fig. 11c–e) presented concerns about the potential for on-target off-tumor toxicities. To evaluate for potential toxicity in a tractable model organism, we generated a human STEAP1 knock-in (hSTEAP1-KI) mouse in which the human *STEAP1* gene was knocked into the mouse *Steap1* gene locus on the C57Bl/6 background (Fig. 4a). A mouse colony was established with genotyping performed by polymerase chain reaction (PCR) of tail DNA (Fig. 4b). Both homozygous and heterozygous hSTEAP1-KI mice exhibited no apparent phenotypic or reproductive abnormalities compared to wildtype littermates. A tissue survey for human STEAP1 expression based on quantitative reverse transcription PCR (qRT-PCR) was performed on male and female heterozygous hSTEAP1-KI (hSTEAP1-KI/+) mice and revealed greatest relative expression in the prostate, followed by the uterus and adrenal gland (Fig. 4c). Further in situ analysis by STEAP1 IHC of male hSTEAP1-KI/+ prostate and adrenal glands revealed human STEAP1 expression confined to luminal epithelial cells of the prostate (Fig. 4d) and expression in the adrenal cortex (Fig. 4e).

A murinized version of the STEAP1 CAR, called STEAP1-mBB ζ CAR, in which the scFv and IgG4 hinge-CH2-CH3 spacer were retained but the CD28 transmembrane domain, 4-1BB costimulatory domain, and CD3 ζ activation domain were replaced with their mouse orthologs was cloned into a gammaretroviral construct (Fig. 4f). In addition, the human EGFRt transduction marker was replaced with a truncated mouse CD19 (mCD19t) to minimize potential immunogenicity. We confirmed the efficient retroviral transduction of T cells enriched from mouse splenocytes (Fig. 4g) and demonstrated the capacity of mouse STEAP1-mBB ζ CAR T cells to induce cytolysis of the RM9 mouse prostate cancer cell line⁵⁶ engineered to express human STEAP1 (RM9-hSTEAP1) by lentiviral transduction (Fig. 4h).

The in vivo efficacy of mouse STEAP1-mBB ζ CAR T cells was validated in a disseminated RM9-STEAP1-fLuc tumor model in NSG mice (Supplementary Fig. 12a). One week after tail vein injection of

RM9-STEAP1-fLuc cells, mice were treated with either 5×10^6 untransduced mouse T cells or mouse STEAP1-mBB ζ CAR T cells by tail vein injection. Mice that received untransduced mouse T cells demonstrated unchecked disease progression, whereas those treated with STEAP1-mBB ζ CAR T cells uniformly exhibited rapid disease regression which was followed by subsequent relapse ten days later (Supplementary Fig. 12b, c). STEAP1-mBB ζ CAR T cell therapy was associated with a statistically significant survival benefit (22 days versus 12 days, $p = 0.0039$ by log-rank test, Supplementary Fig. 12d). Weight loss was evident in both treatment groups as tumor burden increased prior to death (Supplementary Fig. 12e, f). Analysis of mouse splenocytes collected at necropsy showed peripheral persistence of STEAP1-mBB ζ CAR T cells with the detection of mCD3⁺ mCD19t⁺ cells up to 24 days after adoptive transfer (Supplementary Fig. 12g). Lungs were harvested from mice in both treatment groups and STEAP1 IHC showed loss of STEAP1 expression in pulmonary metastases from mice treated with STEAP1-mBB ζ CAR T cells (Supplementary Fig. 12h).

We subsequently expanded clonal RM9-STEAP1-fLuc lines to determine whether the observed tumor antigen escape could be a result of pre-existing heterogeneity in STEAP1 expression. The experiment was repeated with a clonal, disseminated RM9-STEAP1-fLuc tumor model in NSG mice (Supplementary Fig. 13a). In this context, mice treated with STEAP1-mBB ζ CAR T cells demonstrated a prompt and durable complete response (Supplementary Fig. 13b–d). These findings highlight the potency of STEAP1-mBB ζ CAR T cells in eradicating STEAP1⁺ prostate cancer and further suggest that adjunct therapeutic strategies may be needed to overcome resistance in subgroups of advanced prostate cancer patients where heterogeneity of STEAP1 expression is present (Fig. 1e).

STEAP1 CAR T cell therapy is safe in a humanized STEAP1 mouse model

To investigate both the preclinical safety and efficacy of STEAP1-mBB ζ CAR T cell therapy, we inoculated male heterozygous hSTEAP1-KI mice with syngeneic, non-clonal RM9-STEAP1-fLuc cells by tail vein injection (Fig. 5a). After confirmation of metastatic colonization by BLI about a week later, mice received pre-conditioning cyclophosphamide 100 mg/kg by intraperitoneal injection⁵⁷. A day later, mice were randomized to treatment with either 5×10^6 untransduced mouse T cells or mouse STEAP1-mBB ζ CAR T cells by tail vein injection. All mice that received mouse STEAP1-mBB ζ CAR T cells demonstrated a decrease in tumor burden within the first week of treatment initiation based on BLI (Fig. 5b, c). The observed response was short-lived but led to a modest extension of survival (21 days versus 12 days, $p = 0.0138$ by log-rank test, Fig. 5d)—similar to findings from the non-clonal RM9-STEAP1-fLuc experiments in NSG mice (Supplementary Figure 12d). There were no gross toxicities or premature deaths specifically associated with mouse STEAP1-mBB ζ CAR T cell therapy at this dose level where clear evidence of antitumor efficacy was observed. Weight loss was associated with increased tumor burden but common to both treatment arms (Fig. 5e, f).

To further assess potential toxicity of STEAP1-mBB ζ CAR T cell therapy, a similar experiment was performed in parallel in heterozygous hSTEAP1-KI mice bearing RM9-hSTEAP1 tumors and no tumors. Non-tumor bearing mice treated with untransduced T cells or STEAP1-mBB ζ CAR T cells did not demonstrate any differences in survival (Supplementary Fig. 14a) or gross toxicities including loss of body weight (Supplementary Fig. 14b). Because the STEAP1-BB ζ CAR consists of a modified IgG4 spacer which might potentially be immunogenic, we evaluated for a mouse anti-human antibody (MAHA) response by collecting retroorbital bleeds from mice in this experiment. No anti-human IgG and IgM antibodies were detected in the sera of mice at day 8 after treatment with STEAP1-BB ζ CAR T cells (Supplementary Fig. 14c).

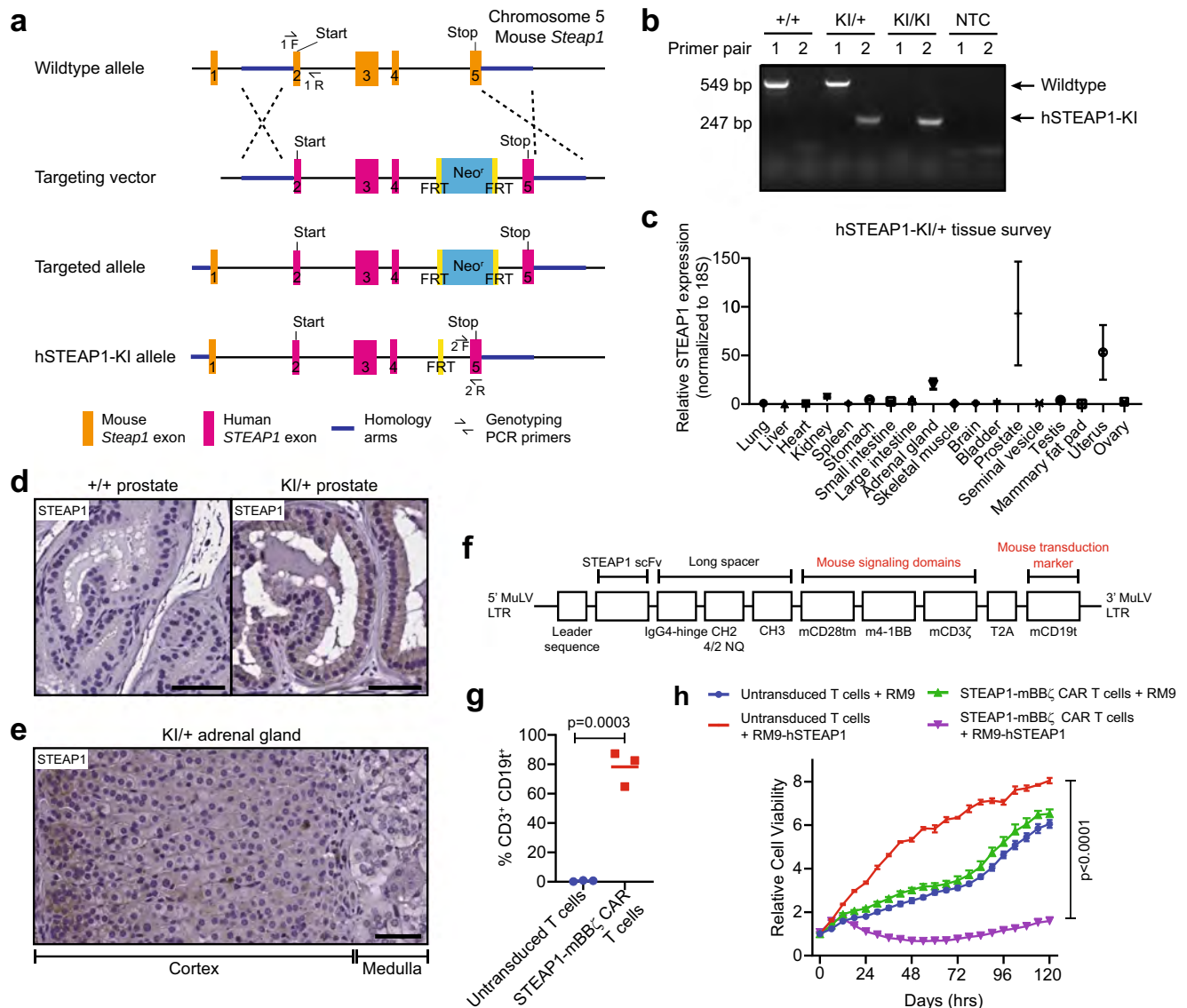
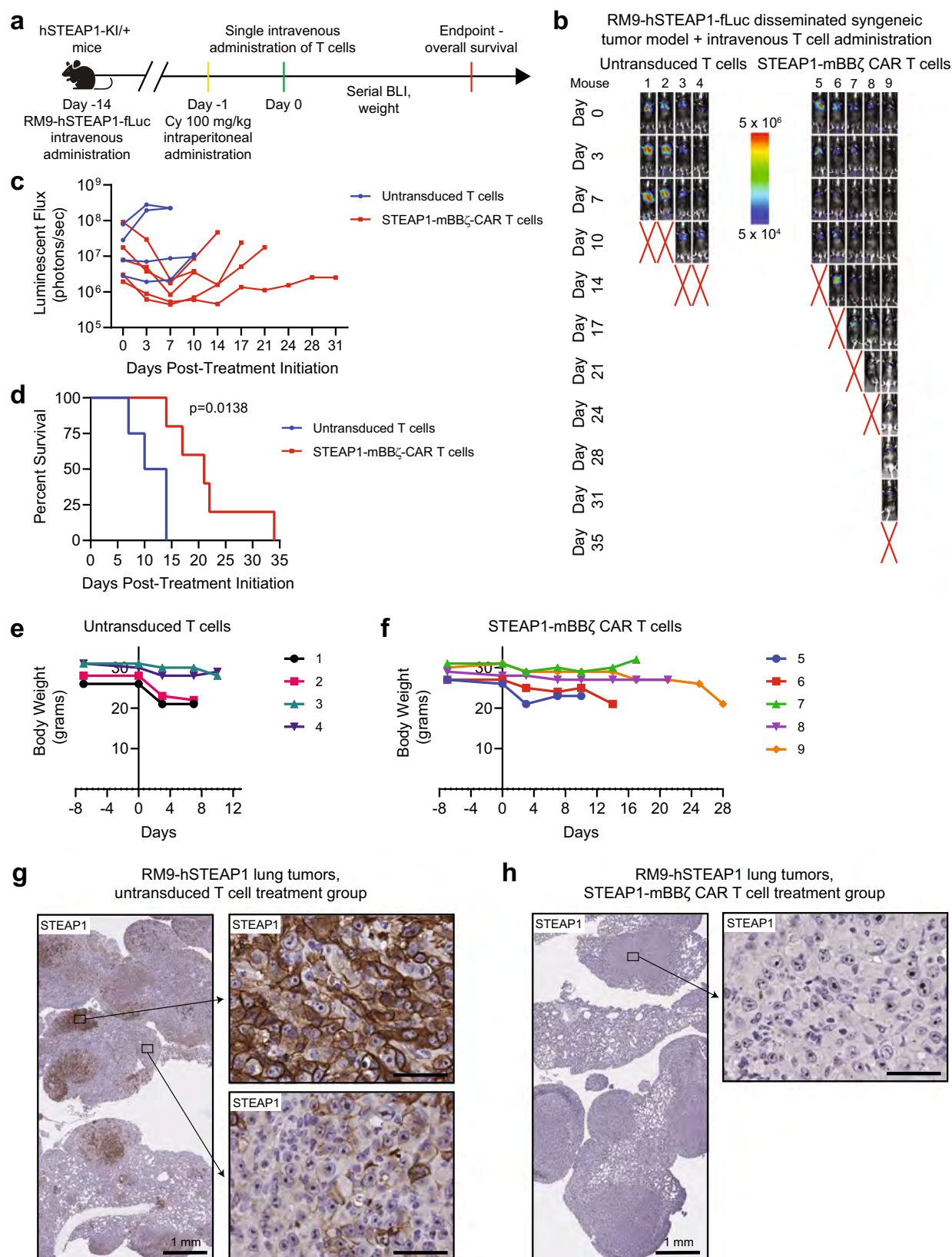


Fig. 4 | Establishing a mouse-in-mouse system with a human *STEAP1* knock-in (hSTEAP1-KI) mouse model and murinized *STEAP1* CAR. **a** Schematic showing the homologous recombination strategy using a targeting vector to knock-in human *STEAP1* exons 2–5 into the mouse *Steap1* locus on the C57Bl/6 background. FRT Flippase recognition target. **b** Visualization of PCR products from tail tip genotyping of wildtype (+/+), heterozygous (KI/+), or homozygous (KI/KI) mice using primer pairs intended to amplify portions of wildtype or hSTEAP1-KI alleles. NTC null template control. Representative gel image from $n = 3$ biologically independent experiments. **c** qPCR for human *STEAP1* expression normalized to 18S expression in a survey of tissues from hSTEAP1-KI/+ mice. $n = 3$ for sex-specific organs and $n = 6$ for common organs. Bars represent mean with SEM. Photo-micrographs of *STEAP1* IHC staining of **(d)** prostate tissues from (left) +/+ and (right) KI/+ mice and **(e)** an adrenal gland from a KI/+ mouse. Scale bars = 50 μm . For

panel **(d, e)** *STEAP1* immunostaining was performed on $n = 3$ biologically independent specimens. **f** Schematic of the retroviral murinized *STEAP1* CAR construct. MuLV murine leukemia virus, mCD19t mouse truncated CD19. **g** Quantification of the efficiency of retroviral transduction of activated mouse T cells from three independent experiments based on the frequency of mouse CD3⁺CD19⁺ cells by flow cytometry ($p = 0.0003$). **h** Relative cell viability of RM9 or RM9-hSTEAP1 target cells over time measured by fluorescence live cell imaging upon co-culture at a 1:1 ratio with mouse *STEAP1*-mBBζ CAR T cells or untransduced T cells ($p < 0.0001$). $n = 4$ biological replicates per condition. Error bars represent mean with SEM. For panel **(g)**, unpaired two-tailed Student's t test with Welch's correction was used. In panel **(h)**, two-way ANOVA with Sidak's multiple comparison test was used. Source data are provided in the Source Data file.

Importantly, heterozygous hSTEAP1-KI mice treated with *STEAP1*-mBBζ CAR T cells demonstrated no obvious tissue disruption or increased infiltration of CD3⁺ T cells in the prostate (Supplementary Fig. 15a, b) or adrenal gland (Supplementary Fig. 15c, d) relative to their counterparts treated with untransduced T cells, suggesting the absence of on-target off-tumor toxicities. Lungs collected at the end of the experiment showed human *STEAP1* expression in RM9-hSTEAP1 pulmonary metastases with regional heterogeneity in mice treated with untransduced mouse T cells (Fig. 5g). On the other hand, tumors from mice treated with mouse

STEAP1-mBBζ CAR T cells again demonstrated an absence of human *STEAP1* expression (Fig. 5h). To evaluate whether mouse *STEAP1*-mBBζ CAR T cell therapy and human *STEAP1* antigen loss may also impact antigen presentation in the RM9-hSTEAP1 tumors, we performed IHC for murine beta-2-microglobulin (B2m) which is a key component of MHC class I molecules. We observed a significant downregulation of B2m expression in progressive tumors after treatment with mouse *STEAP1*-mBBζ CAR T cells compared to untransduced T cells (Supplementary Fig. 15e, f), consistent with our findings in the 22Rv1 model.



Collagen binding domain-IL-12 fusion cytokine elicits antitumor responses through enhanced T cell receptor signaling and antigen presentation

IL-12 is a heterodimeric cytokine consisting of p40 and p35 subunits that regulates T cell responses and leads to the production of IFN- γ . Striking antitumor responses associated with systemic IL-12

administration have been demonstrated in several preclinical models^{58,59} but translation of this therapeutic approach to the clinic stalled due to dose-limiting toxicities and inefficacy⁶⁰⁻⁶³. Alternative strategies to circumvent these issues have been aimed at localizing IL-12 to tumor, either through intratumoral delivery or engineering IL-12 fusion proteins to leverage unique properties of the tumor

Fig. 5 | Determination of the efficacy and safety of mouse STEAP1-mBB ζ CAR T cells in hSTEAP1-KI mice bearing syngeneic, disseminated prostate cancer. **a** Schematic of the tumor challenge experiment for the RM9-hSTEAP1 disseminated model in hSTEAP1-KI/+ mice. Cy cyclophosphamide (for preconditioning). Created with BioRender.com. **b** Serial live BLI of hSTEAP1-KI/+ mice engrafted with RM9-hSTEAP1-fluc metastases and treated with a single intravenous injection of 5×10^6 mouse untransduced T cells or STEAP1-mBB ζ CAR T cells on day 0. Red X denotes deceased mice. Radiance scale is shown. **c** Plot showing the quantification of total flux over time from live BLI of each mouse in **(b)**. **d** Kaplan–Meier survival curves of mice in **(b)** with statistical significance determined by log-rank (Mantel–Cox) test. For panels **(b–d)**, $n = 4$ mice were used in the untransduced T cells group and $n = 5$ mice in the STEAP1-mBB ζ CAR T cells group. Plots of weights for each mouse

(numbered in **(b)**) over time in the **(e)** mouse untransduced T cells treatment group and **(f)** STEAP1-mBB ζ CAR T cell treatment group. **g** Photomicrographs at (left) low and (right) high magnification of STEAP1 IHC staining of RM9-hSTEAP1 lung tumors after treatment with mouse untransduced T cells showing regions of strong homogenous STEAP1 expression and heterogeneous STEAP1 expression. Scale bars = 50 μ m, unless otherwise noted. **h** Photomicrographs at (left) low and (right) high magnification of STEAP1 IHC staining of RM9-hSTEAP1 tumors after treatment with STEAP1-mBB ζ CAR T cells showing no STEAP1 expression. Scale bars = 50 μ m, unless otherwise noted. For panels **(g, h)** STEAP1 immunostaining was performed on $n = 4$ biologically independent specimens. Source data are provided in the Source Data file.

microenvironment. One recently described approach is fusion to the von Willebrand factor A3 domain which serves as a collagen binding domain (CBD, Fig. 6a) and enables binding of fusion proteins to exposed collagen in disordered tumor vasculature⁶⁴. Systemic CBD-IL-12 therapy was shown to remodel the tumor microenvironments of immunologically “cold” murine breast cancer and melanoma models through enhanced IFN- γ signaling and to cooperate with anti-PD-1 immune checkpoint inhibition to induce tumor eradication⁶⁵.

We asked whether CBD-IL-12 could be effective in converting prostate cancer from “cold” to “hot” and inducing antitumor responses. Subcutaneous, syngeneic RM9 and Myc-CaP tumors were established in male C57Bl/6 and FVB mice, respectively, and mice were randomized to treatment with vehicle, anti-PD-1, or CBD-IL-12. Systemic CBD-IL-12 administration induced significant tumor growth inhibition in both syngeneic RM9 and Myc-CaP tumor models which were otherwise poorly responsive to anti-PD-1 therapy (Fig. 6b, Supplementary Fig. 16a). To gain further insight into the mechanisms of action of CBD-IL-12, we performed single-cell RNA-seq (scRNA-seq) analysis of RM9 tumors from mice treated with either vehicle or CBD-IL-12. Uniform Manifold Approximation and Projection plots revealed a substantial reduction in tumor epithelial, fibroblast, and endothelial cell compartments (Supplementary Fig. 16b) consistent with antitumor activity.

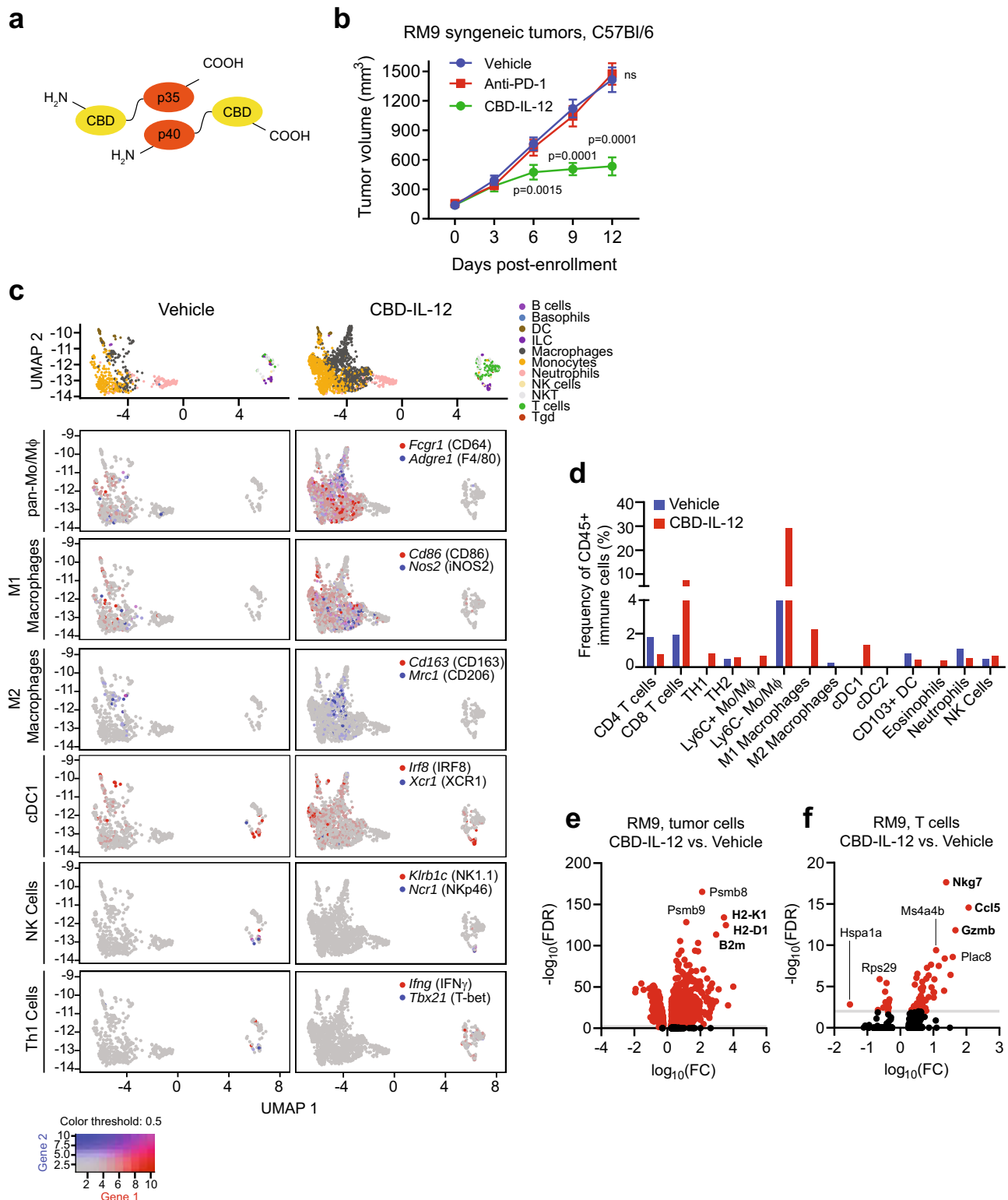
Marker-based profiling of innate and adaptive immune cell subsets (Fig. 6c,d) in the scRNA-seq data identified a substantial increase in CD8⁺ T cells (7.55 vs. 0.76%), antigen cross-presenting cells including XCR1⁺IRF8⁺ conventional type 1 dendritic cells (cDC1, 1.33 vs. 0%), CD86⁺INOS2⁺ M1 polarized macrophages (2.26 vs 0%), and CD64⁺F4/80⁺ monocytes/macrophages (29.2 vs 4.43%) in tumors from the CBD-IL-12 treatment group compared to the vehicle control group. A reduction in immunosuppressive CD163⁺CD206⁺ M2 polarized macrophages (0 vs. 0.25%) and Ly6G⁺ neutrophils (0.52 vs. 1.08%) was also associated with CBD-IL-12 therapy. IHC analysis confirmed that RM9 tumors treated with vehicle generally lacked CD8⁺ T cells while those treated with CBD-IL-12 showed a visibly significant infiltration of CD8⁺ T cells (Supplementary Figure 16c). Tumor cells demonstrated enriched expression of the proteasome and immunoproteasome subunits *Psmb8* and *Psmb9* and major histocompatibility complex class I (MHC I) genes *H2-K1*, *H2-D1*, and *B2m*, consistent with upregulation of antigen processing and presentation machinery (Fig. 6e, Supplementary Figure 16d). In parallel, T cells exhibited enhanced gene expression associated with T cell receptor signaling and immunoregulatory interactions between a lymphoid and a non-lymphoid cell (Fig. 6f, Supplementary Fig. 16e). Cells of the mononuclear phagocyte system (MPS) showed enriched gene expression related to antigen processing and cross-presentation as well as cytokine signaling (Supplementary Fig. 16f, g). These studies established the antitumor activity of CBD-IL-12 in models of prostate cancer through reprogramming of the tumor microenvironment and engagement of both the innate and adaptive immune systems.

Enhanced tumor control with concomitant STEAP1-mBB ζ CAR T cell and CBD-IL-12 therapy

We next hypothesized that broadening the antitumor immune response with CBD-IL-12 therapy could improve the therapeutic efficacy of STEAP1-mBB ζ CAR T cell therapy in prostate cancer by combating tumor antigen heterogeneity and rescue of antigen processing and presentation. We therefore established syngeneic, non-clonal RM9-STEAP1-fluc metastases in male heterozygous hSTEAP1-KI mice and randomized to treatment with either 5×10^6 untransduced mouse T cells or mouse STEAP1-mBB ζ CAR T cells by tail vein injection with or without weekly CBD-IL-12 therapy by retroorbital sinus injection. Groups that received CBD-IL-12 therapy alone or in combination with STEAP1-mBB ζ CAR T therapy did not receive lymphodepleting cyclophosphamide (Fig. 7a). Serial BLI revealed rapid disease progression in mice treated with untransduced T cells and CBD-IL-12 while those receiving STEAP1-mBB ζ CAR T cells in combination with CBD-IL-12 therapy demonstrated a significant delay in tumor progression (Fig. 7b, c). Importantly, combined treatment with mouse STEAP1-mBB ζ CAR T cells and weekly CBD-IL-12 was associated with a statistically significant extension in overall survival when compared to all other treatment groups (Fig. 7d). Plasma cytokine analysis on retroorbital bleeds collected at day 0 and day 8 of treatment showed a significant increase in proinflammatory cytokines IFN- γ , TNF- α , IL-6, and IL-4 levels (Fig. 7e, Supplementary Fig. 17) in mice treated with STEAP1-mBB ζ CAR T cells and CBD-IL-12.

Residual tumors were collected at necropsy and STEAP1 IHC showed antigen loss in tumors from mice treated with both STEAP1-mBB ζ CAR T cells alone and in combination with CBD-IL-12 (Fig. 8a). In addition, we observed an increase in tumor B2m expression (Fig. 8a) associated with CBD-IL-12 therapy. CD3⁺ T cells were also increased (Fig. 8b) in tumor conditions subjected to IL-12 therapy but, while a trend was appreciated, we did not find a statistically significant increase in intratumoral T cells between the STEAP1-mBB ζ CAR T cell and combined STEAP1-mBB ζ CAR T cell and CBD-IL-12 treatment groups.

Residual tumors including those from mice treated with combined STEAP1-mBB ζ CAR T cell and CBD-IL-12 collected at maximal treatment response (nadir) at day 10 and at compassionate endpoints for tumor progression (relapse) were dissociated to single cells and immune cell subsets were characterized by multiparametric flow cytometry (Supplementary Figure 18). In concordance with our scRNA-seq results, CBD-IL-12 treatment either alone or in combination with STEAP1-mBB ζ CAR T cells led to a significant increase in CD11b⁺Ly6C⁺F4/80⁺MHC-II⁺ macrophages (Fig. 8c, d, Supplementary Fig. 19a). CD11b⁺Ly6C⁺F4/80⁺MHC-II⁺ cells represent mature antigen-presenting macrophages⁶⁶ and this population was preferentially enriched and demonstrated increased induced nitric oxide synthase (iNOS) expression as a marker of proinflammatory M1 polarization with CBD-IL-12 therapy. We also observed an expansion of the cDC1 population and a reduction in the conventional type 2 dendritic cell (cDC2) population (Fig. 8e, Supplementary Fig. 19b). cDC1 have been



implicated in the activation of antitumor cytotoxic CD8⁺ T cells and T cell migration to tumor while cDC2 are important for the activation of CD4⁺ T cells including Tregs⁶⁷. Consistent with the cDC2 findings, profiling of CD4⁺FOXP3⁺ Tregs revealed a decreased in Tregs associated with CBD-IL-12 therapy (Supplementary Figure 19c). Of interest, we observed that in relapsed tumors after combined STEAP1-mBBζ CAR T cell and CBD-IL-12 therapy there was a decrease in cDC1 and increase in Tregs, implicating reduced priming of cytotoxic CD8 T cells and enrichment of immunosuppressive Treg signaling as

potential mechanisms of therapeutic resistance. We found no significant difference in the ratios of KLRG1⁺ and KLRG1⁻ natural killer cells across treatment groups (Supplementary Fig. 19d). Frequencies of F40/80⁺SiglecF⁺ eosinophils were increased while Ly6C⁺ neutrophils were decreased with the addition of CBD-IL-12 to STEAP1-mBBζ CAR T cell therapy (Fig. 8f, g). Notably, the frequency of these tumor-associated neutrophils was heightened with treatment with STEAP1-mBBζ CAR T cells compared to untransduced T cells (54 vs. 34%), diminished in conditions with CBD-IL-12 therapy, but increased

Fig. 6 | Systemic collagen-binding domain IL-12 (CBD-IL-12) cytokine fusion therapy inhibits prostate cancer tumor growth and reprograms the tumor immune microenvironment. **a** Schematic of CBD-IL-12, composed of the p35 and p40 subunits fused to the CBD from von Willebrand factor domain A3. **b** Volumes of RM9 subcutaneous tumors in syngeneic C57Bl/6 mice over time with treatment with vehicle, anti-PD-1 (clone 29 F.1A12) 200 Mg by intraperitoneal injection every 5 days, or CBD-IL-12 25 Mg by intravenous injection every 5 days starting on day 0. $n = 7$ mice in vehicle and CBD-IL-12 treated groups and $n = 8$ mice in anti-PD1 treated group. $p < 0.0001$ at day 9 and 12. Bars represent mean with SEM. P -values are derived from two-way ANOVA with Dunnett's multiple comparisons test, ns not significant. **c** Uniform Manifold Approximation and Projection (UMAP) plots of different immune cell subsets (top) from single-cell RNA-seq (scRNA-seq) analysis of five RM9 tumors each aggregated from mice treated with vehicle or CBD-IL-12. UMAP plots colored with gene expression of immune cell subset-specific markers

for pan-monocytes/macrophages, M1 and M2 polarized macrophages, conventional type 1 dendritic cells (cDC1), natural killer (NK) cells, and T helper type 1 (Th1) cells. **d** Plots showing the frequency of specific immune cell populations (relative to CD45⁺ immune cells) identified by scRNA-seq analysis including CD4⁺ and CD8⁺ T cells, Th1 (Infg⁺Tbx21⁺) and Th2 (cMAF⁺Gata3⁺) cells, Ly6C⁺ monocytes/macrophages (Ly6C⁺Adgre1⁺), M1 macrophages (CD80⁺CD86⁺INOS2⁺), M2 macrophages (CD163⁺Mrc1⁺cMAF⁺), cDC1 (XCR1⁺IRF8⁺), conventional type 2 dendritic cells (cDC2, CD1⁺IRF4⁺), migratory CD103⁺ dendritic cells (Itgae⁺), eosinophils (SiglecF⁺), neutrophils (Ly6G⁺), and NK cells (Klrb1c⁺Ncr1⁺) in tumors treated with vehicle or CBD-IL-12. Volcano plots showing differential gene expression in (e) tumor cells and (f) T cells from RM9 tumors of mice treated CBD-IL-12 relative to those treated with vehicle. FC fold change, FDR false discovery rate. Source data are provided in the Source Data file.

upon tumor relapse after combined STEAP1-mBBZ CAR T cell and CBD-IL-12 therapy (19 to 42%). These findings suggest that the immunosuppressive properties⁶⁶ of tumor-associated neutrophils may play a prominent role in mediating treatment resistance and tumor progression. Overall, these analyses indicate that CBD-IL-12 treatment reverts the hostile immunosuppressive tumor milieu to a proinflammatory state and broadens antitumor activity in conjunction with adoptively transferred STEAP1-mBBZ CAR T cell therapy.

We further performed TCR repertoire analysis using multiplex PCR-based TCR beta chain sequencing⁶⁸ on lungs bearing tumors collected from each treatment group at necropsy. We observed a significant decrease in Simpson clonality in samples from mice treated with CBD-IL-12 alone and in combination with STEAP1-mBBZ CAR T cell therapy which was indicative of increased intratumoral T cell diversity (Fig. 8h). These findings establish that adding CBD-IL-12 as an adjunct to STEAP1 CAR T cell therapy may be beneficial through remodeling of the prostate cancer tumor microenvironment, enhancing antigen processing and presentation, and engaging host immunity to promote epitope spreading.

Discussion

The effectiveness of CAR T cell therapy and other immune-based targeted therapeutics is highly dependent on consistent antigen expression on all or most cells comprising the tumor population within an individual patient. However, antigen heterogeneity is pronounced in solid tumors including prostate cancer, where progression to mCRPC and treatment resistance are associated with the emergence of divergent disease subtypes marked by distinct transcriptional programs^{69–71} and cell surface antigen expression¹⁷. While PSMA is considered one of the foremost biomarkers in prostate cancer with significant overexpression found across the spectrum of disease progression, our work corroborates findings from a recent publication¹⁴ indicating that PSMA expression is heterogeneous in lethal mCRPC. We show that STEAP1 is more broadly expressed than PSMA in this setting but is by no means expressed uniformly at high levels in all mCRPC tissues. No single antigen-targeted therapy including CAR T cell therapy may be able to overcome pre-existing tumor antigen heterogeneity in mCRPC. Thus, it is of critical importance to thoroughly credential additional therapeutic targets such as STEAP1 in mCRPC that may enable combinatorial therapies that exert insurmountable therapeutic pressure. These include dual antigen-targeted (e.g., PSMA and STEAP1) CAR T cell therapies or multimodal strategies combining CAR T cell therapies with ADCs, T-BsAbs, or other treatments that potentially promote antigen-independent and -dependent tumor killing.

We engineered a STEAP1-targeted CAR T cell therapy that is highly antigen-specific and functionally localized the epitope recognized by the CAR to the second ECD of STEAP1. Our STEAP1-BBZ CAR T cells demonstrate substantial antitumor activity against multiple disseminated prostate cancer models both in human-in-mouse and mouse-in-mouse studies. Importantly, our STEAP1-BBZ CAR is capable

of inducing T cell activation and target cell cytolysis even in low antigen density conditions, as evidenced by reactivity against the PC3 prostate cancer model. However, this sensitivity of STEAP1-BBZ CAR T cells to low levels of STEAP1 expression may be advantageous from the perspective of enhancing antitumor efficacy but could also accentuate liabilities from on-target off-tumor toxicity. Systemic expression of STEAP1 has previously been reported as virtually absent in normal human tissues^{18,72} except the prostate gland where membranous expression in prostate epithelial cells has been described²⁷. To delve into the safety of STEAP1-BBZ CAR T cell therapy in the preclinical setting, we generated a humanized STEAP1 mouse model. The hSTEAP1-KI mouse model recapitulated human STEAP1 expression in the prostate gland and showed expression in the adrenal cortex. Reassuringly, STEAP1-BBZ CAR T cell therapy at a dose sufficient to induce antitumor activity did not lead to evident systemic toxicities in hSTEAP1-KI mice including on-target off-tumor toxicities at sites of human STEAP1 expression.

A recurring mechanism of prostate cancer relapse and progression after STEAP1-BBZ CAR T cell therapy in our studies was tumor antigen escape. On one hand, this finding underscores the overall potency of STEAP1-BBZ CAR T cell therapy. However, it is unclear whether the loss of tumor STEAP1 expression is solely due to inherent tumor antigen heterogeneity or whether there is also adaptive downregulation of STEAP1 expression. A recent publication showed that promoter methylation of *STEAP1* modulates STEAP1 expression and epigenetic deregulation by DNA methyltransferase and histone deacetylase inhibition was sufficient to significantly upregulate STEAP1 expression⁷³. Treatment with epigenetic inhibitors in combination with STEAP1 CAR T cell therapy could simultaneously enhance tumor STEAP1 expression and reprogram CAR T cells to favorable exhaustion-resistant differentiation states^{74,75}, thereby mitigating tumor antigen loss and enhancing antitumor efficacy in prostate cancer. Our study also implicates STEAP1 as playing a functional role in regulating cell cycle progression and cellular metabolism in prostate cancer. STEAP1 is unique from other STEAP family members (STEAP2, 3, and 4) in that it lacks an intracellular oxidoreductase domain²² which is necessary for metalloredox activity. As a result, STEAP1 homotrimers, but not heterotrimers with other STEAP proteins, lack enzymatic function to reduce Fe³⁺ to Fe²⁺ and Cu²⁺ to Cu¹⁺. Whether and how the involvement of STEAP1 in metal ions and cellular metabolism promotes cancer progression has yet to be determined and is worthy of further investigation.

The immunologically 'cold' tumor microenvironment of prostate cancer is a major barrier to the efficacy of cancer immunotherapies. For example, exploratory studies associated with a phase I clinical trial of PSMA CAR T cell therapy armored to express dominant-negative transforming growth factor- β receptor (TGF β R-DN) in mCRPC showed that the expression of immunosuppressive signaling molecules in the tumor microenvironment increases after CAR T infusion¹³. In our studies, a critical finding was the association between the loss of STEAP1

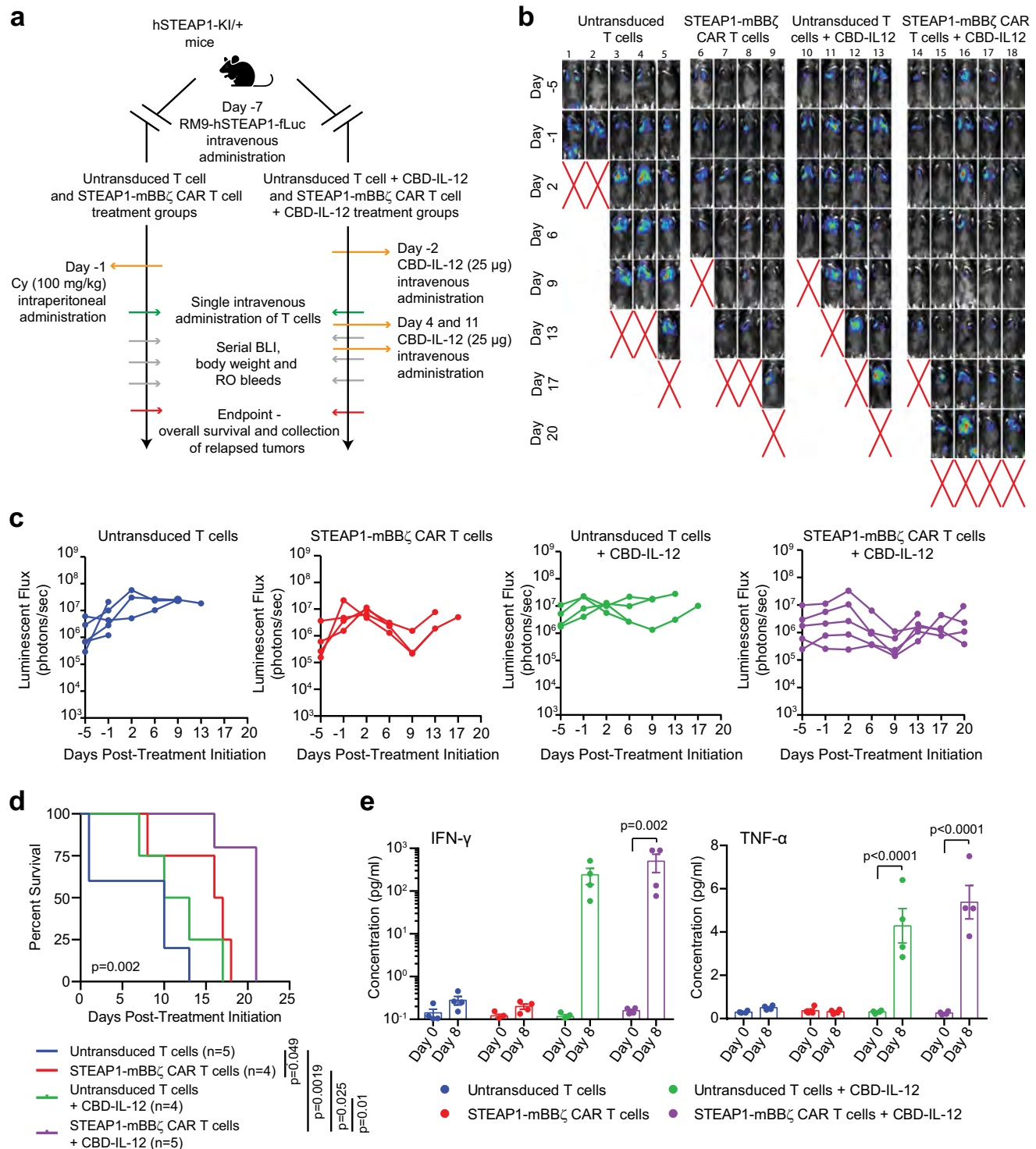
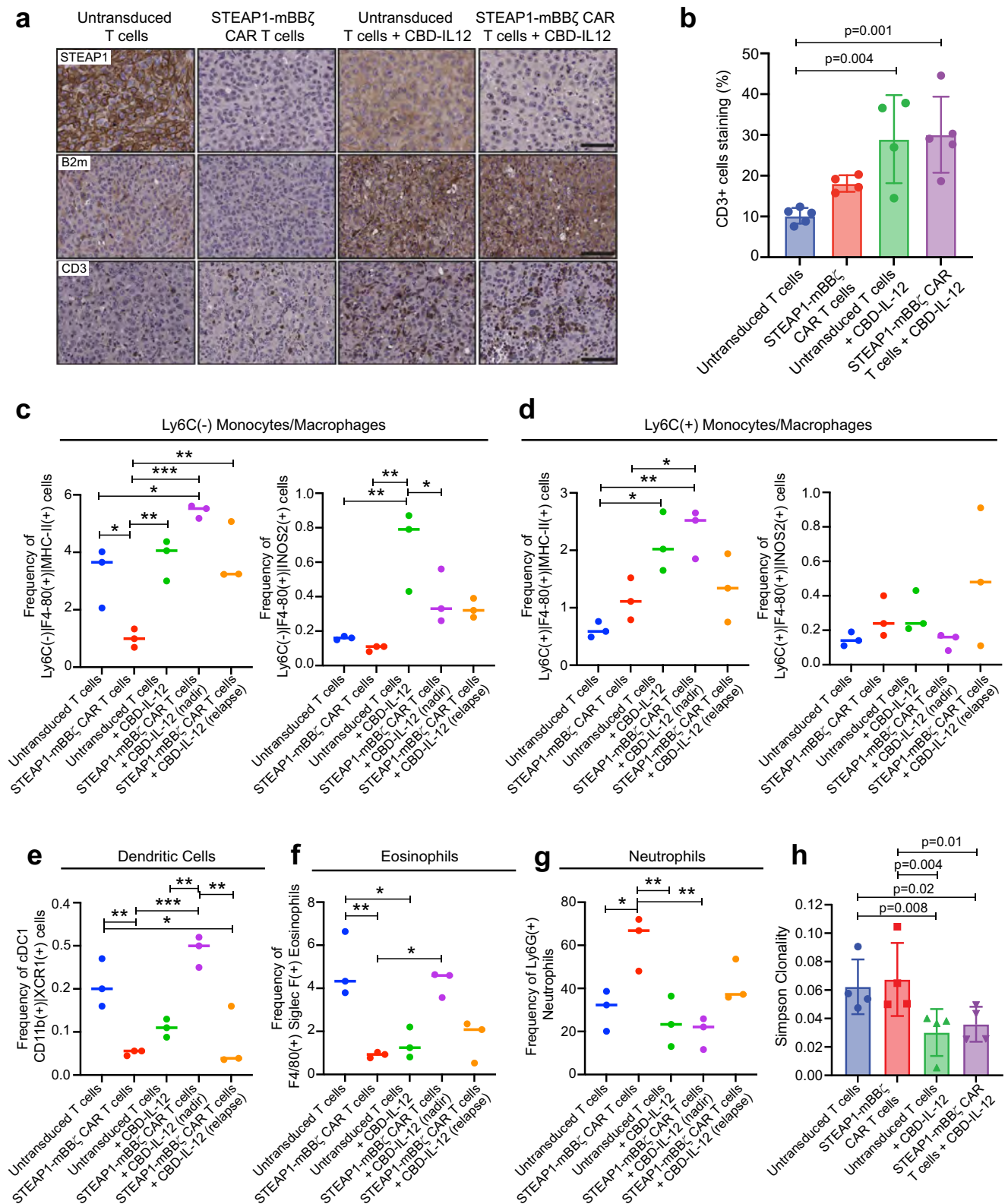


Fig. 7 | Combining CBD-IL-12 with STEAP1-mBB ζ CAR T cell therapy enhances overall survival and inflammatory cytokine levels. **a** Schematic of the tumor challenge experiment for the RM9-hSTEAP1 disseminated model in hSTEAP1-KI/+ mice investigating the combination of CBD-IL-12 with STEAP1-mBB ζ CAR T cell therapy. Cy cyclophosphamide (for preconditioning). Created with BioRender.com. **b** Serial live BLI of hSTEAP1-KI/+ mice engrafted with RM9-hSTEAP1-fLuc metastases and treated with a single intravenous injection of 5×10^6 mouse untransduced T cells or STEAP1-mBB ζ CAR T cells on day 0 with or without CBD-IL-12 treatment weekly. Red X denotes deceased mice. The radiance scale is shown. **c** Plot showing the quantification of total flux over time from live BLI of each mouse in

(b). **d** Kaplan-Meier survival curves of mice in (b) with statistical significance determined by log-rank (Mantel-Cox) test ($p = 0.002$). **e** Plots showing serum cytokine levels of IFN- γ (left, $p = 0.002$) and TNF- α (right, $p < 0.0001$) based on ProcartaPlex immunoassays from retroorbital bleeds of hSTEAP1-KI/+ mice (n = 4 mice per group) bearing RM9-hSTEAP1-fLuc metastases prior to (day 0) and after treatment (day 8) with untransduced mouse T cells or mouse STEAP1-mBB ζ CAR T cells with or without CBD-IL-12 therapy. Error bar represents mean with SEM. For panel (e), p-values were derived from two-way ANOVA with Sidak's multiple comparisons test. Source data are provided in the Source Data file.



expression and downregulation of antigen processing and presentation, both in STEAP1 knockout cells and STEAP1 antigen loss tumors after STEAP1-BBζ CAR T cell therapy. Thus, STEAP1 antigen loss in prostate cancer promotes not only direct resistance to STEAP1-BBζ CAR T cell therapy but may also limit host adaptive antitumor immunity. Loss of tumor target antigen expression and conversion to a more immunosuppressive state with increased infiltration of Tregs and

higher expression of immunosuppressive molecules has also been observed in a phase I study of EGFR-vIII CAR T cells in participants with recurrent glioblastoma⁷⁶. Additional work will be necessary to understand the functional mechanisms underlying STEAP1 antigen loss and more generally how dynamic effects of adoptive CAR T cell therapy may contribute to antigen loss and immunoediting by modulating tumor-immune-stromal interactions in solid tumors.

Fig. 8 | Combining CBD-IL-12 with STEAPI-mBB ζ CAR T cell therapy reprograms the tumor immune microenvironment and promotes antigen presentation and epitope spreading. **a** Photomicrographs of STEAPI (top), B2m (middle), and CD3 (bottom) IHC staining of RM9-hSTEAPI lung tumors after treatment with mouse untransduced T cells or STEAPI-mBB ζ CAR T cells with or without CBD-IL-12 treatment. Scale bars = 50 μ m. **b** Bar plot showing IHC quantification of CD3 positive cells infiltrating the metastatic lung tumors ($n = 4$ tumors per group). One-way ANOVA $p = 0.0021$. Error bar represents mean with SD. Plots showing the frequencies of **(c)** Ly6C⁺F4/80⁺MHC-II⁺ (left, one-way ANOVA $p = 0.0005$) and Ly6C⁺F4/80⁺iNOS2⁺ (right, one-way ANOVA $p = 0.0017$) macrophages, **(d)** Ly6C⁺F4/80⁺MHC-II⁺ (left, one-way ANOVA $p = 0.0038$) and Ly6C⁺F4/80⁺iNOS2⁺ (right, one-way ANOVA $p = \text{ns}$) macrophages, **(e)** CD11b⁺XCRI⁺cDC1 (one-way ANOVA $p = 0.0003$), **(f)** F4/80⁺SiglecF⁺ eosinophils (one-way ANOVA $p = 0.0008$), and **(g)** Ly6G⁺ neutrophils (one-way ANOVA $p = 0.0035$) normalized to total CD45⁺ cells as

determined by multiparametric flow cytometry after treatment with untransduced T cells, STEAPI-mBB ζ CAR T cells, untransduced T cells and CBD-IL-12, and STEAPI-mBB ζ CAR T cells and CBD-IL-12 at maximal treatment response (nadir) and tumor relapse (relapse). **h** Bar plots representing Simpson clonality as a measure of ‘evenness’ of the TCR repertoire analyzed by TCRB sequencing on tumor infiltrating cells collected from mice in **(a)**. $n = 4$ tumors per group. P -values for untransduced T cells + CBD-IL-12 compared to untransduced T cells and STEAPI-mBB ζ CAR T cells are 0.008 and 0.02, respectively; and STEAPI-mBB ζ CAR T cells + CBD-IL-12 compared to untransduced and STEAPI-mBB ζ CAR T cells are 0.004 and 0.01, respectively. Error bar represents mean with SD. For panels **(c–h)** $n = 3$ tumors per group, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, p -values in panels **(b–h)** are from one-way ANOVA with Dunn’s multiple comparisons test. Source data are provided in the Source Data file.

To broaden the antitumor response, we investigated the systemic administration of CBD-IL-12 in combination with STEAPI-BB ζ CAR T cell therapy in a disseminated, syngeneic RM9-hSTEAPI tumor model in hSTEAPI-KI mice to approximate the immunosuppressive nature of mCRPC based on prior characterization of RM9 as a poorly immunogenic model^{57,77}. Co-treatment with CBD-IL12 led to improvements in overall survival, cytokine production, tumor antigen presentation, and intratumoral T cell diversity consistent with epitope spreading. Close investigation of the tumor immune microenvironment revealed that adding CBD-IL-12 to STEAPI-BB ζ CAR T cell therapy induces tumor stromal reversion and enhances activated macrophages and cDC1 while reducing tumor-associated neutrophils. However, cure was not achieved in our studies, and we highlight potential compensatory, adaptive mechanisms of resistance and tumor progression including increased frequencies of immunosuppressive neutrophils and cDC2 which may promote Treg induction. It is important to note that further optimization of the dose and administration schedule of CBD-IL-12 may be needed to maximize antitumor responses. However, these results support the investigation of combinatorial immunotherapeutic approaches such as arming CAR T cells to express recombinant cytokines⁷⁸ (e.g., IL-2, IL-12, IL-15, or IL-18), concurrent treatment with immunomodulators (e.g., anti-PD-1/PD-L1 or anti-CTLA4), or tumor-directed radiotherapy to remodel the prostate cancer tumor microenvironment and enhance CAR T cell effector function.

While this manuscript was in preparation, a study from a group in Norway reported the preclinical development of a STEAPI CAR T cell therapy with antitumor activity in a subcutaneous 22Rv1 model in immunocompromised mice⁷⁹. The reported STEAPI CAR differs from STEAPI-BB ζ CAR as it incorporates a synthetic scFv called Oslo1 and a CD8 α hinge and transmembrane domain. Another differentiating feature is that STEAPI-BB ζ CAR T cells are prepared as a defined product with a normal ratio of CD4/CD8 T cells while the Oslo1 STEAPI CAR T cells are not. Mechanisms of resistance to and safety studies related to the Oslo1 STEAPI CAR T cell therapy were not reported. However, it will be of interest to see how these differences in CAR engineering and cell product composition may impact antitumor efficacy, persistence, and safety as both the Oslo1 STEAPI CAR T cell therapy and our STEAPI-BB ζ CAR T cell therapy programs are translated to the clinic.

The findings of our studies have led to a partnership with the National Cancer Institute (NCI) Experimental Therapeutics (NExT) Program with the goal of translating STEAPI-BB ζ CAR T cell therapy to a first-in-human trial for men with mCRPC. Safety and efficacy signals from this early phase clinical trial will help determine whether there may also be value in investigating this therapeutic approach for other cancer types that highly express STEAPI.

Methods

All studies were conducted in accordance with the ethical guidelines expressed in the World Medical Association Declaration of Helsinki.

Cell lines

22Rv1 (CRL-2505), LNCaP (CRL-1740), PC3 (CRL-1435), DU145 (HTB-81), NCI-H660 (CRL-5813), C4-2B (CRL-3315), RM9 (RL-3312), HEK293T (CRL-3216), and Myc-CaP (CRL-3255) were obtained from the American Type Culture Collection. LNCaP95 cells were a gift from Stephen R. Plymate (University of Washington, Seattle). MSKCC EF1 were derived from the MSKCC PCa4 organoid line provided by Yu Chen (Memorial Sloan Kettering Cancer Center), as previously described¹⁷. Cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin, and 4 mmol/L GlutaMAX (Thermo Fisher). PLAT-E (RV-101) Retroviral packaging cell line was obtained from Cell Biolabs, Inc. and were cultured in DMEM supplemented with 10% FBS media.

22Rv1 STEAPI ko and PC3 STEAPI ko cells were generated by transient transfection of 22Rv1 cells with a pool of PX458 (Addgene, #48138) plasmids each expressing one of four different sgRNA targeting sequences predicted from the Broad Institute Genetic Perturbation Platform sgRNA Designer⁸⁰: (1) 5'-ATAGTCTGTCTTACCCATG-3'; (2) 5'-CCTTTGTAGCATAAGGACAC-3'; (3) 5'-ATCCACTTATCCAAACAAATG-3'; and (4) 5'-CATCAACAAAGTCTTGCCAA-3'. 48–72 h after transfection, GFP-positive cells were singly sorted on a Sony SH800 Cell Sorter into a 96-well plate and clonally expanded.

gBlocks encoding firefly luciferase (fLuc), hSTEAPI, mSTEAPI, mSTEAPI hECD1, mSTEAPI hECD2, mSTEAPI hECD3, hSTEAPIB isoform 1, hSTEAPIB isoform 2, and hSTEAPIB isoform 3 were cloned into the EcoRI site of FU-CGW⁸¹ by HiFi DNA Assembly. The FU-hSTEAPI-CGW plasmid was modified to excise the GFP cassette by digestion with AgeI and BsrGI. A PCR product encoding firefly (fLuc) was then inserted by HiFi DNA assembly to generate the FU-hSTEAPI-C-fLuc-GW plasmid. These lentiviruses were produced and titered as previously described⁸². Lentiviruses were used to introduce stable transgene expression in the cell lines noted above.

Animal studies

All mouse studies were performed in accordance with protocols approved by the Fred Hutchinson Cancer Center Institutional Animal Care and Use Committee and regulations of Comparative Medicine.

For studies using immunocompromised mice, six- to eight-week-old male NSG (NOD-SCID-IL2Ry-null) mice were obtained from The Jackson Laboratory. For the 22Rv1 subcutaneous tumor and intratumoral T cell administration model, 2×10^6 22Rv1 cells were suspended in 100 μ l ice-cold Matrigel matrix (Corning) and injected subcutaneously into the flanks of NSG mice. Tumors were measured twice weekly using electronic calipers and tumor volume (TV) calculated based on the equation $TV = \frac{1}{2} (L \times W^2)$. When TV was ~ 75 – 100 mm³, 5×10^6 untransduced or STEAPI CAR T cells at a defined CD4/CD8 composition of 1:1 and suspended in 100 μ l of PBS was injected intratumorally. Mice were sacrificed 25 days after intratumoral T cell therapy. For disseminated human prostate cancer and intravenous T cell administration models, between 5×10^5 and 10^6 prostate cancer cells

were suspended in 100 μ l of PBS and injected into the tail veins of NSG mice. Tumor burden was monitored by live bioluminescence imaging on an IVIS Spectrum (PerkinElmer) after intraperitoneal injection of XenoLight D-luciferin (PerkinElmer). When metastatic colonization was confirmed by imaging, 5×10^6 human untransduced or STEAP1-mBBZ CAR T cells at a defined CD4/CD8 composition of 1:1 and suspended in 100 μ l of PBS was injected by tail vein. Metastatic tumors and spleen were harvested when mice were euthanized at compassionate endpoints. In the disseminated C4-2B model, lungs and livers were collected from a subset of mice and placed individually in six-well plates in DMEM media supplemented with 10% FBS and GlutaMAX. Ex vivo bioluminescence imaging was performed by introducing XenoLight D-luciferin into the media and quantifying signal on an IVIS Spectrum. Metastatic tumors were formalin-fixed and paraffin-embedded. Splenocytes were harvested from spleens to perform flow cytometry to evaluate the peripheral persistence of CAR T cells.

For animal studies using hSTEAP1-KI mice, heterozygous hSTEAP1-KI mice were generated by crossing homozygous hSTEAP1-KI mice with wildtype C57Bl/6 mice. Genotyping of all hSTEAP1-KI mice was performed by PCR of 10 ng of tail DNA using the Taq 2X Master Mix (New England Biolabs) and visualization of PCR products by gel electrophoresis on a 2% agarose gel. Primers used for genotyping PCR reactions are (1) wildtype: forward 5'-CTAGGTGGCTGAAGCCGTA-3' and reverse 5'-GCGATGACCAAAAGTGACTTC-3', (2) hSTEAP1-KI: forward 5'-CAGATGAGGTAGGATGGGATAAAC-3' and reverse 5'-CCTCAAGCATGGCAGGAATAG-3'. Thermocycler conditions for genotyping PCR are 95 °C $\times$ 30 sec; (95 °C $\times$ 30 sec, 58 °C $\times$ 30 sec, 68 °C $\times$ 70 sec) $\times$ 35 cycles; 68 °C $\times$ 5 min; and 12 °C hold.

For disseminated RM9-hSTEAP1-fluc mouse prostate cancer and intravenous T cell administration models, 5×10^5 RM9-hSTEAP1-fluc cells were suspended in 100 μ l of PBS and injected into the tail veins of either NSG or heterozygous hSTEAP1-KI mice. Tumor burden was monitored by live bioluminescence imaging on an IVIS Spectrum (PerkinElmer) after intraperitoneal injection of XenoLight D-luciferin (PerkinElmer). When metastatic colonization was confirmed by imaging, 5×10^6 mouse untransduced or STEAP1-mBBZ CAR T cells suspended in 100 μ l of PBS was injected by tail vein. Retroorbital blood was collected using heparinized capillary tubes into polystyrene tubes containing an EDTA/PBS solution. After collection, retroorbital blood samples were incubated at room temperature for 15–20 min and centrifuged in a tabletop centrifuge at 2000 $\times$ g for 10 min. Plasma was collected and stored at -80 °C. Lungs, spleen, prostate, and adrenal glands were harvested when mice were euthanized at compassionate endpoints. Lungs, prostate, and adrenal glands were formalin-fixed and paraffin-embedded. Splenocytes were harvested from spleens to perform flow cytometry to evaluate the peripheral persistence of CAR T cells.

For animal studies to assess the antitumor efficacy of systemic CBD-IL-12 treatment in prostate cancer, 10^6 syngeneic RM9 and Myc-CaP murine prostate cancer cells were subcutaneously injected into the flanks of C57Bl/6 and FVB mice, respectively. Tumors were measured using electronic calipers twice weekly. Once TV was ~ 100 mm³, mice were randomized to treatment with (a) PBS control, (b) 200 Mg anti-PD-1 (BioXCell, clone 29 F.1A12) by intraperitoneal injection every 5 days, and (c) 25 μ g CBD-IL-12 by retroorbital sinus injection every 5 days. Tumors were harvested at the end of the experiment, dissociated viably to single cells, and subjected to single cell RNA-sequencing. Tumors were also collected and processed for paraffin embedding for IHC analysis.

For animal studies testing combined CBD-IL-12 and STEAP1-mBBZ CAR T cell therapy, heterozygous hSTEAP1-KI mice were inoculated with RM9-hSTEAP1-fluc cells by tail vein injection as described above. When metastatic colonization was confirmed, mice were randomized into four groups: (a) untransduced T cells, (b) STEAP1-mBBZ CAR T cells, (c) untransduced T cells + CBD-IL-12, and (d) STEAP1-mBBZ CAR

T cells + CBD-IL-12. 48 h prior to T cell infusion, groups c and d were pre-treated with 25 μ g CBD-IL-12 by retroorbital sinus injection and then weekly thereafter. 24 h prior to T cell infusion, groups a and b received preconditioning cyclophosphamide 100 mg/kg. 5×10^6 mouse untransduced or STEAP1-mBBZ CAR T cells suspended in 100 μ l of PBS was injected by tail vein. Tumor burden monitoring and collection of retroorbital blood and tissue samples were performed as described above.

For all mice experiments, animals were routinely monitored for disease progression and overt toxicity such as $>10\%$ decline in body weights, lethargy, immobility, fur ruffling, and fur loss. For the subcutaneous mouse tumor model, mice were euthanized when the tumor size approached 2.0 cm in diameter which is the maximum tumor size allowed for a mouse bearing a single tumor. For metastatic mouse models, mice were sacrificed when tumor burden based on bioluminescence imaging reached $>1 \times 10^{12}$ photons/sec/cm²/steradian or institutional compassionate euthanasia criteria were met. Maximal tumor size/burden was not exceeded.

Immunohistochemical studies

Tissue sections were deparaffinized in xylene and rehydrated in 100, 95, 75% ethanol, and finally TBS with 0.1% Tween 20 (TBST). Antigen retrieval was conducted in Citrate-Based Antigen Unmasking Solution (Vector Labs) using a pressure cooker at 95 °C for 30 min. Tissue sections were blocked with Dual Endogenous Enzyme-Blocking Reagent (Agilent Technologies) and incubated for 10 min followed by three washes with TBST. Slides were incubated with primary antibody in a humidified chamber at 37 °C for one hour. Primary antibodies and dilutions used for this study are rabbit anti-STEAP1 polyclonal antibody (LS Bio, LS-C291740, 1:500), rabbit anti-CD3 antibody (Thermo Fisher, MA5-14524, 1:100), mouse anti-PSMA antibody (Dako, Clone 3E6, M3620, 1:50), rabbit anti-Androgen Receptor antibody (Abcam, Clone EPRI535(2), ab133273), mouse anti-SYP/Synaptophysin Antibody (Santa Cruz, sc-17750 (D4), 1:50), rabbit anti-beta-2-microglobulin antibody (Clone EPR21752-214, Abcam, ab218230, 1:1000) and mouse anti-HLA Class 1 ABC antibody (Clone EMR8-5, Abcam, ab70328, 1:3000). Slides were washed with TBST and incubated with Power-Vision Poly-HRP anti-rabbit IgG or anti-mouse IgG (Leica Biosystems) in a humidified chamber at 37 °C for 30 min. Slides were washed with TBST and incubated with 3,3'-Diaminobenzidine (DAB) (Sigma Aldrich) at room temperature for 10 min. DAB was quenched in deionized water. Slides were stained in Dako hematoxylin (Agilent Technologies) at room temperature for one minute and washed with deionized water for five minutes. Slides were dehydrated in 75, 95, 100% ethanol, and finally xylene. Slides were mounted using Permount mounting medium (Fisher Chemical), and cover slipped. Stained slides were scanned using Ventana DP200 (Roche). QuPath 0.2.3 was used to analyze the IHC images.

Prostate cancer tissue microarray analysis

University of Washington mCRPC Tissue Acquisition Necropsy (TAN) tissue microarrays (Prostate Cancer Biorepository Network) were used for immunohistochemical studies. Human tissues studies were approved by the Institutional Review Board of the University of Washington (Protocol #2341). All rapid autopsy tissues represented on the tissue microarray were collected from patients who provided informed written consent under the Prostate Cancer Donor Program at the University of Washington. Each core stained with either STEAP1 or PSMA was scored by an experienced pathologist (M.P.R) and assigned an intensity score of 0, 1, 2, or 3 and frequency of positive cell staining ranging from 0 to 100%. H-scores were generated for each core by multiplying the intensity score by the frequency of positive cell staining resulting in a minimum of 0 and maximum of 300. The average H-score of replicate cores represented in the tissue microarray was determined for each mCRPC tissue.

Immunoblotting

Protein extracts were collected in a 9M urea lysis buffer and quantified using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher). Protein samples were fractionated with SDS-PAGE using Bolt 4–12% Bis-Tris Plus Gels (Thermo Fisher) and transferred to nitrocellulose membranes using the Invitrogen Mini Blot module (Thermo Fisher). Membranes were blocked with 5% non-fat milk in PBS + 0.5% Tween 20 (PBST) on a shaker at room temperature for 30 min. Membranes were then incubated with primary antibody on a shaker at 4 °C overnight. Primary antibodies used for this study are mouse anti-STEAP1 (Clone B-4, Santa Cruz, sc-271872, 1:1000) for human STEAP1, Polyclonal Rabbit anti-STEAP1 antibody (BioRad, AHP1438) for murine STEAP1, and GAPDH (Clone GT239, GeneTex, GX627408, 1:5000). Membranes were washed with PBST and incubated with goat anti-mouse IgG (H + L) secondary antibody conjugated with horseradish peroxidase (Thermo Fisher, 31430, 1:10,000) on a shaker at room temperature for 1 h. Membranes were washed with PBST, incubated with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) at room temperature for three minutes, and visualized on a ChemiDoc MP Imaging System (Bio-Rad Laboratories).

Absolute STEAP1 quantification

Flow cytometric quantification of STEAP1 antigen density across human prostate cancer cell lines was performed using Quantum Simply Cellular microspheres (Bangs Laboratories) per the manufacturer's protocol. Vandortuzumab (Creative Biolabs) was used as the primary antibody and rat anti-human IgG Fc antibody conjugated to APC (BioLegend) was used as the secondary antibody. Stained cells and beads were analyzed on a BD FACSCanto II (BD Biosciences). The Geo Mean or Median channel values for each population were recorded into the provided QuickCal spreadsheet yielding a regression coefficient of 0.998.

Chimeric antigen receptors (CAR) expression plasmids

gBlocks (Integrated DNA Technologies) encoding the GM-CSF leader, DSTP3086S scFv (VL-[G4S3]-VH), IgG4 spacers, CD28 transmembrane domain, 4-1BB costimulatory domain, CD3ζ chain, EGFRt or mCD19t, and WPRE were cloned into the EcoRI site of pCCL-c-MNDU3-X (gift from Donald Kohn, Addgene plasmid #81071) or pMYs (Cell Biolabs) by HiFi DNA Assembly (New England Biolabs). Sequences involving cloning junctions and open reading frames were validated by Sanger sequencing at the Fred Hutch Genomics & Bioinformatics Shared Resource.

CAR lentivirus and retrovirus production

For STEAP1-BBζ CAR lentivirus production, HEK 293 T cells (ATCC) were thawed, cultured, and expanded in DMEM media supplemented with 10% FBS and GlutaMAX. HEK 293 T cells were seeded on plates coated with Cultrex Poly-L-Lysine (R&D Systems) prior to transfection with the pCCL-c-MNDU3 STEAP1-BBζ CAR lentiviral plasmid and the packaging plasmids pMDL, pVSVg, and pREV using the TransIT-293 transfection reagent (Mirus Bio). About 18 h after transfection, sodium butyrate and HEPES were added to each plate to a final concentration of 20 mM each. Eight hours later, media was aspirated from the plates and each plate was washed with PBS. DMEM media supplemented with 10% FBS, GlutaMAX, and 20 mM HEPES was added to each plate. Lentiviral supernatant was collected at 48 h after transfection, vacuum filtered through a 0.22 μm filter, and concentrated by ultracentrifugation in polypropylene Konical tubes (Beckman Coulter) at 85,929 g at 4 °C for two hours in an Optima XE 90 (Beckman Coulter). Lentiviral pellets were resuspended in the minimal residual media present after aspirating off supernatant, aliquoted in cryovials and stored at -80 °C.

For STEAP1-mBBζ CAR retrovirus production, PLAT-E cells (Cell Biolabs) were thawed and cultured in DMEM media supplemented with

10% FBS, GlutaMAX, 1 μg/ml puromycin, and 10 μg/ml blasticidin. One day prior to seeding cells for transfection, PLAT-E cells were washed and seeded in antibiotic-free DMEM media supplemented with 10% FBS and GlutaMAX. PLAT-E cells were transfected with the pMYs STEAP1-mBBζ CAR retroviral construct using the FuGENE HD transfection reagent (Promega). 48 and 72 h after transfection, supernatants containing retrovirus were passed through a 0.22 μm syringe filter prior to use in transduction.

Human CAR T cell manufacturing

Peripheral blood mononuclear cells (PBMCs) from three de-identified healthy donor used in this study was reviewed by the Institutional Review Board of the Fred Hutchinson Cancer Center and deemed Not Human Subjects Research. PBMCs were collected from participants who provided informed written consent under the Co-Operative Center for Excellence in Hematology at Fred Hutchinson Cancer Center. PBMCs were thawed and washed with pre-warmed TCM base media consisting of AIM-V media (Gibco) supplemented with 55 mM beta-mercaptoethanol, human male AB plasma (Sigma), and GlutaMAX. PBMCs were centrifuged in a tabletop centrifuge at 600 g for 5 min. Cells were resuspended in TCM base media and counted on a hemacytometer. Dynabeads CD8 and CD4 Positive Isolation Kits were used per manufacturer's protocol to separate CD8 and CD4 T cells. After bead detachment, CD8 T cells were seeded in CD8 media (TCM base media supplemented with 50 U/ml human IL-2 and 0.5 ng/ml human IL-15) and CD4 T cells were seeded in CD4 media (TCM base media supplemented with 0.5 ng/ml human IL-15 and 5 ng/ml human IL-7). CD8 and CD4 T cells were activated and expanded with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher) per manufacturer's protocol. After two to four days, CD8 and CD4 T cells were counted and transduced with STEAP1-BBζ CAR lentivirus at a relative multiplicity-of-infection of 10 based on the infectious titer on HEK 293 T cells. Lentiviral transduction was performed in the presence of 10 μg/ml protamine sulfate. 48 h after transduction, T cells were collected, activation beads removed, and transduction efficiency of the T cells evaluated by flow cytometry. CAR-modified CD4 and CD8 T cells were counted every two days and maintained at a density of 10⁶ cells/ml in their respective CD4 and CD8 media.

Mouse CAR T cell manufacturing

Splenocytes were harvested from the manual dissociation of spleens obtained from heterozygous hSTEAP1-KI mice. Splenocytes were passed through a 70 μm strainer and pelleted by centrifugation at 600 g for six minutes. Cells were resuspended in RBC lysis buffer (BioLegend) and incubated on ice for five minutes. Cells were washed with PBS and pelleted by centrifugation. Murine CD3 T cells were isolated using Mouse CD3 + T Cell Enrichment Columns (R&D Systems) per the manufacturer's protocol. T cells were cultured in RPMI 1640 media containing 10% FBS, 50 U/ml human IL-2, 10 ng/ml murine IL-7, and 50 μM beta-mercaptoethanol. T cells were activated and expanded with Dynabeads Mouse T-Activator CD3/CD28 (Thermo Fisher) per manufacturer's protocol. 48 and 72 h later, mouse T cells were transduced with filtered pMYs STEAP1-mBBζ CAR retroviral supernatants via spinoculation on a tabletop centrifuge at 2000 × g at 30 °C for two hours. On day six of culture, beads were magnetically removed, and T cell transduction efficiency was determined by flow cytometry prior to use in functional assays.

Immunophenotyping CAR T cell products and assessment of peripheral persistence

1.5 × 10⁵ human untransduced or STEAP1-BBζ CAR T cells, mouse untransduced or STEAP1-BBζ CAR T cells, or splenocytes were incubated with fluorophore conjugated antibodies on ice for 20 min. Cells were washed with PBS after antibody staining and acquired on a BD

FACSCanto II or BD Symphony 4. Data were analyzed on FlowJo v.10.8 (Treestar).

Antibodies used for immunophenotyping transduced human T cells were mouse anti-human CD3 conjugated to APC (1:100, clone SK7, Thermo Fisher, 47-0036-42), mouse anti-human CD8 conjugated to FITC (1:100, clone RPA-T8, BD Biosciences, 555366), rabbit anti-human EGFR (1:100, clone C225, cetuximab) conjugated to PE (Novus Biologicals, NBP2-52671PE).

Antibodies used for immunophenotyping murine transduced splenocytes were rat anti-mouse CD8a conjugated to FITC (1:100, clone 53-6.7, BioLegend, 100706) and rat anti-mouse CD19 conjugated to PE (1:100, clone 6D5, BioLegend, 115508).

For immunophenotyping differentiation and exhaustion state of STEAP1-BB ζ CAR T cells from different donors were mouse anti-human CD3 conjugated to BUV395 (5 μ l per condition, clone UCHT1BD, Biosciences, 563548), mouse anti-human CD4 conjugated to BV605 (0.5 μ l per condition, clone SK3, BioLegend, 344645), mouse anti-human CD8 conjugated to BUV805 (5 μ l per condition, clone SK1, BD Biosciences, 564912), mouse anti-human CD45RO conjugated to BV510 (2 μ l per condition, clone UCHL1, BioLegend, 304246), mouse anti-human CD45RA conjugated to BV711 (0.1 μ l per condition, clone HI100, BioLegend, 304138), mouse anti-human PD-1 conjugated to APC (2 μ l per condition, clone A17188B, BioLegend, 621610), mouse anti-human CD95 conjugated to BUV615 (2 μ l per condition, clone DX2, BD Biosciences, 752346), mouse anti-human CXCR3 conjugated to BV421 (2 μ l per condition, clone G025H7, BioLegend, 353716), mouse anti-human CD62L conjugated to BV785 (2 μ l per condition, clone SK11, BD Biosciences, 565311), and mouse anti-human LAG-3 conjugated to BV421 (2 μ l per condition, clone T47-530, BD Biosciences, 565721).

Tumor immune microenvironment analysis using multi-parametric flow cytometry

Tumor infiltrating lymphocytes from RM9-hSTEAP1 tumors treated with untransduced T cells and STEAP1-mBB ζ CAR T cells with or without CBD-IL-12 were isolated using mechanical dissociation of tumor chunks followed by dispase/collagenase (10 mg/ml) digestion at 37 °C for 1 h. Dissociated cells were treated briefly with trypsin for 5 min followed by syringe dissociation and incubation in 1x RBC lysis buffer for 5 min. Isolated single cells were frozen or processed for downstream analysis. For tumor immune cell phenotyping analysis, 2.5×10^6 cells were counted and resuspended in PBS followed by incubation in Fc Block buffer (TruStain FcX, 101319) for 15 min at room temperature. Cells were subsequently washed twice with PBS and were stained with Fixable Viability Dye (Zombie UV, 1:1000) for 15 min. After washing, cells were stained with a cocktail of anti-mouse cell-surface antibodies including pan-CD45-BUV805 (1:320, clone 30-F11, BD Biosciences); Dump APCF-750 including antibodies against B220/CD45R (1:200, clone RA3-6B2, eBioscience), CD19 (1:200, clone 6D5, BioLegend), CD3 (1:200, clone 145-2C11, BioLegend), CD4 (1:200, clone RM4-5, BioLegend), and CD8a (1:200, clone 53-6.7, BioLegend); CD103-BUV395 (1:200, clone 2E7, BD Biosciences); CD24-BUV496 (1:100, clone MI/69, BD Horizon); Ly6G-BUV737 (1:160, clone IA8, BD Biosciences); NK1.1-BV421 (1:80, clone PK136, BioLegend); KLRG1-BV510 (1:100, clone 2F1, BD Biosciences); CD11b-BV605 (1:100, clone MI/70, BioLegend); XCR1-BV785 (1:100, clone ZET, BioLegend); F4/80 AF488 (1:100, clone T45-2342, BD Biosciences); Ly6C PerCP-Cy5.5 (1:200, clone HK1.4, BioLegend); CD69 PE (1:100, clone HL2F3, BioLegend); Siglec-F PE-CF594 (1:400, clone E50-2440, BD Biosciences); and MHC-II A700 (1:400, clone M5/114.15.2, BioLegend). Cells were incubated with the antibody cocktail for 30 min followed by two washes with FACS buffer. Cells were incubated overnight in the Perm wash buffer for membrane permeabilization at 4 °C. On the following day, cells were washed once and stained for intracellular proteins including anti Ki-67 BV650 (1:100, clone B56, BD Biosciences), FOXP3 e450 (1:100, clone

FJK-16s, eBioscience) and iNOS APC (1:100, clone CXNFT, eBiosciences) for 30 min. Cells were washed and were analyzed on Cytex® Aurora System. The detailed gating strategy for analysis of different immune cell subsets including neutrophils, eosinophils, monocytes/macrophages, and dendritic cells is provided in Supplementary Fig. 18. The frequencies of individual populations were plotted and statistically analyzed using GraphPad Prism v9.4.1.

Immunologic co-culture assays

CAR T cell functional assays were performed by co-culturing prostate cancer cells engineered to express GFP with either human or mouse untransduced or STEAP1 CAR T cells at variable effector-to-target (E:T) ratios in 96-well plates. For cytotoxicity assays, 96-well clear bottom black wall plates (Corning) were coated with Cultrex Poly-L-Lysine for 30 min and seeded with prostate cancer cells. Plates were incubated at 37 °C for one hour. Effector cells were then counted and seeded into wells with tumor cells at specified E:T ratios. The plates were placed into a BioTek BioSpa 8 Automated Incubator (Agilent Technologies) and read by brightfield and fluorescence imaging on a BioTek Cytation 5 Cell Imaging Multi-Mode Reader (Agilent Technologies) every six hours for a total of six days. Target cells were quantified based on the number of GFP⁺ objects identified per scanned area using BioTek Gen5 Imager Software (Agilent Technologies). To assess T cell activation based on cytokine release, 25–50 μ l of co-culture supernatants were collected at 24 and 48 h of co-culture and stored at –30 °C.

Mitogen stimulation of T cells

T cells were stimulated with 5 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) and 250 ng/ml of ionomycin (Sigma Aldrich) or with 5 μ g/ml of phytohemagglutinin-L (PHA-L, Sigma Aldrich). Supernatants were collected at 24 h for use as a positive control for IFN- γ ELISA studies or T cells were used as a positive control for induction of the exhaustion markers PD-1 and LAG-3 as assessed by flow cytometry.

Enzyme-linked immunosorbent assay

To determine IFN- γ levels in co-culture supernatants, samples were thawed and sandwich ELISA for human or mouse IFN- γ levels was performed using the BD Human IFN-gamma ELISA Set (BD Biosciences, 555142) or BD Mouse IFN-gamma ELISA Set (BD Biosciences, 555138) according to the manufacturer's protocol. Plates were read at 450 nm and 560 nm wavelengths using a BioTek Cytation 3 Cell Imaging Multi-Mode Microplate Reader (Agilent Technologies). Plasma samples isolated from retroorbital bleeds were used for ProcartaPlex immunoassays (Thermo Fisher) to quantify levels of mouse IFN- γ , IL-2, IL-6, IL-4 and TNF- α according to the manufacturer's protocol. Samples were assayed on a Luminex 100/200 System (Luminex). Anti-human IgG and IgM antibodies from the sera of mice treated with untransduced T cells or STEAP1-mBB ζ CAR T cells were quantified using MAHA (Mouse Anti-Human Antibody) ELISA Assay Kit (Eagle Biosciences, MAH11-K01).

Bulk RNA-sequencing and analysis

Total RNA was isolated from 22Rv1 wt, 22Rv1 STEAP1 ko, and 22Rv1 STEAP1 ko + rescue cells using the Purelink™ RNA Mini Kit (Thermo Scientific). Samples with RNA integrity number (RIN) greater than 8.0 were processed for library preparation using the Illumina TruSeq Stranded mRNA kit and sequencing on an Illumina NovaSeq 6000 with 2 $\times$ 100 bp paired-end reads by the Fred Hutch Genomics and Bioinformatics Shared Resource.

For RNA-seq on the 22Rv1 metastatic tumor samples from animals treated with untransduced T cells or STEAP1-BB ζ CAR T cells, total RNA was isolated using the RNeasy FFPE Kit (Qiagen). DV200 were assessed by TapeStation (Agilent) and tumor samples with DV200 > 30% were selected for library preparation using the Illumina TruSeq RNA Exome

kit and sequencing on an Illumina NovaSeq 6000 with 2 × 50 bp paired-end reads by the Fred Hutch Genomics and Bioinformatics Shared Resource.

Data quality check for RNA-seq results were performed using NovaSeq Control Software (v1.8.0) and demultiplexing was performed with the Illumina Bcl2fastq2 (v2.20) program. FASTQ files were processed using the UCSC TOIL (v5.7.1) processing pipeline⁸³. Reads were assessed for quality control using FASTQC and aligned to the hg38 human reference genome. A count matrix filtered for coding genes using HUGO and containing raw log2 transformed gene expression values was generated. Differential gene expression analysis was performed using DESeq2 (v3.15) with default parameters⁸⁴. Pathway analysis was performed using Gene Set Enrichment Analysis (v4.3.1)⁸⁵ and the cell cycle progression (CCP) score was calculated as previously described⁵⁴. Bioinformatic analysis was performed using R (v4.0.3) and R studio (v1.3.1093).

Single-cell RNA-sequencing and analysis

Single-cell libraries of RM9 tumor cells from mice treated with vehicle or CBD-IL-12 were generated per the 10x Genomics Chromium platform and sequenced on an Illumina NovaSeq 6000 in the Fred Hutch Genomics and Bioinformatics Shared Resource. Reads were aligned to the GRCm38/mm10 mouse reference genome and quality control and data processing was performed using Seurat V4⁸⁶. Annotation of cell type was performed using the Bioconductor package SingleR v1.10.0⁸⁷ based on the ImmGenData reference.

TCR repertoire analysis

Genomic DNA from metastatic lung tumors were isolated using the GeneJET Genomic DNA Purification Kit (Thermo Fisher). Subsequent TCRV-β sequencing was performed on the immunoSEQ platform (Adaptive Biotechnologies). Productive (in-frame) TCR rearrangements were used for the clonality assessment based on Simpson clonality index using immunoSEQ Analyzer 3.0 (Adaptive Biotechnologies). Non-parametric multiple comparison Dunn's test was used to estimate the significance between means of each group and adjusted p-values are reported.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Sequencing data pertaining to this study is available from Gene Expression Omnibus (GEO) as SuperSeries [GSE214585](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214585). RNA-seq data from the parental 22Rv1, 22Rv1 STEAP1 ko, and 22Rv1 STEAP ko + rescue cells lines and 22Rv1 tumors treated with untransduced T cells or STEAP1-BBζ CAR T cells (resulting in STEAP1 antigen loss) is available from accession number [GSE214583](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214583). Single-cell RNA-seq (scRNA-seq) data for RM9 tumors treated with vehicle or CBD-IL-12 is available from accession number [GSE214584](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214584). T cell receptor (TCR) beta sequencing profiles are available from accession number [GSE228884](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE228884) and also as part of the ImmuneCODE data resource available to be downloaded from the Adaptive Biotechnologies immuneACCESS site under the immuneACCESS Terms of Use at: <https://doi.org/10.21417/VB2023NC>. Source data are provided with this manuscript in the Source Data file. All remaining data related to the study are included in the article, Supplementary Information and Source Data file. Source data are provided with this paper.

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Competing interests

T.E.P. and J.K.L. are inventors on U.S. Provisional Patent Application No. 63/309389 entitled “Chimeric Antigen Receptors Binding STEAP1”

related to this work. J.I. is a co-founder and shareholder of Arro-wimmune, Inc. J.I. is a scientific advisor of Libo Pharma Corp. The remaining authors declare no competing interests.

Additional information

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Supplementary Information

Targeting advanced prostate cancer with STEAP1 chimeric antigen receptor T cell and tumor-localized IL-12 immunotherapy

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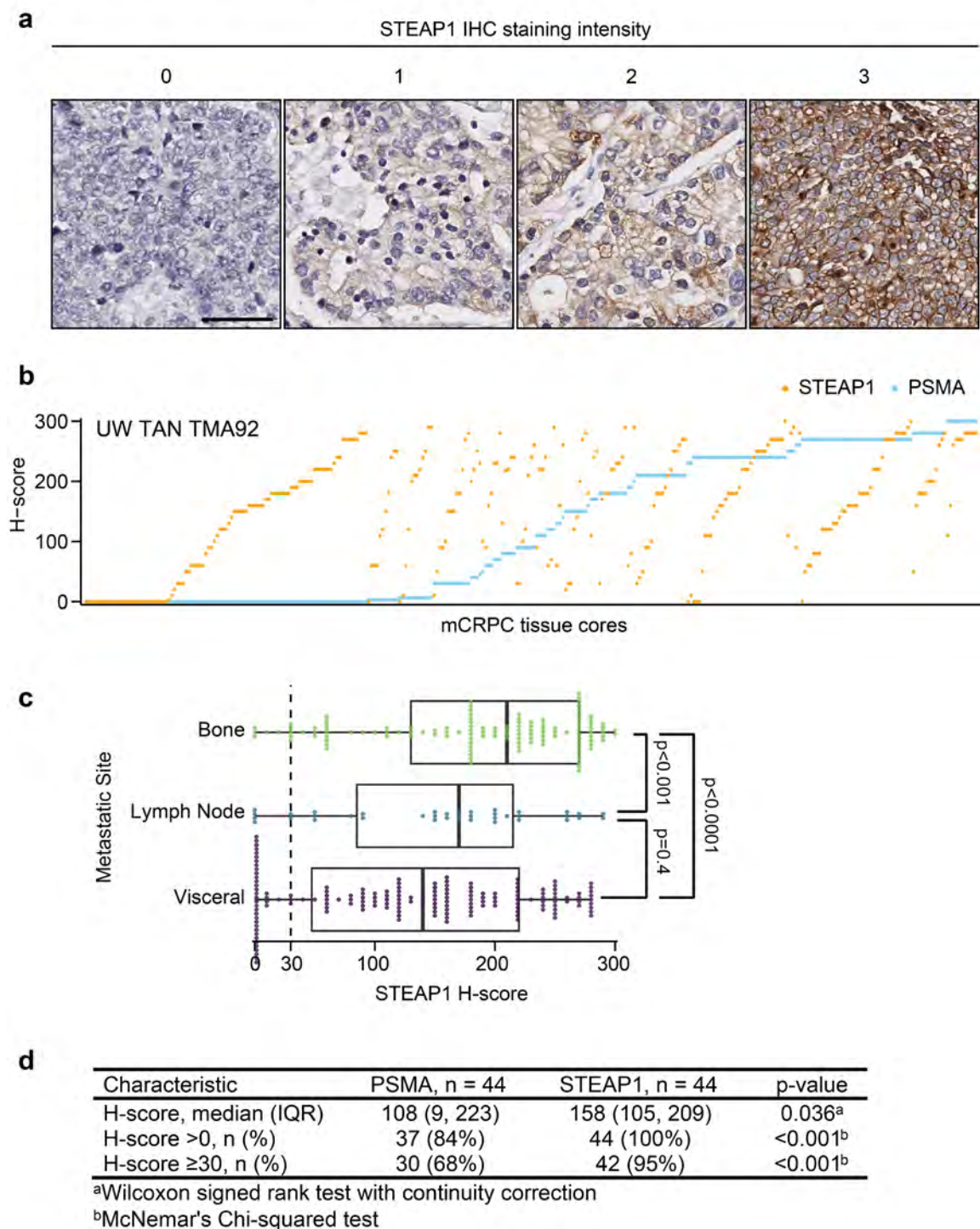
Supplementary Information includes:

Supplementary Figures S1 to S19

Other Supplementary Materials for this manuscript include:

Source Data file

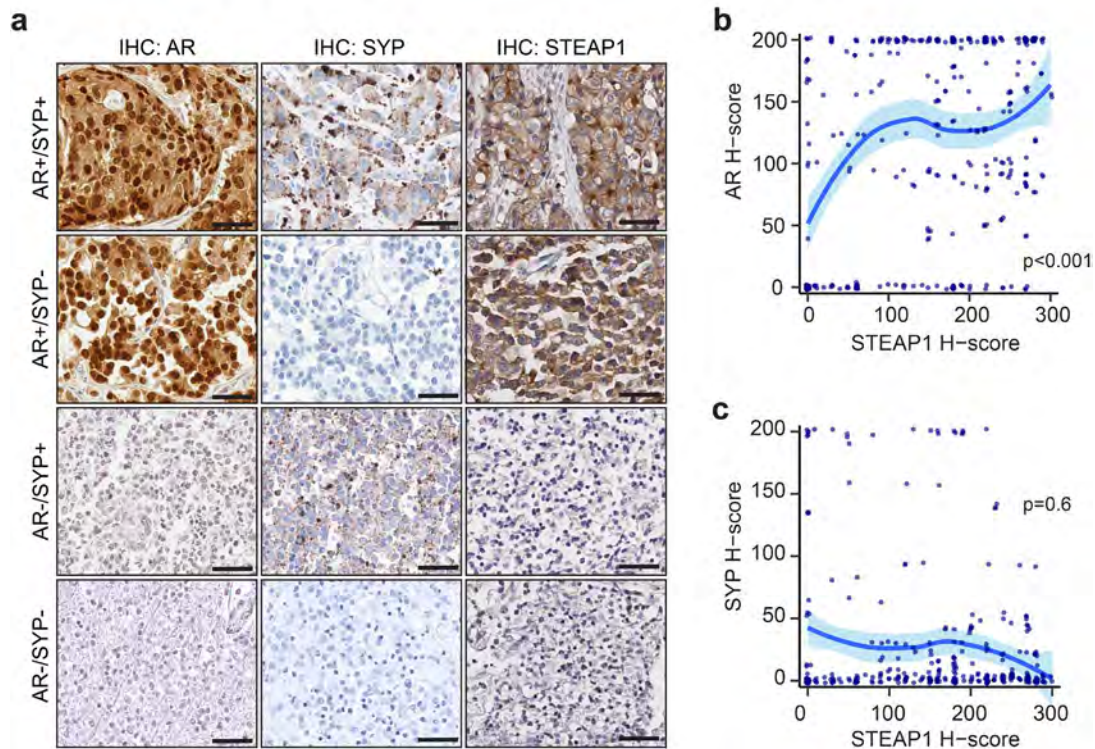
Supplementary Figure 1



Supplementary Figure 1. Characteristics of STEAP1 expression in lethal mCRPC tissues. (a) Photomicrographs of select mCRPC tissue cores after STEAP1 IHC staining to highlight the plasma membrane staining consistent with staining intensity scores of 0, 1, 2, and 3. Scale bars = 50 μ m. (n=332 mCRPC cores were stained for STEAP1) (b) Plot showing paired H-scores of STEAP1 (blue) and PSMA (orange) immunohistochemical (IHC) staining of all cores from each mCRPC tissue. (c) Boxplots of STEAP1 H-scores of mCRPC tissue cores based on their metastatic site (n=332 tissue cores) show the median (thick vertical line), interquartile range spanning the 25th to the 75th

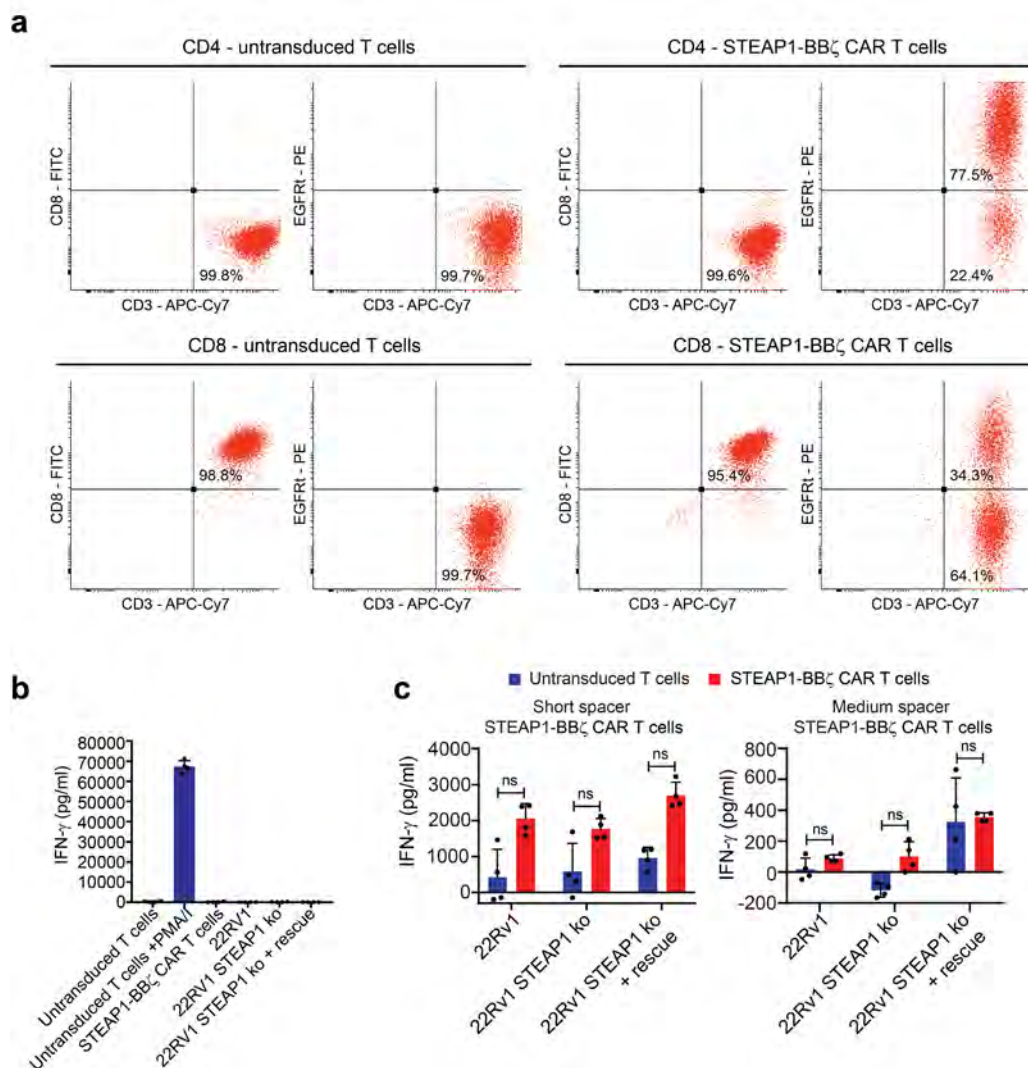
percentiles (thin rectangle), and the smallest and largest values that lie within 1.5 times the interquartile range of the 25th and 75th percentiles (horizontal “whiskers”). Superimposed dots show STEAP1 H-scores for individual tissue cores. Two-sided p-values were obtained from a linear mixed model with untransformed H-scores with random effect for patients. No adjustments were made for multiple comparisons. P-values for bone vs. visceral and bone vs. lymph nodes are <0.0001 . **(d)** Patient-level analysis of median H-scores (averaged within patients) using a Wilcoxon signed rank test and for the frequency of STEAP1 or PSMA IHC staining above the specified H-score thresholds ($p < 0.001$ for H-score ≤ 30 and > 0) using McNemar’s Chi-squared test. No adjustments were made for multiple comparisons. All p-values were two-sided. Source data are provided in the Source Data file.

Supplementary Figure 2



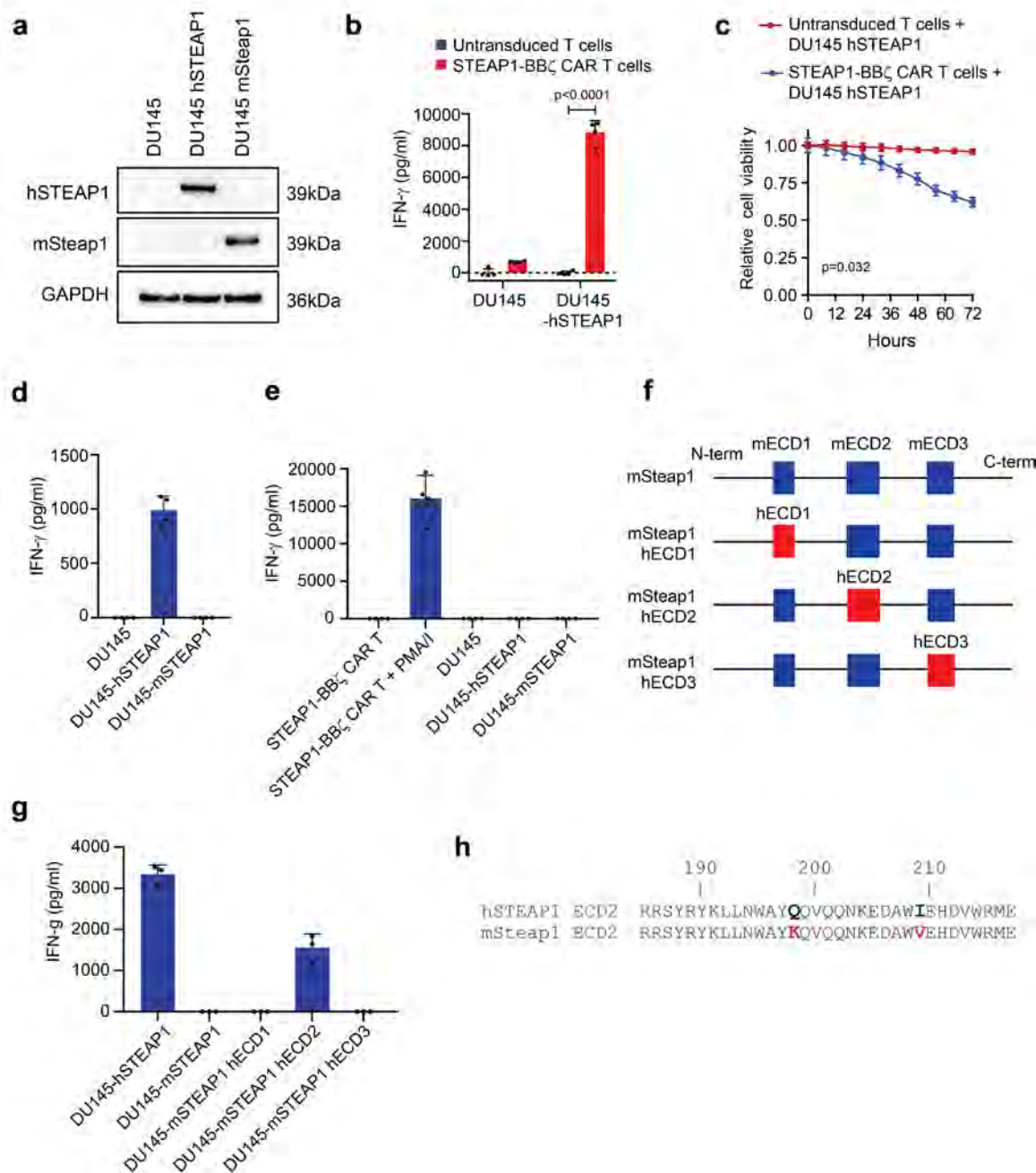
Supplementary Figure 2. STEAP1 expression positively correlates with androgen receptor expression in mCRPC. (a) Photomicrographs of androgen receptor (AR), synaptophysin (SYP), and STEAP1 IHC staining in representative sections from AR+/SYP+, AR+/SYP-, AR-/SYP+ and AR-/SYP- mCRPC tissues (n=332 mCRPC cores were stained for AR, SYP, STEAP1 and PSMA). Scatter plots of STEAP1 H-score and (b) AR H-score ($p < 0.001$) or (c) SYP H-score ($p = 0.6$) for each mCRPC tissue core (points), jittered to reduce overplotting, and a fitted local polynomial regression (smooth curve) with pointwise 95% confidence limits (semitransparent ribbon). Two-sided p-values in panels (b) and (c) were obtained from separate fitted linear mixed models with random effect for patients, applied to log-transformed H-scores after adding 1 to each H-score (to avoid taking the log of zero). No adjustments were made for multiple comparisons. Source data are provided in the Source Data file.

Supplementary Figure 3



Supplementary Figure 3. Validation of the antigen-specific activation and target cell cytotoxicity of STEAP1-BB ζ CAR T cells. (a) Representative flow cytometry plots showing immunophenotyping of CD4 and CD8 T cell products at day 9 of expansion from untransduced control and lentiviral STEAP1 CAR transduction conditions. IFN- γ quantification by ELISA from (b) control culture conditions including untransduced T cells with Phorbol-Myristate-Acetate and Ionomycin (PMA/I) and only target cells conditions. (c) IFN- γ quantification by ELISA from co-cultures of either untransduced T cells or short (left) and medium (right) spacer length STEAP1-BB ζ CAR T cells with parental 22Rv1, 22Rv1 STEAP1 knockout (ko) cells, and 22Rv1 STEAP1 ko with STEAP1 rescue cells at a 1:1 ratio at 24 hours. $n=4$ biological replicates per condition. Bars represent SEM. P-values were estimated using unpaired two-tailed Student's t test with Welch's correction. Source data are provided in the Source Data file.

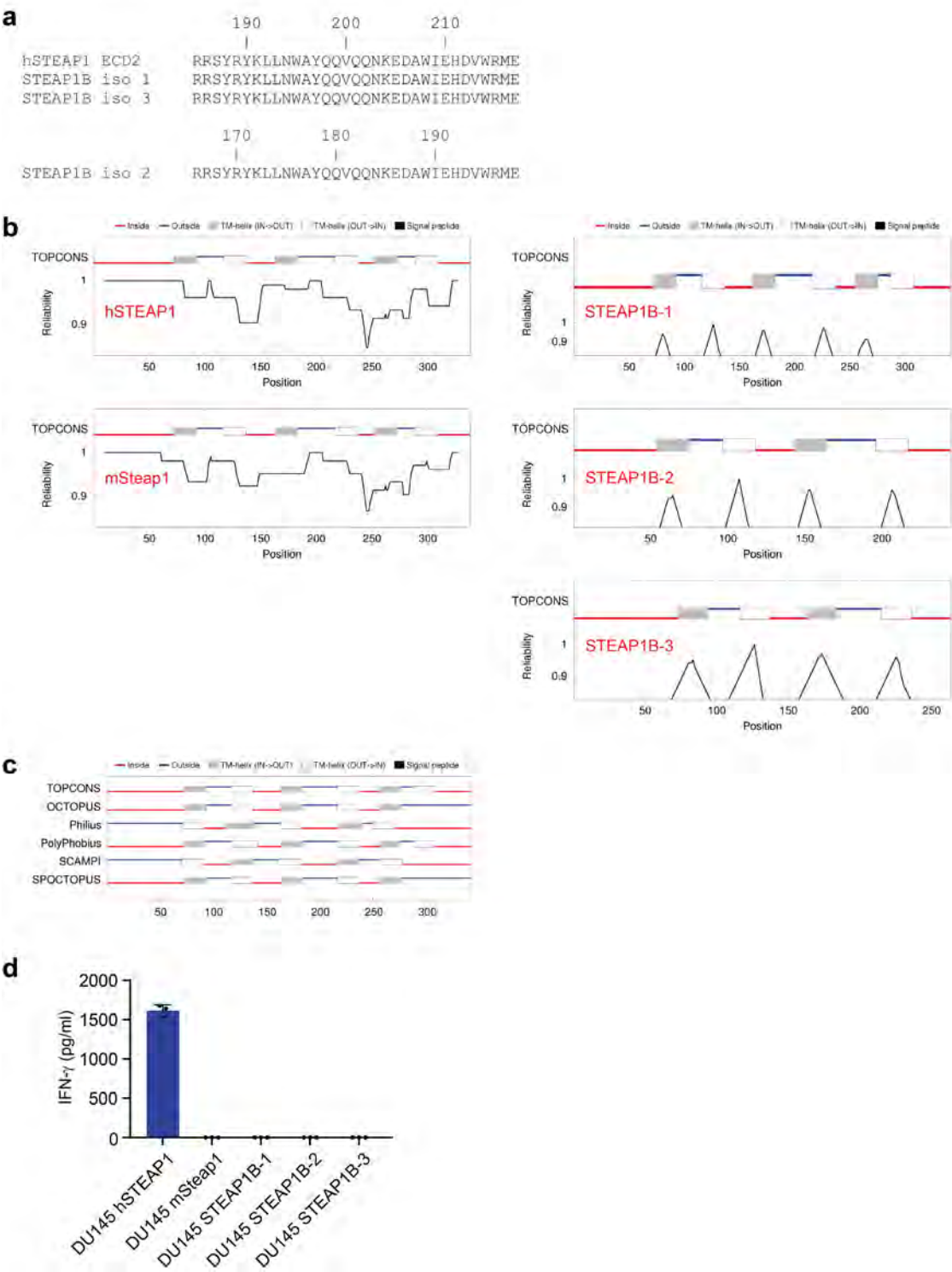
Supplementary Figure 4



Supplementary Figure 4. Determination of the STEAP1 ectodomain specificity of STEAP1-BB ζ CAR T cells using mouse/human Steap1 chimeras. (a) Immunoblot analysis confirming the lack of human STEAP1 (hSTEAP1) and mouse Steap1 (mSteap1) expression in the DU145 cell line and their respective expression in the lentivirally engineered DU145 hSTEAP1 and DU145 mSteap1 lines. GAPDH is used as a protein loading control. n=3 biologically independent experiments were performed. (b) IFN- γ quantification by ELISA from co-cultures of STEAP1-BB ζ CAR T cells with parental DU145 and DU145 hSTEAP1 cells. n=4 replicates per condition. Bars represent SD. (c) Relative cell viability of DU145 and DU145 hSTEAP1 target cells over time measured by fluorescence live cell imaging upon co-culture with STEAP1-BB ζ CAR T cells or untransduced T cells at a 1:1 ratio. n=4 biological replicates per condition. Bars represent mean with SEM. P-values were estimated using unpaired two-

tailed Student's t test with Welch's correction. **(d)** IFN- γ quantification by ELISA from co-cultures of STEAP1-BB ζ CAR T cells with the DU145, DU145 hSTEAP1, and DU145 mSteap1 cell lines at a 1:1 ratio or **(e)** control culture conditions at 24 hours. n = 4 biological replicates per condition. For panels **(d-e)** bars represent mean with SEM. **(f)** Schematic of the mSteap1 protein with extracellular domains (mECDs, blue) highlighted and the mouse/human Steap1 chimeric proteins each with individual replacement of a mECD with the counterpart hSTEAP1 extracellular domain (hECD, red). **(g)** IFN- γ quantification by ELISA from co-cultures of STEAP1-BB ζ CAR T cells with each of the DU145 lines engineered to express hSTEAP1, mSteap1, or mouse/human Steap1 chimeric proteins at a 1:1 ratio at 24 hours. n=4 biological replicates per condition. Bars represent mean with SD. **(h)** Alignment of the amino acid sequences of the hSTEAP1 ECD2 and mSteap1 ECD2 showing non-conserved residues at positions 198 and 209. Source data are provided in the Source Data file.

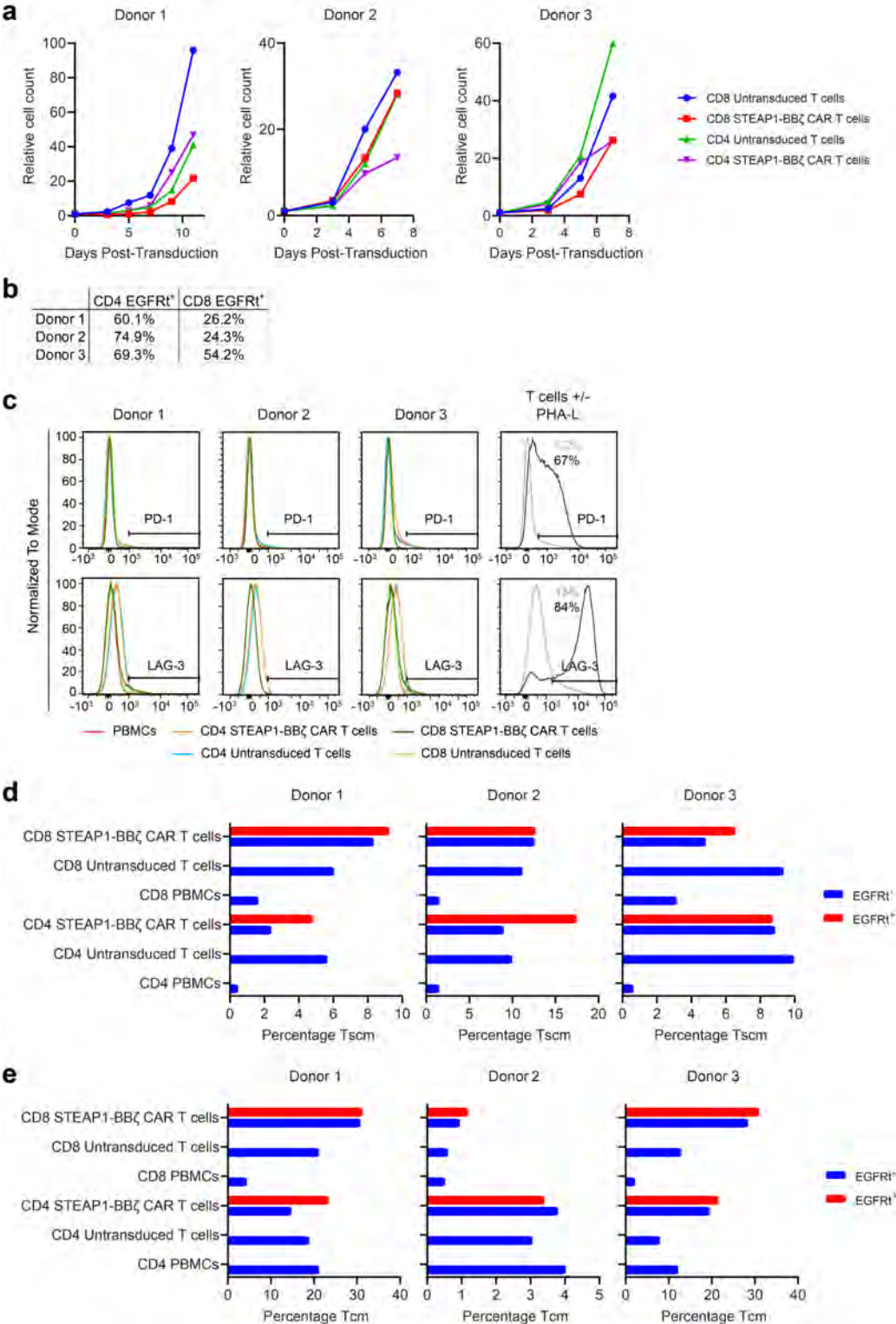
Supplementary Figure 5



Supplementary Figure 5. Evaluation of the reactivity of STEAP1-BB ζ CAR T cells to STEAP1B isoforms. (a) Alignment of the amino acid sequence of the hSTEAP1 ECD2 to human STEAP1B isoforms 1, 2, and 3 showing complete sequence conservation. (b) Plots of TOPCONS consensus predictions of membrane protein topology and reliability scores for hSTEAP1, mSteap1, and the three human STEAP1B isoforms. (c) Plots of membrane protein topology predictions for human STEAP1B

isoform 1 showing disagreement between different algorithms. **(d)** IFN- γ quantification by ELISA from co-cultures of STEAP1-BB ζ CAR T cells with each of the DU145 lines engineered to express hSTEAP1, mSteap1, or human STEAP1B isoforms at a 1:1 ratio at 24 hours. n=4 biological replicates per condition. Bars represent mean with SD. Source data are provided in the Source Data file.

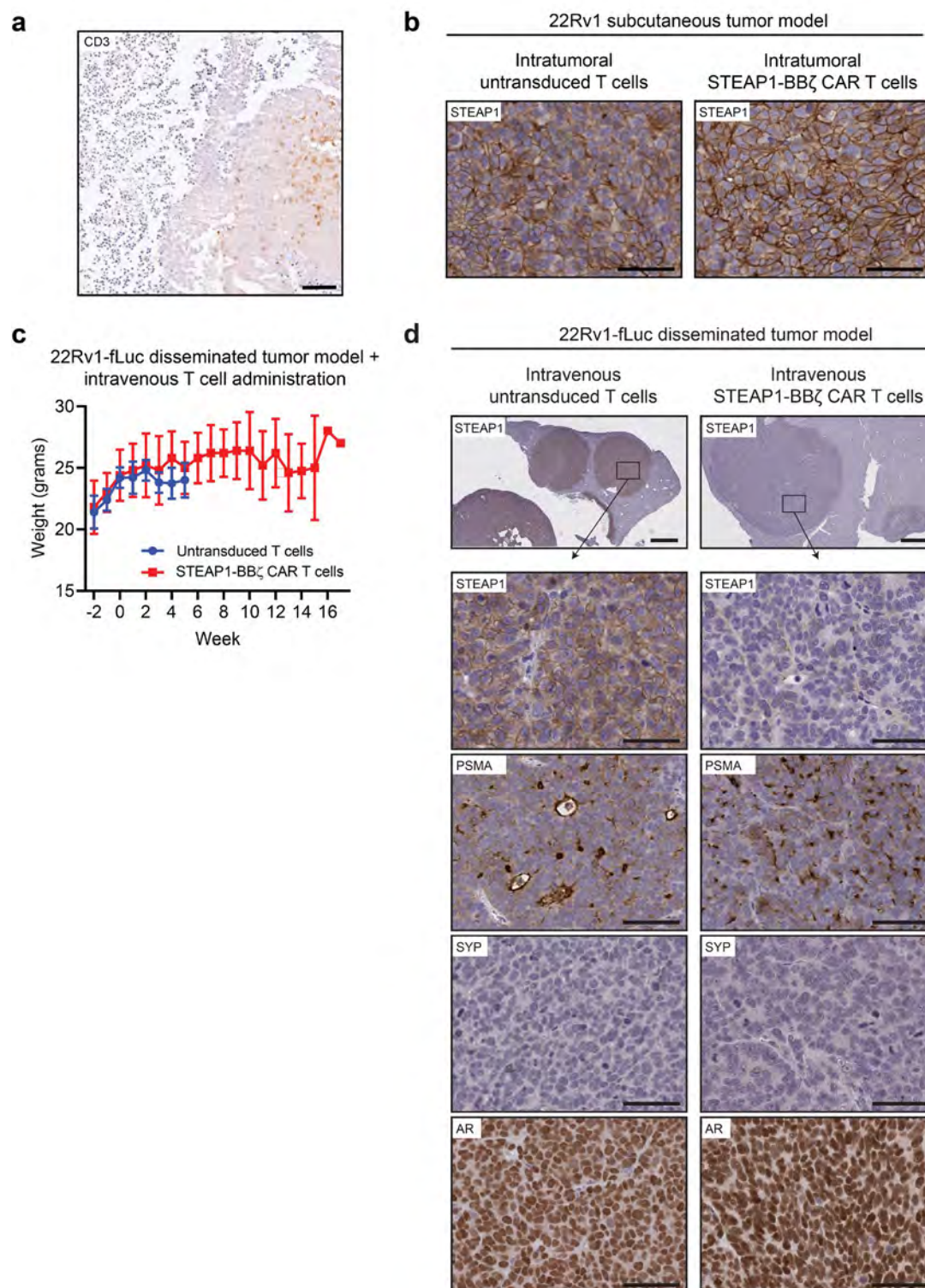
Supplementary Figure 6



Supplementary Figure 6. Characterization of the expansion, transduction efficiency, and differentiation states of STEAP1-BBζ CAR T cell products. (a) Plots showing the relative expansion of untransduced and STEAP1-BBζ CAR T cell subsets derived from three independent donors over time in culture. (b) Table showing the percentage of T cell subsets transduced with STEAP1-BBζ CAR lentivirus that express EGFRt as measured by flow cytometry seven days after transduction. (c) Flow

cytometry histogram plots showing the expression of the T cell exhaustion markers PD-1 and LAG-3 in PBMCs as well as untransduced and STEAP1-BBζ CAR T cell subsets. T cells treated with PHA-L are used as a positive control for induction of PD-1 and LAG-3 expression. Bar graphs showing the percentages of (d) stem cell memory T cells (Tscm: CD62L⁺ CD45RA⁺ CD95⁺ CXCR3⁺) and (e) central memory T cells (Tcm: CD62L⁺ CD45RA⁻) in T cell subsets from PBMCs as well as untransduced and STEAP1-BBζ CAR T cell products. Source data are provided in the Source Data file.

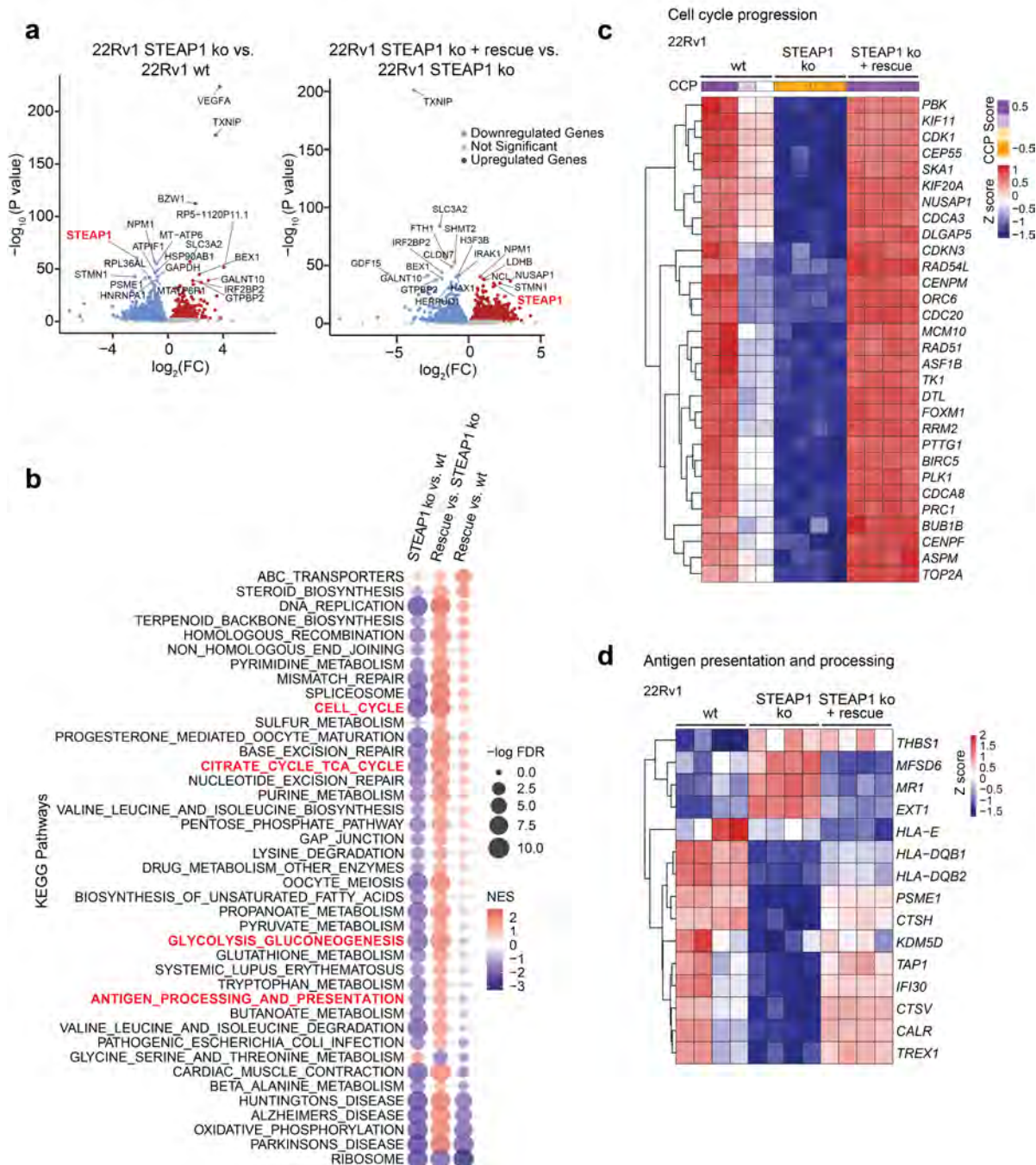
Supplementary Figure 7



Supplementary Figure 7. Antitumor activity of STEAP1-BBζ CAR T cell therapy in human 22Rv1 prostate cancer cell line xenograft models. (a) Photomicrograph of CD3 IHC staining of a 22Rv1 subcutaneous tumor 25 days after intratumoral treatment with STEAP1-BBζ CAR T cells. Scale bars = 100 μm. **(b)** Representative photomicrographs of STEAP1 IHC staining of 22Rv1-fLuc subcutaneous tumors after intratumoral treatment with (left) untransduced T cells or (right) STEAP1-BBζ CAR T cells.

Scale bars = 50 μ m. For panel **(a-b)** immunostaining was performed on n=4 biologically independent specimens. **(c)** Plot of average weights of NSG mice engrafted with 22Rv1-fLuc metastatic tumors over time after treatment with either untransduced T cells or STEAP1-BB ζ CAR T cells. n=5 mice per group. Bars represent mean with SD. **(d)** Representative low magnification (top) photomicrographs of STEAP1 IHC staining of 22Rv1-fLuc metastatic liver tumors after intravenous treatment with (left) untransduced T cells or (right) STEAP1-BB ζ CAR T cells. Scale bars = 1 mm. Higher magnification photomicrographs of STEAP1, PSMA, SYP and AR IHC staining of regions shown below. Scale bars = 50 μ m. Source data are provided in the Source Data file.

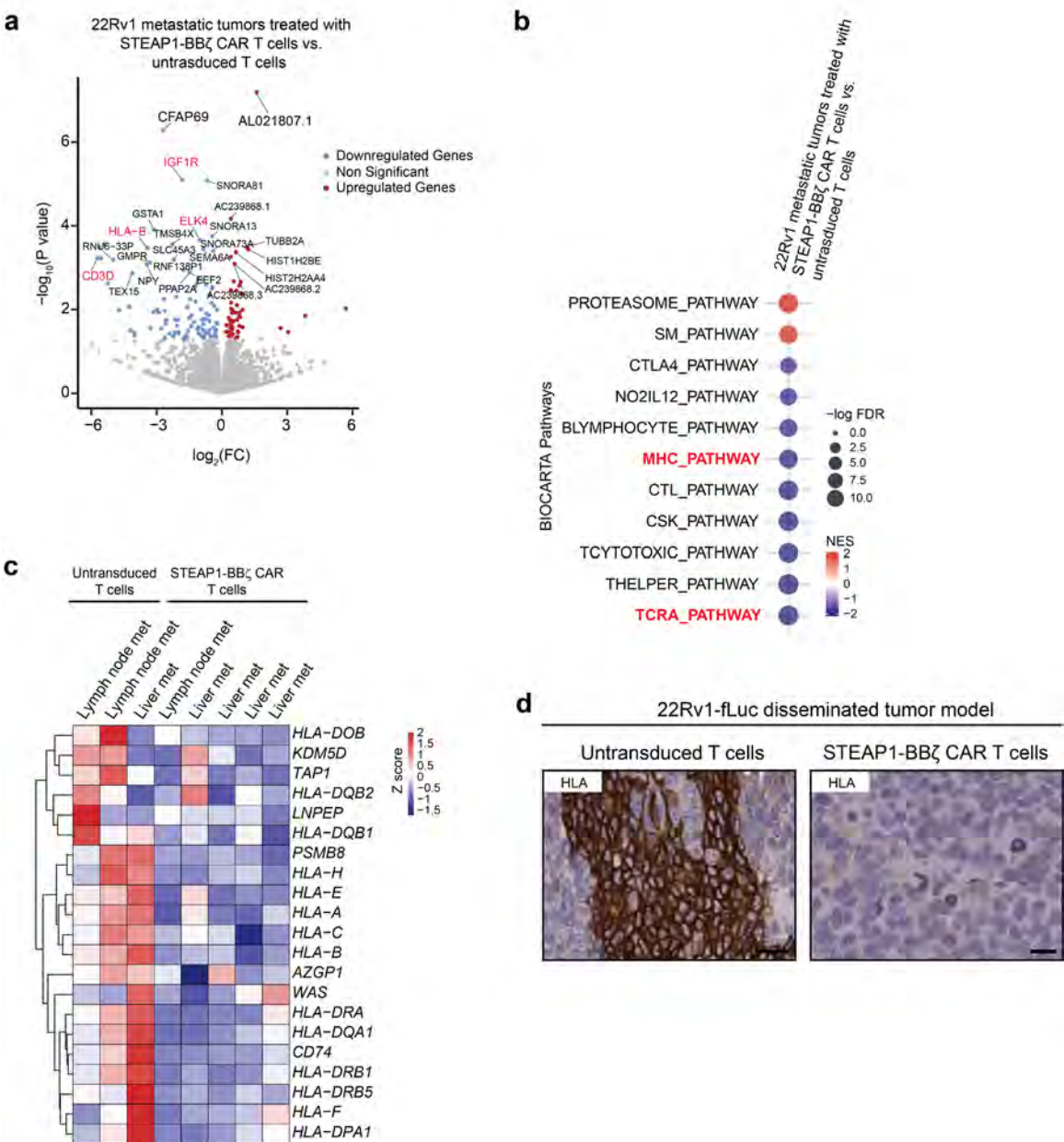
Supplementary Figure 8



Supplementary Figure 8. Transcriptome and pathway analysis of isogenic 22Rv1 cell lines with STEAP1 knockout and addback. (a) Volcano plots depicting differentially expressed genes in 22Rv1 STEAP1 knockout (ko) cells compared to 22Rv1 wildtype (wt) cells (left) and in 22Rv1 STEAP1 ko cells with addback of STEAP1 (+ rescue) compared to 22Rv1 STEAP1 ko cells (right). Red and blue dots represent upregulated and downregulated differentially expressed transcripts (DETs), respectively, with adjusted $p < 0.05$ and $\log_2$ (fold change) > 0.6 . Top 20 deregulated genes are marked. Wald-test was used for calculate the p-values with Benjamin-Hochberg correction. (b) Bubble plot showing KEGG pathways that are positively and negatively enriched in conditions mentioned in (a). The size of the bubbles represents $-\log$ FDR indicating the significance of enriched pathways while the color scheme represents the negative or positive normalized enrichment score (NES) predicted by GSEA. (c)

Heatmap representing the 31 gene signature used to evaluate the cell cycle progression (CCP) score in 22Rv1 wt, 22Rv1 STEAP1 ko, and 22Rv1 STEAP1 ko + rescue cells. The color schemes represents the Z score and the CCP score for each sample. **(d)** Heatmap showing differentially expressed genes (FDR<0.05) associated with antigen presentation and processing. For panels **(a-d)** data represent n=4 independent biological samples per conditions which were processed for RNA-seq.

Supplementary Figure 9

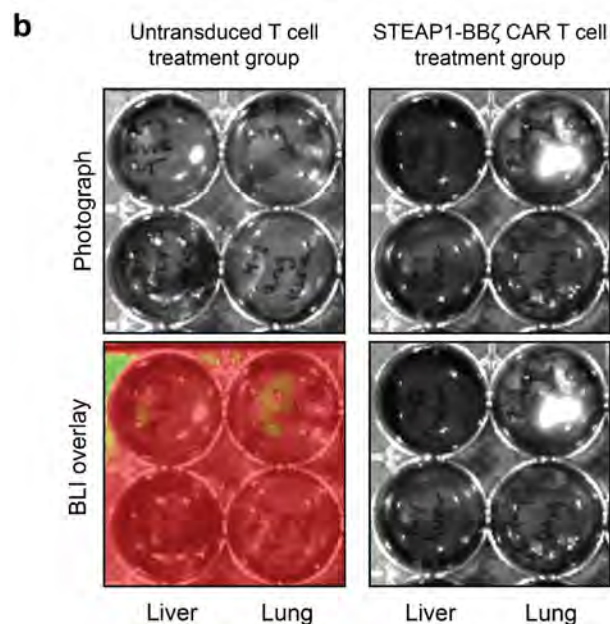
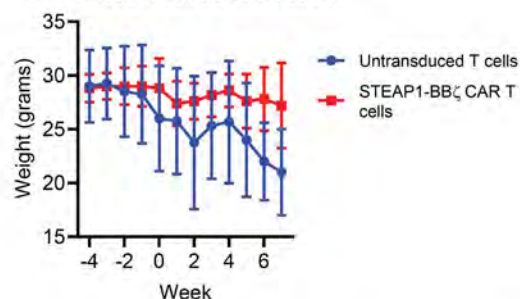


Supplementary Figure 9. Antigen presentation and processing is negatively enriched in 22Rv1 metastatic tumors with STEAP1 antigen loss after treatment with STEAP1-BB ζ CAR T cell therapy. (a) Volcano plots depicting differentially expressed genes in 22Rv1 metastatic tumors from mice treated with STEAP1-BB ζ CAR T cells compared to those treated with untransduced T cells. Red and blue dots represent upregulated and downregulated differentially expressed transcripts (DETs), respectively, with adjusted $p < 0.05$ and $\log_2$ (fold change) > 0.6 . The top 20 deregulated genes are marked and those associated with antigen processing and presentation are highlighted with gene names in red. Wald-test was used for calculate the p-values with Benjamin-Hochberg correction. (b) Bubble plot showing BIOCARTA pathways that are positively and negatively enriched in conditions mentioned in (a). The size of the bubbles represents $-\log$ FDR indicating the significance of enriched pathways while the color scheme represents the negative or positive NES predicted by GSEA. (c) Heatmap representing the genes associated with antigen presentation and processing in 22Rv1 metastatic tumor samples from mice treated with untransduced T cells and STEAP1-BB ζ CAR T cells.

(d) Representative photomicrographs of IHC staining for HLA-A,B,C in 22Rv1 metastatic liver tumors from mice treated with untransduced T cells (left) or STEAP1-BB ζ CAR T cells (right). Scale bars = 20 μ m. Immunostaining was performed on n=3 biologically independent specimens. Source data are provided in the Source Data file.

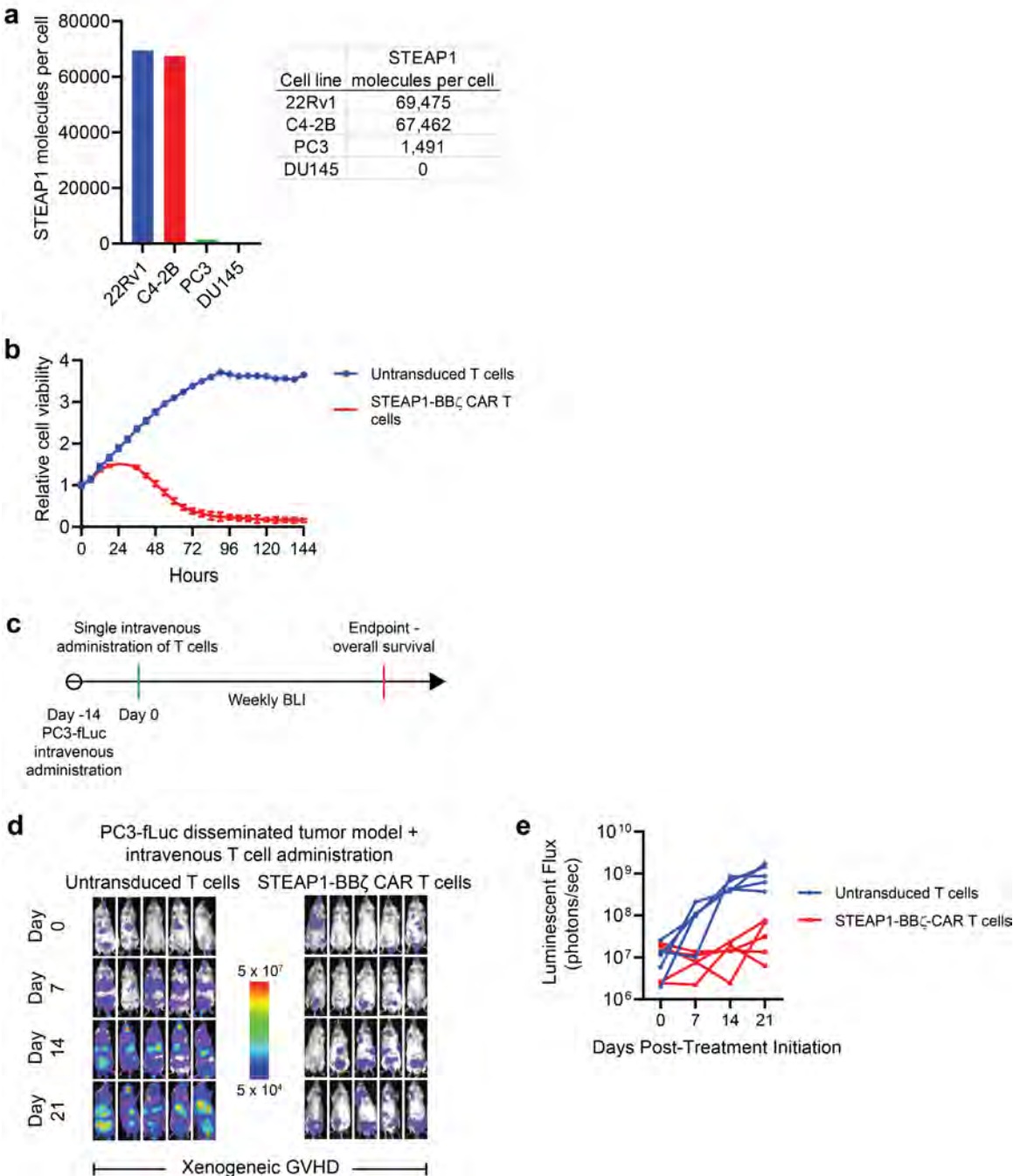
Supplementary Figure 10

a C4-2B-fLuc disseminated tumor model + intravenous T cell administration



Supplementary Figure 10. Antitumor activity of STEAP1-BBζ CAR T cell therapy in a disseminated human C4-2B prostate cancer cell line xenograft model. (a) Plot of average weights of NSG mice engrafted with C4-2B-fLuc metastatic tumors over time after treatment with either untransduced T cells (n=4) or STEAP1-BBζ CAR T cells (n=5). Bars represent SD. (b) *Ex vivo* photographic images (top) and BLI overlay (bottom) of individual liver and lung tissues from NSG mice engrafted with C4-2B-fLuc metastatic tumors and treated with either (left) untransduced T cells or (right) STEAP1-BBζ CAR T cells. Panel (b) shows n=4 individual tumors in each group. Radiance scale is shown. Source data are provided in the Source Data file.

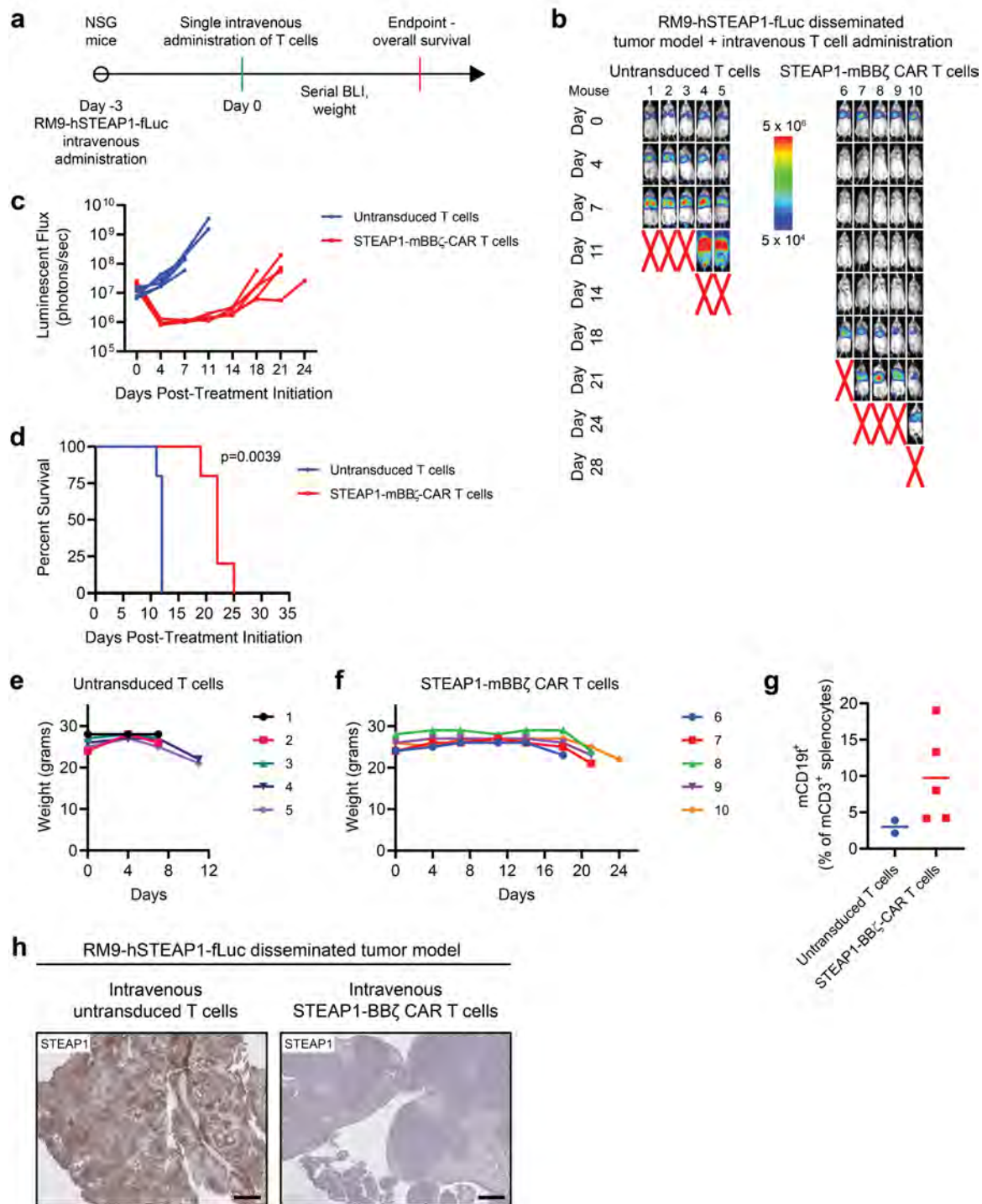
Supplementary Figure 11



Supplementary Figure 11. Antitumor activity of STEAP1-BB ζ CAR T cell therapy in a disseminated human PC3 prostate cancer cell line xenograft model. (a) Plot and table showing absolute quantification of STEAP1 molecules per cell across select human prostate cancer cell lines including PC3 as determined by flow cytometry using external standard beads. (b) Relative cell viability of PC3 target cells over time measured by fluorescence live cell imaging upon co-culture with untransduced T cells or STEAP1-BB ζ CAR T cells at a 1:1 ratio. n=4 biological replicates per condition and bars represent SEM. (c) Schematic of tumor challenge experiments for the PC3 disseminated model. (d) Serial live bioluminescence imaging (BLI) of NSG mice engrafted with PC3-fLuc metastases and treated with a single intravenous injection of 5×10^6 untransduced T cells or STEAP1-BB ζ CAR T cells at a normal CD4/CD8 ratio on day 0. Mice were euthanized due to the onset of severe xenogeneic

graft-versus-host (GVHD) in both treatment arms in week 4. Radiance scale is shown. **(e)** Plot showing the quantification of total flux over time from live BLI of each mouse in **(d)**. For panels **(d-e)** n=5 mice in each condition were used. Source data are provided in the Source Data file.

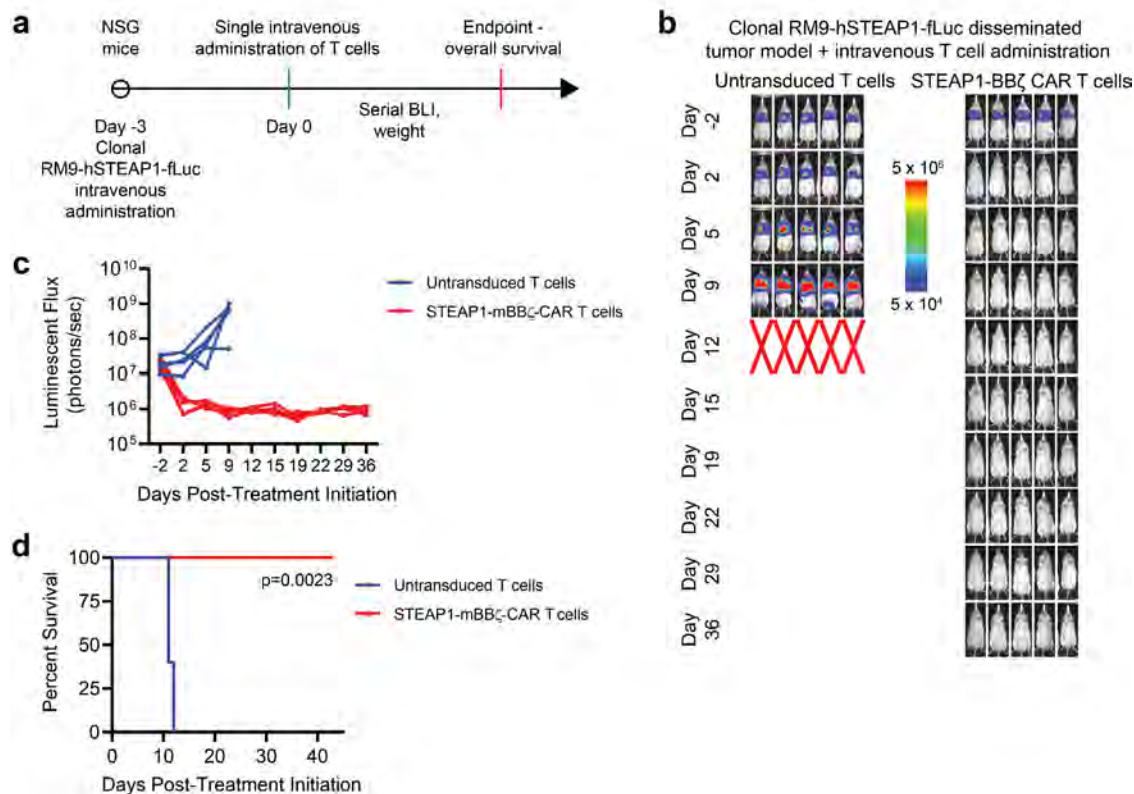
Supplementary Figure 12



Supplementary Figure 12. Antitumor activity of STEAP1-mBBζ CAR T cell therapy in a disseminated, non-clonal mouse RM9-hSTEAP1 prostate cancer allograft model in NSG mice. (a) Schematic of tumor challenge experiments for the non-clonal RM9-hSTEAP1 disseminated model. (b) Serial live BLI of NSG mice engrafted with RM9-hSTEAP1-fLuc metastases and treated with a single intravenous injection of 5×10^6 untransduced mouse T cells or mouse STEAP1-mBBζ CAR T cells on day 0. Red X denotes deceased mice. Radiance scale is shown. (c) Plot showing the quantification of total flux over time from live BLI of each mouse in (b). (d) Kaplan-Meier survival curves

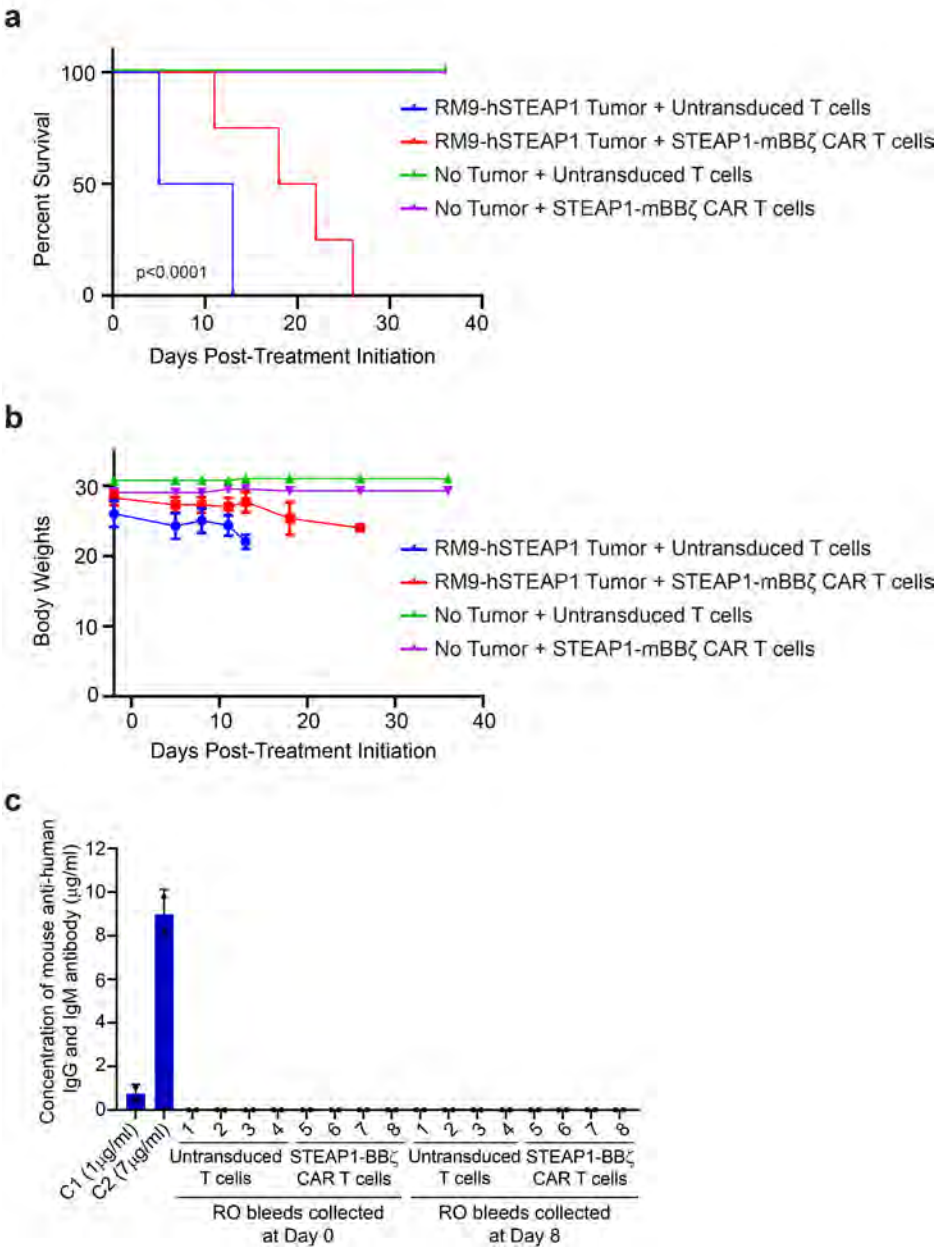
of mice in **(b)** with statistical significance determined by log-rank (Mantel-Cox) test. Plots of weights for each mouse numbered in **(b)** over time in the **(e)** untransduced T cell treatment group and **(f)** STEAP1-mBB ζ CAR T cell treatment group. For panels **(b-f)** n=5 mice per condition were used. **(g)** Quantification of mCD3⁺mCD19⁺ STEAP1-mBB ζ CAR T cells by flow cytometry from splenocytes of mice treated with untransduced T cells (n=2) and STEAP1-BB ζ CAR T cells (n=5) collected at necropsy. **(h)** Photomicrographs at low and magnification of STEAP1 IHC staining of RM9-hSTEAP1 lung tumors after treatment with (left) mouse untransduced T cells or (right) STEAP1-BB ζ CAR T cells. Scale bars = 500 μ m. Immunostaining was performed on n=4 biologically independent specimens. Source data are provided in the Source Data file.

Supplementary Figure 13



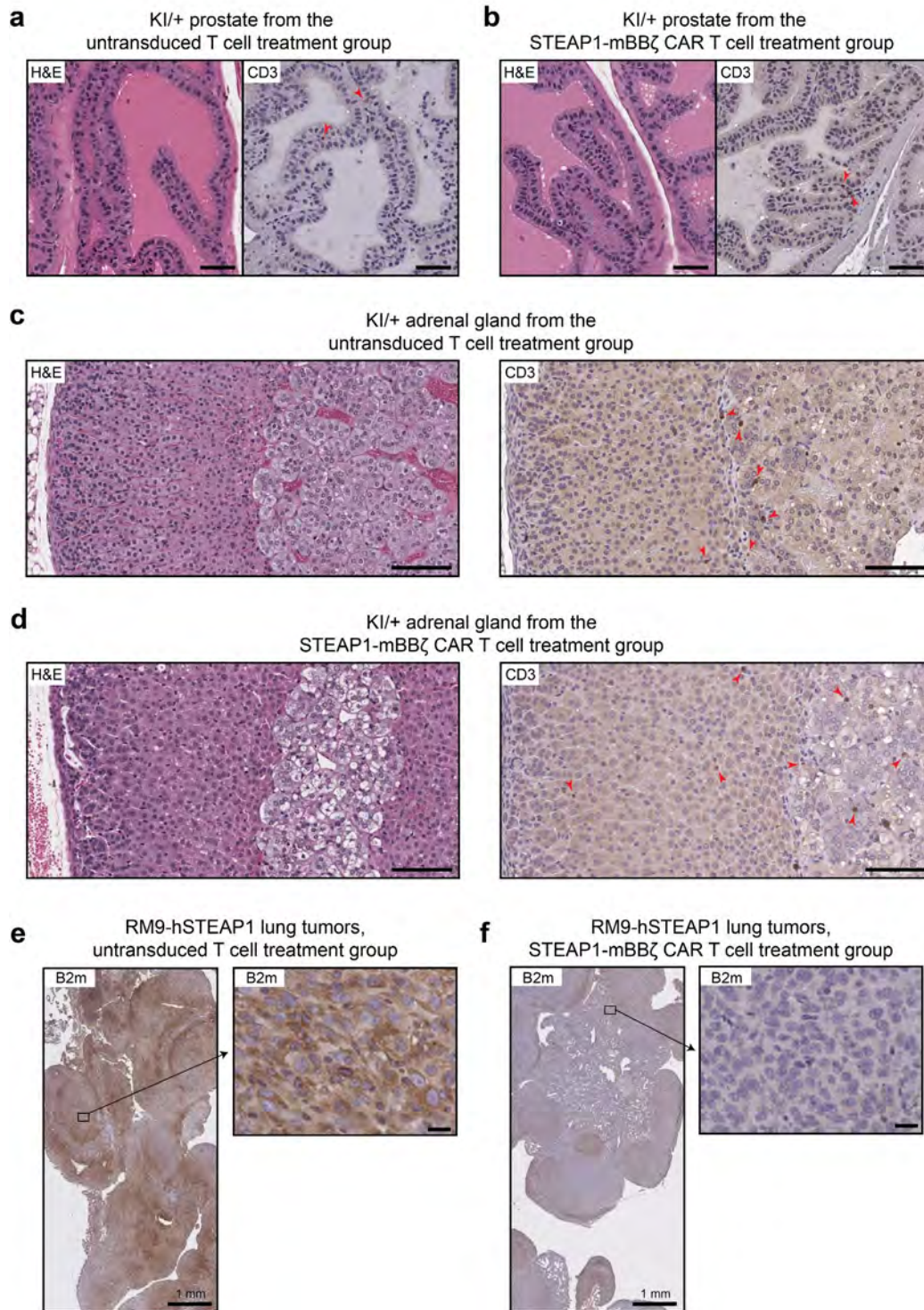
Supplementary Figure 13. Antitumor activity of STEAP1-mBBζ CAR T cell therapy in a disseminated, clonal mouse RM9-hSTEAP1 prostate cancer allograft model in NSG mice. (a) Schematic of tumor challenge experiments for the clonal RM9-hSTEAP1 disseminated model. **(b)** Serial live BLI of NSG mice engrafted with RM9-hSTEAP1-fLuc metastases and treated with a single intravenous injection of 5×10^6 untransduced mouse T cells or mouse STEAP1-mBBζ CAR T cells on day 0. Red X denotes deceased mice. Radiance scale is shown. **(c)** Plot showing the quantification of total flux over time from live BLI of each mouse in (b). **(d)** Kaplan-Meier survival curves of mice in (b) with statistical significance determined by log-rank (Mantel-Cox) test. For panels (b-d) n=5 mice in each condition were used. Source data are provided in the Source Data file.

Supplementary Figure 14



Supplementary Figure 14. Characterization of the safety of STEAP1-mBB ζ CAR T cell therapy in non-tumor bearing hSTEAP1-KI mice. (a) Kaplan-Meier survival curves of mice bearing RM9-hSTEAP1 tumors or no tumors treated with untransduced T cells or STEAP1-mBB ζ CAR T cells. Statistical significance determined by log-rank (Mantel-Cox) test ($p=0.0001$). (b) Plots of average body weights of mice in each treatment group over time. $n=4$ mice per condition. Bars represent SD. (c) ELISA quantification of anti-human IgG and IgM antibodies from serum collected at day 0 and 8 from RM9-hSTEAP1 tumor bearing mice treated with untransduced T cells and STEAP1-mBB ζ CAR T cells ($n=4$ mice per condition). Bar represents the mean with SD. Source data are provided in the Source Data file.

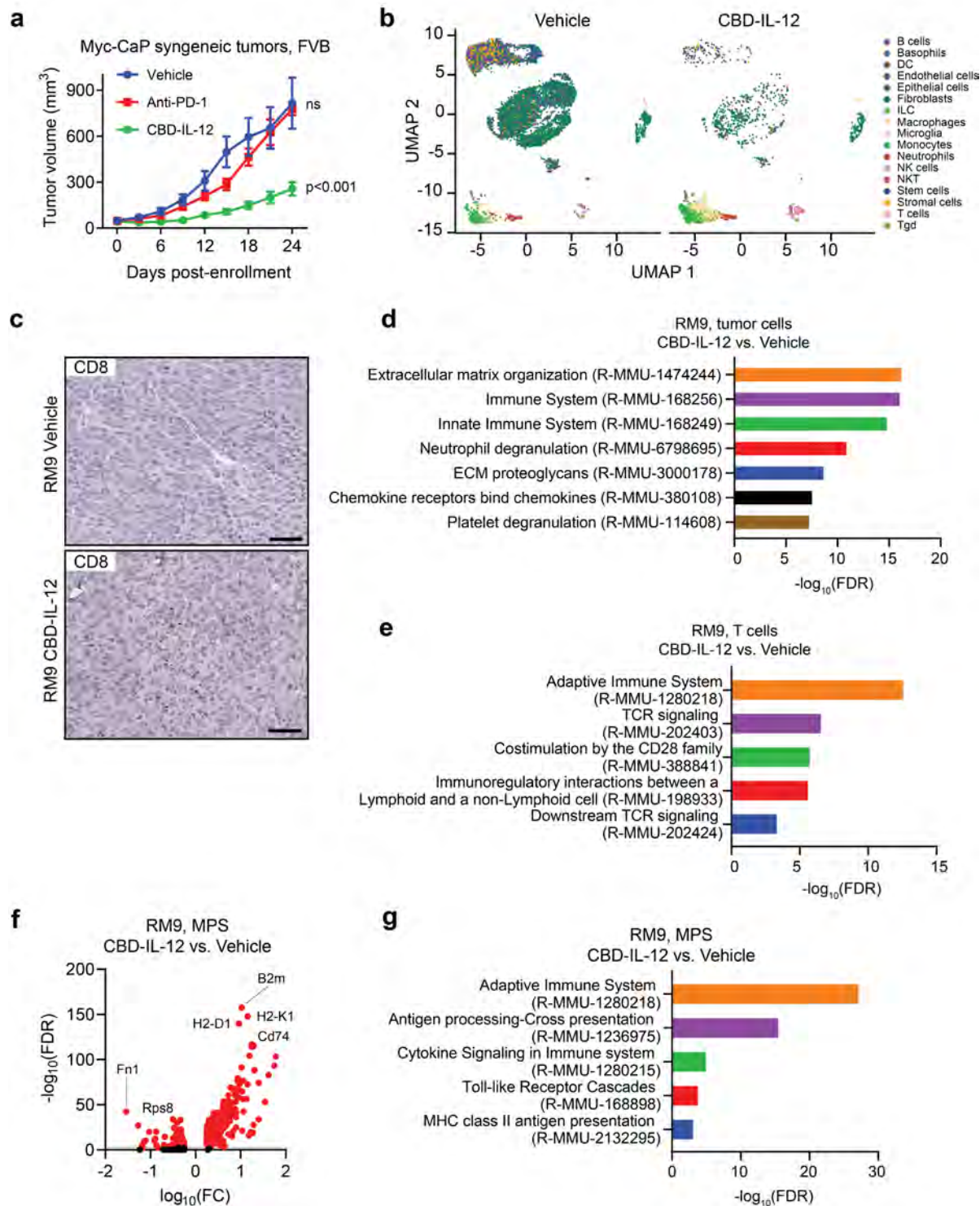
Supplementary Figure 15



Supplementary Figure 15. Preserved tissue architecture and absence of increased T cell infiltration in the prostates or adrenal glands of hSTEAP1-KI/+ mice treated with mouse STEAP1-mBBζ CAR T cells. Representative photomicrographs of hematoxylin & eosin (H&E) and CD3 IHC staining of hSTEAP1-KI/+ prostates from mice treated with (a) untransduced T cells and (b) STEAP1-mBBζ CAR T cells. Red arrowheads indicate rare CD3⁺ cells. Scale bars = 50 μm. Representative photomicrographs of H&E and CD3 IHC staining of hSTEAP1-KI/+ adrenal glands from mice treated with (c) untransduced T cells and (d) STEAP1-mBBζ CAR T cells. Red arrowheads indicate CD3⁺ cells.

Scale bars = 100 μm . Photomicrographs at low (left) and high (right) magnification of B2m IHC staining of RM9-hSTEAP1 lung tumors from mice after treatment with **(e)** mouse untransduced T cells and **(f)** STEAP1-mBB ζ CAR T cells. Scale bars = 20 μm , unless otherwise noted. For panels **(a-f)** immunostaining was performed on n=4 biologically independent specimens.

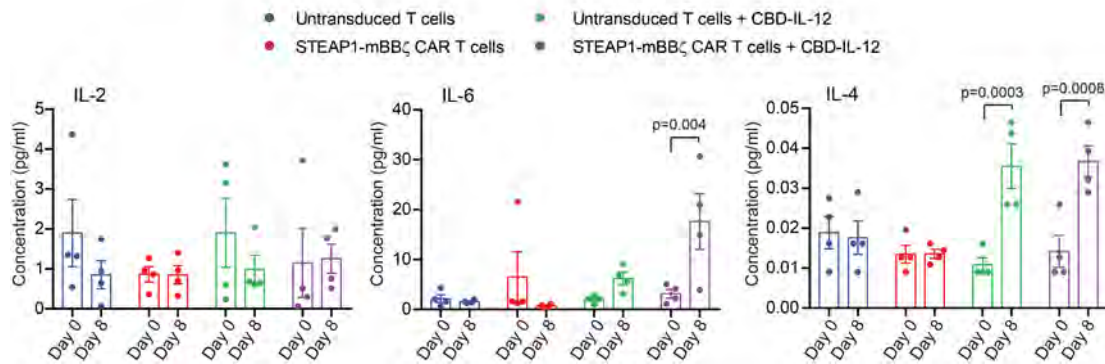
Supplementary Figure 16



Supplementary Fig. 16. CBD-IL-12 reprograms the tumor immune microenvironment of prostate cancer models. (a) Volumes of Myc-CaP subcutaneous tumors in syngeneic FVB mice over time with treatment with vehicle (n=7 mice), anti-PD-1 (clone 29F.1A12) 200 μg (n=5 mice) by intraperitoneal injection every 5 days, or CBD-IL-12 25 μg (n=6 mice) by intravenous injection every 5 days starting on day 0. Bar represents the mean with SEM. P-values ($p < 0.0001$ for vehicle vs. CDB-IL-12) were obtained from two-way ANOVA with Dunnett's multiple comparisons test. (b) Uniform Manifold

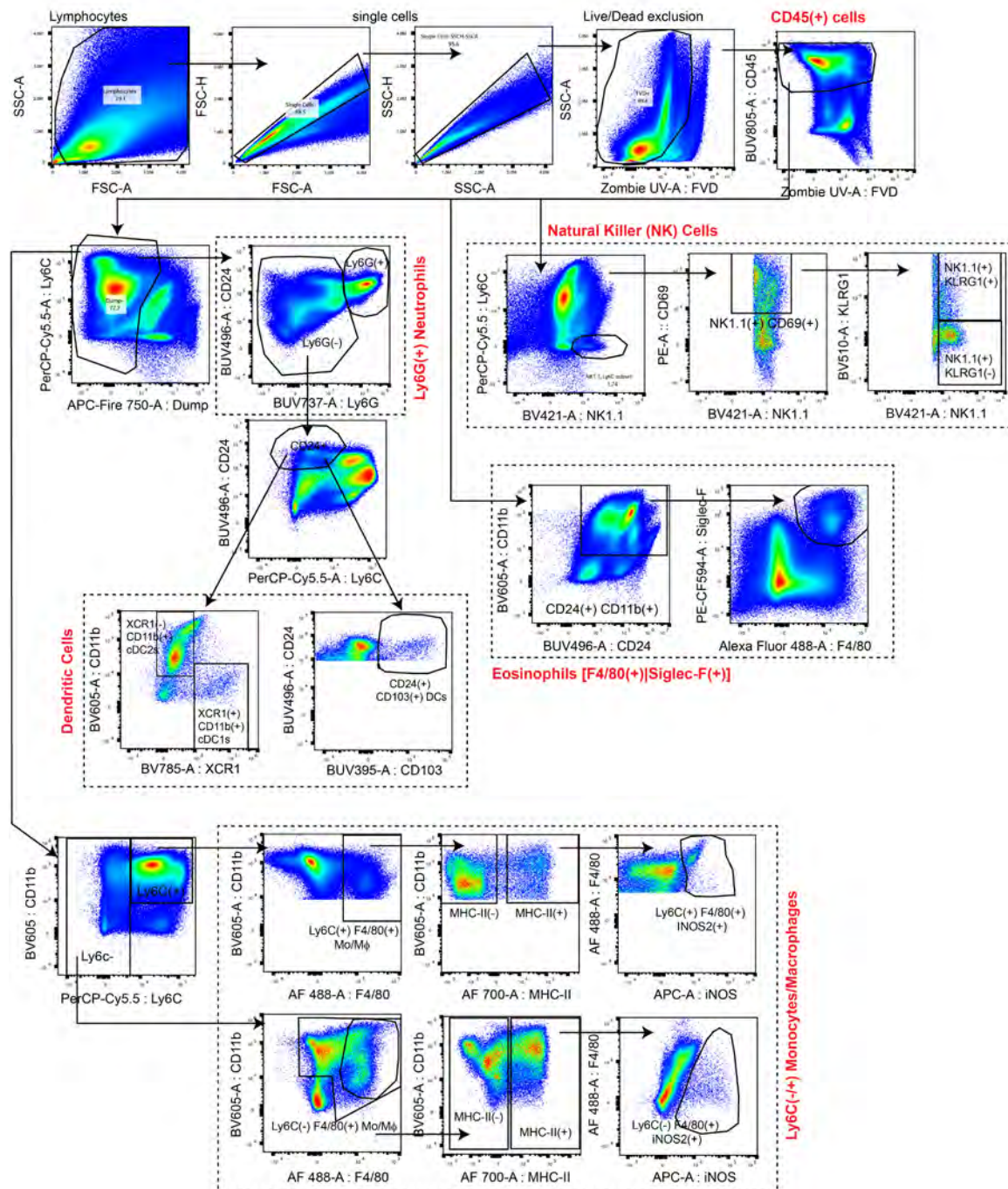
Approximation and Projection (UMAP) plots of single-cell RNA-seq (scRNA-seq) analysis of five RM9 tumors each aggregated from mice treated with vehicle or CBD-IL-12. **(c)** Photomicrographs of CD8 IHC staining of RM9 tumors from mice treated with (left) vehicle or CBD-IL-12 (right). Scale bars = 50 μm . CD8 immunostaining was performed on n=3 biologically independent specimens. Bar graphs showing Reactome pathways identified from differentially enriched genes ($\log_2$ fold change >1, $q < 0.05$) expressed in **(d)** tumor cells or **(e)** T cells. FDR = false discovery rate. **(f)** Volcano plot showing differential gene expression in mononuclear phagocyte system (MPS) cells from RM9 tumors of mice treated CBD-IL-12 relative to those treated with vehicle. FC = fold change. **(g)** Bar graphs showing Reactome pathways identified from differentially enriched genes ($\log_2$ fold change >1, $q < 0.05$) expressed in MPS cells. Source data are provided in the Source Data file.

Supplementary Figure 17



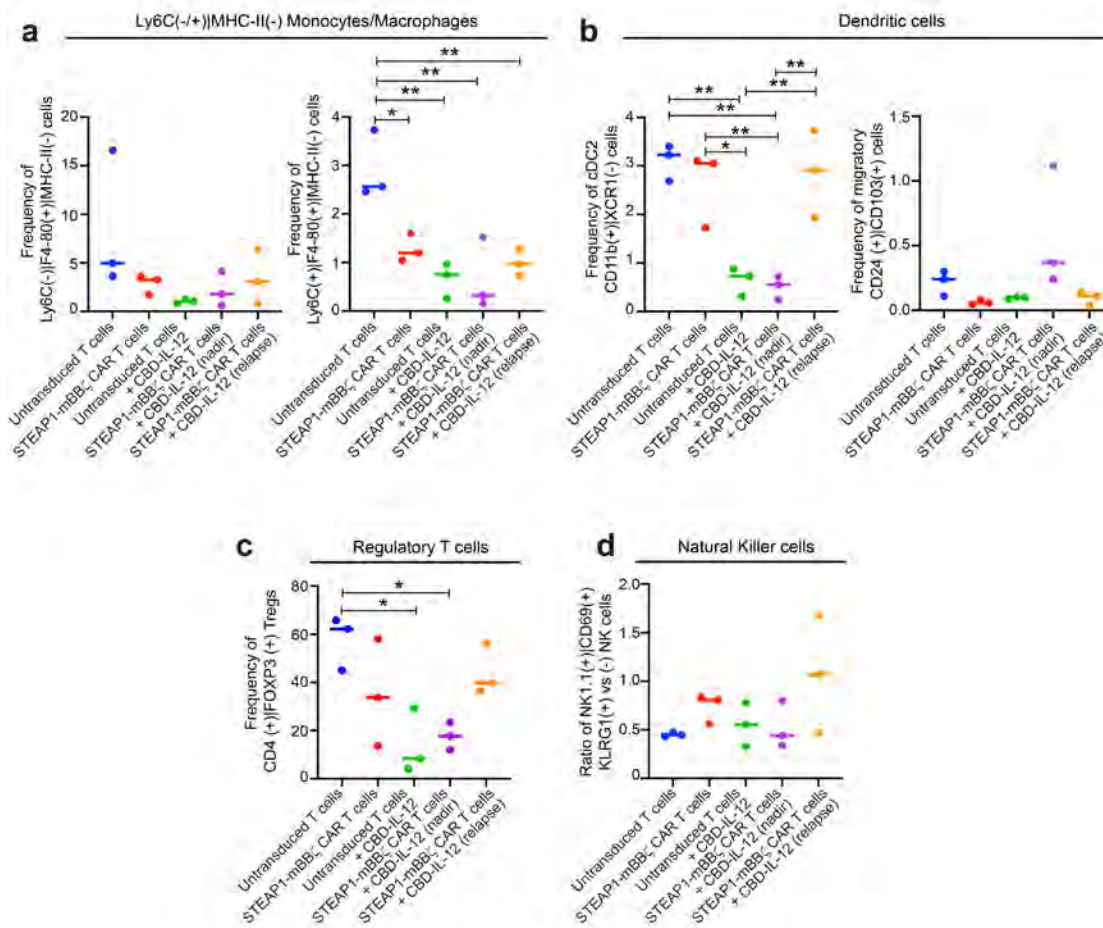
Supplementary Fig. 17. Plasma cytokine analysis before and after mouse STEAP1-mBB ζ CAR T cell therapy with or without concurrent CBD-IL-12 therapy in hSTEAP1-KI mice. Plots showing serum cytokine levels (IL-2, IL-6, and IL-4) based on ProcartaPlex immunoassays from retroorbital bleeds of hSTEAP1-KI/+ mice bearing RM9-hSTEAP1-fLuc metastases prior to (day 0) or after (day 8) treatment with untransduced mouse T cells or mouse STEAP1-mBB ζ CAR T cells with or without CBD-IL-12 treatment. n=4 for each condition. Bar represents the mean with SEM. P-values are derived from two-way ANOVA with Sidak's multiple comparisons test. Source data are provided in the Source Data file.

Supplementary Figure 18



Supplementary Figure 18. Gating strategy for the immunophenotyping of tumor immune cell subsets. Lymphocytes were gated on forward (FSC-A) and side (SSC-A) scatter, followed by gating on single cells and live cell population based on Fixable Viability Dyes (FVD) stain. Live cells were gated for CD45⁺ immune cells followed by analysis of F4/80⁺Siglec-F⁺ eosinophils. CD45⁺ cells were selected for a dump gate based on staining for B220, CD19, CD3, CD4, and CD8a. Dump-negative cells were gated for Ly6C⁺/F4/80⁺MHC-II⁺/ monocytes or macrophages, Ly6G⁺ tumor associated neutrophils, CD24⁺ dendritic cells, and NK1.1⁺CD69⁺KLRG1⁺ natural killer cells.

Supplementary Figure 19



Supplementary Figure 19. Combining CBD-IL-12 with STEAP1-mBB ζ CAR T cell therapy enhances tumor infiltration of innate immune cells. Plots showing the frequencies of (a) Ly6C⁻F4/80⁺MHC-II⁻ (left, one-way ANOVA p=ns) and Ly6C⁺F4/80⁺MHC-II⁻ (right, one-way ANOVA p=0.0017) macrophages, (b) CD11b⁺XCR1⁻cDC2 (left, one-way ANOVA p=0.0004) and CD103⁺migratory dendritic cells (right, one-way ANOVA p=0.08), and (c) CD4⁺FOXP3⁺ regulatory T cells (Tregs) normalized to total CD45⁺ cells as determined by multiparametric flow cytometry after treatment with untransduced T cells, STEAP1-mBB ζ CAR T cells, untransduced T cells and CBD-IL-12, and STEAP1-mBB ζ CAR T cells and CBD-IL-12 at maximal treatment response (nadir) and tumor relapse (relapse) group (one-way ANOVA p=0.015). (d) Plot showing the ratio of KLRG1⁺ to KLRG1⁻ natural killer cells (one-way ANOVA p=0.3) in the treatment groups in (a). For panels (a-d), n=3 tumors per condition, * denotes p<0.05; ** denotes p<0.01, *** denotes p<0.001. P-values are derived from one-way ANOVA with Dunn's multiple comparisons test. Source data are provided in the Source Data file.