

Defining an Optimal Dual-Targeted CAR T-cell Therapy Approach Simultaneously Targeting BCMA and GPRC5D to Prevent BCMA Escape-Driven Relapse in Multiple Myeloma



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ABSTRACT

Chimeric antigen receptor (CAR) T-cell therapy for multiple myeloma targeting B-cell maturation antigen (*TNFRSF17*; BCMA) induces high overall response rates; however, relapse occurs commonly. A reservoir of multiple myeloma cells lacking sufficient BCMA surface expression (antigen escape) may be implicated in relapse. We demonstrate that simultaneous targeting of an additional antigen—here, G protein-coupled receptor class-C group-5 member-D (*GPRC5D*)—can prevent BCMA escape-mediated relapse in a model of multiple myeloma. To identify an optimal approach, we compare subtherapeutic doses of different forms of dual-targeted cellular therapy. These include; (i) parallel-produced and pooled mono-targeted CAR T cells, (ii) bicistronic constructs expressing distinct CARs from a single vector, and (iii) a dual-scFv “single-stalk” CAR design. When targeting BCMA-negative disease, bicistronic and pooled approaches had the highest efficacy, whereas for dual-antigen-expressing disease, the bicistronic approach was more efficacious than the pooled approach. Mechanistically, expressing two CARs on a single cell enhanced the strength of CAR T-cell/target cell interactions.

SIGNIFICANCE: Myeloma frequently relapses post-CAR T-cell therapy; antigen escape-mediated relapse can be mitigated with upfront dual-targeting (BCMA/GPRC5D). A bicistronic vector encoding two CARs avoids the challenge of parallel manufacturing separate CAR T-cell products, while providing superior efficacy; this dual-targeted approach may enhance the durability of responses to cellular therapy for myeloma.

See related commentary by Simon and Riddell, p. 130.

INTRODUCTION

Treatment options for multiple myeloma have substantially improved over the last decade, resulting in improved overall survival (1, 2); however, despite this progress, patients are rarely cured. The natural history of multiple myeloma

involves multiple relapses with progressively shorter durations of remission, until the patient develops refractory disease (3, 4). Addressing relapsed/refractory multiple myeloma (RRMM) necessitates the development of novel treatment approaches; one such approach under development, with early clinical data demonstrating unprecedented response

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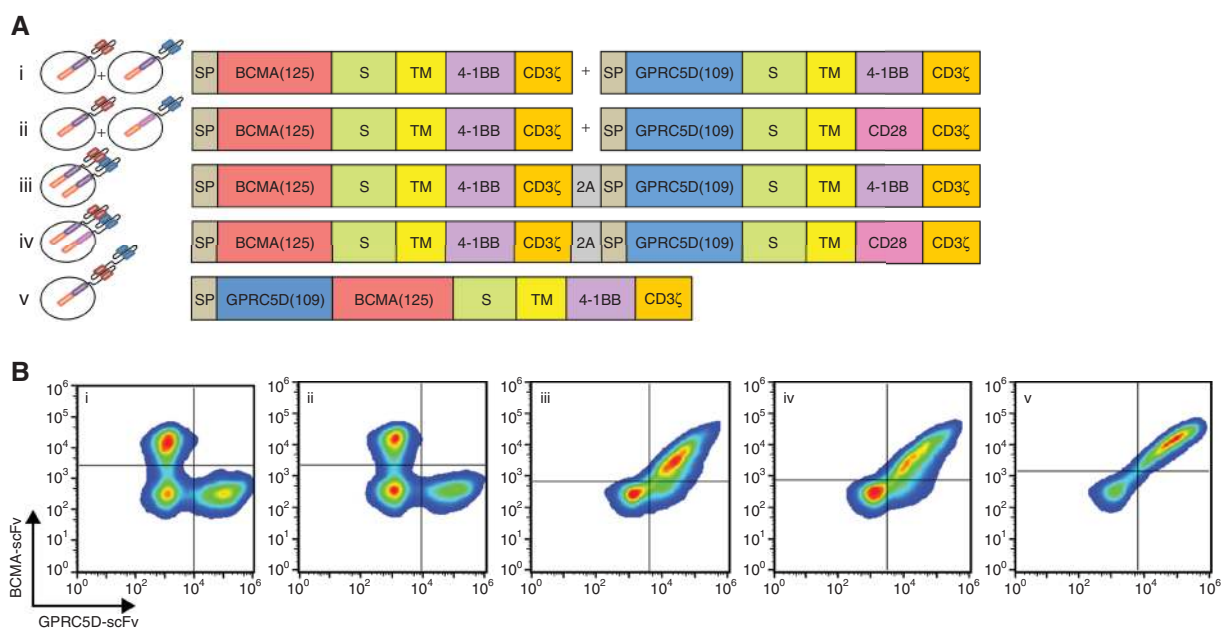


Figure 1. Dual-targeted CAR T cells express both scFVs efficiently and specifically lyse target antigen-positive cells. **A**, BCMA/GPRC5D dual-targeted CAR strategies evaluated. (i, ii) simultaneous 1:1 infusion of independent CAR T cells manufactured in parallel; (iii–iv) bicistronic dual-CAR expression on T cells via a construct with a “self-cleaving” 2A peptide; (v) tandem-scFv, “single stalk” CAR design. SP, signal peptide; S, spacer; TM, transmembrane domain. **B**, Expression of individual BCMA-targeted and GPRC5D-targeted scFVs on the surface of primary donor T cells by flow cytometry with reagents specific to either the BCMA-targeted scFv or the GPRC5D-targeted scFv. (continued on next page)

rates in this population of patients with heavily pretreated multiple myeloma, is immunotherapy (5).

Most clinically advanced immune therapies for multiple myeloma target B-cell maturation antigen (BCMA; ref. 6). These therapies include BCMA-targeted antibody–drug conjugates (7, 8), bispecific T-cell engager antibody-based therapies targeting BCMA and CD3 (9, 10), and BCMA-targeted chimeric antigen receptor (CAR) T-cell therapy (11–15). Despite high response rates with these BCMA-targeted immune approaches, including CAR T-cell therapies, most patients still go on to relapse (13–15). BCMA-negative or BCMA-low multiple myeloma cells are implicated as a reservoir of treatment-resistant disease preceding relapse in recent clinical investigations of cellular therapies, and may be one of several mechanisms responsible for relapse (13, 14). In this way, BCMA escape could limit the potential of CAR T-cell therapy to deliver durable responses.

An approach to mitigate BCMA escape-mediated relapse is through simultaneous targeting of an additional antigen. G protein-coupled receptor class C group 5 member D (GPRC5D), an antigen we previously described as a plasma cell specific target for the immunotherapy of multiple myeloma, is an attractive target to pair with BCMA (16). Individual approaches for CD19-based dual-targeted CAR T-cell therapy with various partners for B-cell acute lymphoblastic leukemia (ALL) have been investigated in isolation (17–19). However, multiple dual-targeting approaches are feasible and have yet to be comprehensively compared. We therefore pursued head-to-head investigation of dual-targeted CAR T-cell strategies to elucidate an optimal dual-targeted approach, using multiple myeloma as a model, with the goal to prevent BCMA escape-mediated relapse.

RESULTS

Expression and Activity of Dual-CAR Constructs

Potential approaches for dual-targeted adoptive cellular therapy that were explored include bicistronic CAR vectors, a dual-single-chain variable fragment (scFv) single-stalk CAR, and use of pairs of mono-targeted CAR T cells that were produced in parallel and then pooled. Where possible, we analyzed dual-41BB and mixed 41BB/CD28 containing CAR strategies (Fig. 1A). To enhance clinical translatability, in each approach we left unperturbed the BCMA(125)-41BB ζ CAR amino acid sequence, which is under clinical evaluation in a multicenter study (JCARH125, NCT03430011; refs. 12, 20). Dual-CAR vectors were manually codon optimized to minimize the potential for DNA recombination. Expression of BCMA- and/or GPRC5D-targeted scFVs on gene-modified primary human T cells was assessed using scFv-specific flow cytometric reagents. While BCMA- and GPRC5D-targeted CAR T cells produced in parallel and then pooled contain two separate populations of uniquely targeted CAR T cells (Fig. 1B, i–ii), all single-vector dual-targeted approaches expressed both scFVs on the predominant T-cell population in a 1:1 ratio (Fig. 1B, iii–v). Using an antibody to the common IgG4/IgG2-based spacer domain (21), we found similar transduction efficiencies (60%–70%) and staining intensities of transduced cells across all constructs, despite the fact that bicistronic vectors encode two independent CARs (Fig. 1C). All CARs specifically induced lysis of 3T3-artificial antigen-presenting cells (aAPC) expressing cognate target antigen, but not aAPCs lacking cognate target antigen (Fig. 1D). Donor human T cells expressing each of the CAR constructs induced lysis in 3 of 3 multiple myeloma cell lines evaluated (OPM2, RPMI8226, and MM1S),

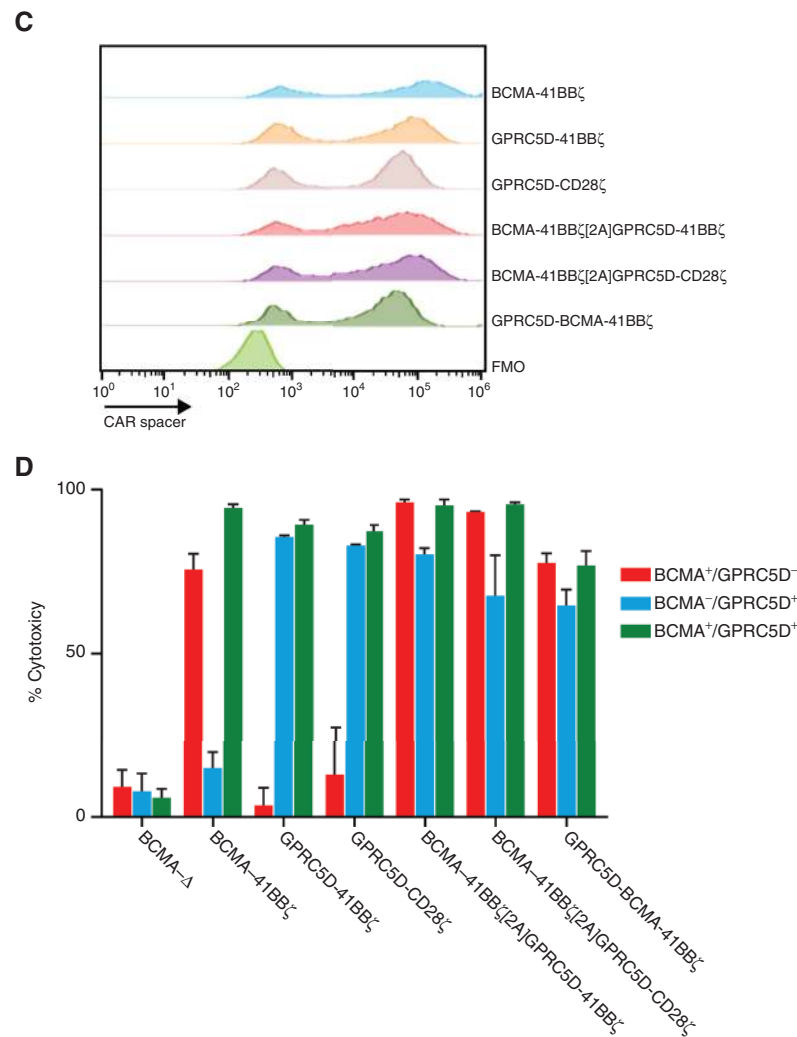


Figure 1. (Continued) C, Total CAR expression and transduction efficiency as measured by flow cytometry with antibody to the spacer domain shared among all CARs. FMO, fluorescence-minus-one. **D**, Cytotoxicity measured by ATP-dependent bioluminescence after 24-hour coculture of CAR T cells with a monolayer of 3T3-aAPC ffLuc $^+$ cells expressing the indicated antigen(s); normalized to aAPCs alone. Mean \pm SD from representative biological triplicate shown.

with increasing cytotoxicity at higher ratios of CAR T cells to tumor cells (Supplementary Fig. S1A–S1C). A CRISPR/Cas9-mediated BCMA-knockout (KO) OPM2 multiple myeloma cell line was generated, with BCMA KO confirmed by flow cytometry and resistance to BCMA-targeted CAR T-cell cytotoxicity (Supplementary Fig. S2A and S2B). Dual-targeted CAR T cells can lyse these OPM2 BCMA-KO cells with similar efficiency to mono GPRC5D-targeted CAR T cells (Supplementary Fig. S2B).

Upfront Treatment with Dual-Targeted CAR T Cells Is Efficacious *In Vivo* and Prevents Progression in an Antigen Escape-Mediated Relapse Model

Efficacy of dual-targeted CAR T-cell therapies was evaluated in a bone marrow-tropic xenograft model of multiple myeloma (22), where NOD/SCID gamma (NSG) mice are injected intravenous with wild-type OPM2 cells (which endogenously express both BCMA and GPRC5D). The multiple myeloma

cells were allowed to engraft and expand for 14 days to a high burden of disease; then the mice were treated with a single high dose of CAR T cells (3×10^6), this dose of CAR T cells was previously shown to generate long-term survival in this murine model (16). Control mice were treated with T cells bearing a CAR devoid of signaling domains (BCMA- Δ CAR); these mice reliably developed hind limb paralysis at approximately 35 days after tumor cell injection. In contrast, all experimental CAR T-cell approaches with this high dose of CAR T cells, including dual-targeted approaches, eradicated disease and generated long-term tumor-free survival (Fig. 2A–C; median overall survival not reached in each experimental arm vs. 32 days in the BCMA- Δ arm; $P < 0.0001$ for each experimental arm vs. control). A few mice in the experimental groups were euthanized because of xenogeneic graft-versus-host disease (GvHD), but these mice (with a single exception) had remained tumor free.

To test the ability of the dual-targeted CAR T-cell approaches to prevent BCMA escape-mediated relapse, we challenged

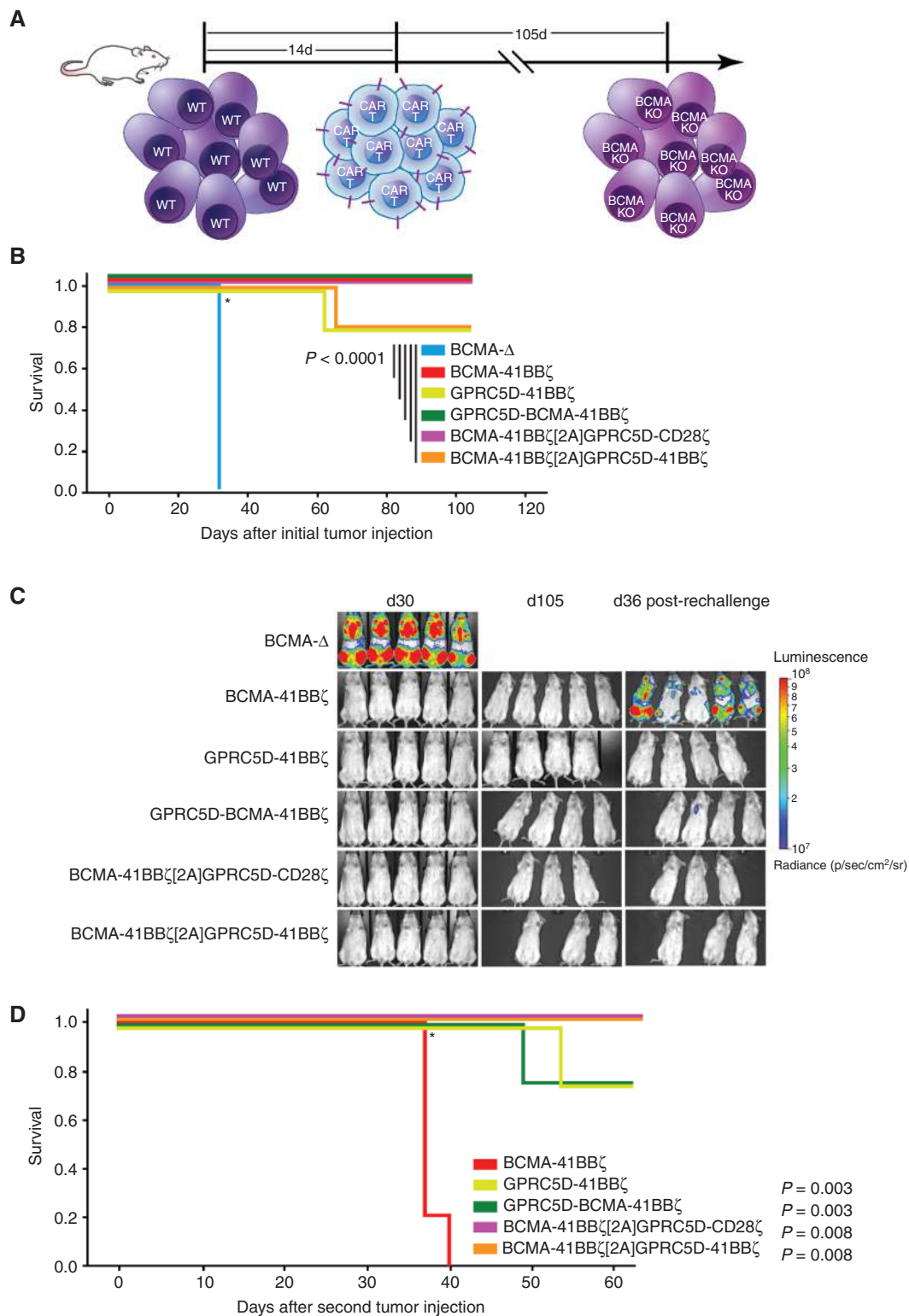


Figure 2. Upfront treatment with dual-targeted CAR T cells prevents BCMA escape-mediated relapse in a rechallenge model. **A**, Experimental scheme, 2×10^6 cells of the human bone marrow tropic myeloma cell line OPM2-WT were injected via tail vein into NSG mice and allowed to engraft and expand for 14 days. Mice were randomized to treatment with donor T cells (3×10^6) gene-modified as indicated. Mice in the treatment arms that showed signs of xenogeneic GvHD were euthanized, and on day 105 the remaining long-term surviving mice were challenged with OPM2-BCMA KO cells (2×10^6). **B**, Kaplan-Meier curves of overall survival after OPM2-WT injection. *P* values are indicative of each arm compared with BCMA- Δ control. **C**, Bioluminescent imaging of tumors over time. **D**, Kaplan-Meier curves after OPM2-BCMA KO injection. *P* values are indicative of each arm compared with BCMA-41BB ζ arm.

long-term surviving mice from the above experiment with tumor cells that had CRISPR-mediated knockout of BCMA. Specifically, mice that did not require euthanasia from xenogeneic GVHD were challenged on day 105, without second CAR T-cell treatment. The mice previously treated with BCMA-41BB ζ mono-targeted CAR T cells developed progressive disease with BCMA-negative tumor, while all groups that were treated with dual-targeted CAR T cells, or GPRC5D-targeted CAR T cells, were protected from this rechallenge (Fig. 2C and D; median overall survival was 37 days after challenge in the BCMA mono-targeted arm vs. not reached in the other groups; $P < 0.01$ for each experimental arm vs. BCMA mono-targeted arm).

Assessment of Optimal Dual-Targeted CAR T-cell Therapy Approach

As all the dual-targeted CAR T-cell approaches prevented BCMA escape-mediated relapse in the above model, we compared their efficacy at lower doses in a model of established tumor including BCMA-negative disease. To develop the model, we intravenously injected mice with a mixture of 5% to 10% BCMA-KO tumor cells (marked with firefly luciferase) and 90% to 95% wild-type (WT) tumor cells with endogenous BCMA and GPRC5D expression (marked with membrane-tethered Cypridina luciferase; Supplementary Fig. S3A). Because the two luciferases have different substrates, the two populations of tumor cells can be imaged separately in the same mice. Mice were treated with subtherapeutic moderate (5×10^5) doses of CAR T cells. As expected, in control BCMA- Δ CAR T-cell-treated mice, both WT and BCMA-KO tumors progressed, and in BCMA-41BB ζ CAR T-cell-treated mice, WT tumor was controlled, while BCMA-KO tumors progressed (Supplementary Fig. S3B). BCMA-41BB ζ + GPRC5D-CD28 ζ pooled CAR T-cell-treated mice showed control of WT tumor, while they did not control BCMA-KO disease. Interestingly, BCMA-41BB ζ [2A]GPRC5D-CD28 ζ CAR T cells also did not control BCMA-KO disease, despite signaling through the BCMA-41BB ζ CAR in the same cells. In contrast to this, limited efficacy of CARs containing a CD28 costimulatory domain, all dual-targeted CAR approaches exclusively containing 4-1BB costimulatory domain(s) induced deep responses to both WT and BCMA-KO disease (Supplementary Fig. S3B).

To distinguish the optimal 4-1BB-containing design, we further investigated the 4-1BB-containing strategies in our established BCMA-escape model at an even lower dose of CAR T cells. At the lowest dose of CAR T cells evaluated (2.5×10^5), mice treated with BCMA-41BB ζ + GPRC5D-41BB ζ pooled CAR T cells or BCMA-41BB ζ [2A]GPRC5D-41BB ζ CAR T cells induced deeper remissions and increased survival compared with single-stalk GPRC5D-BCMA-41BB ζ CAR T cells, where all mice progressed with tumor, including 100% of mice with BCMA-KO disease (median overall survival 50 days for the single-stalk group vs. 69 days for pooled or bicistronic arms; $P = 0.022$ and $P = 0.002$, respectively; Fig. 3A–C). We next investigated the efficacy of dual-targeting approaches in the presence of tumor exclusively expressing both antigens, still using low doses of CAR T cells (2.5×10^5). In contrast to the experiments where BCMA-KO disease was a main driver of relapse, in experiments in the absence of BCMA-KO disease, mice treated with bicistronic BCMA-41BB ζ [2A]GPRC5D-41BB ζ CAR T cells had enhanced depth of response

and survival compared with mice treated with the pooled BCMA-41BB ζ + GPRC5D-41BB ζ CAR T-cell strategy (median overall survival 70 days for the pooled arm vs. 85 days for BCMA-41BB ζ [2A]GPRC5D-41BB ζ arm; $P = 0.004$; Fig. 4A–C).

Expression of Two CARs on the T-cell Surface Enhances the Avidity for Dual-Antigen-Expressing Target Cells

We hypothesized that the dual-41BB bicistronic strategy had enhanced efficacy compared to the pooled CAR T-cell strategy specifically for dual-antigen-positive tumors, because the “multi-valent” approach increased the avidity for target cells. To test this hypothesis, we assessed cell–cell interactions of mono-targeted or dual-targeted CAR T cells with aAPCs expressing both antigens. BCMA⁺/GPRC5D⁺ aAPCs were allowed to adhere to a microfluidics chip; subsequently CAR T cells were flowed in. Strength of cell–cell interactions was quantified while exposing cells to an increasing acoustic force ramp (23, 24). At higher forces, bicistronic BCMA-41BB ζ [2A]GPRC5D-41BB ζ CAR T cells maintained cell–cell interactions better than either BCMA-41BB ζ or GPRC5D-41BB ζ mono-targeted CAR T cells (bicistronic vs. BCMA or GPRC5D mono-targeted, $P = 0.027$ and $P = 0.011$, respectively; Supplementary Fig. S4A and S4B).

DISCUSSION

Relapse is a challenge facing CAR T-cell therapy for hematologic malignancies, in particular multiple myeloma, where the overall response rate is high but response durability for many patients is limited (15). Antigen escape is likely one of several clinically relevant mechanisms of relapse after BCMA targeted CAR T-cell therapy for multiple myeloma (13, 14). We demonstrate, using murine models, that simultaneous dual-targeting of BCMA and GPRC5D antigens with CAR T-cell therapy can prevent BCMA-escape-mediated relapse (Fig. 2), as well as treat established BCMA-negative disease (Fig. 3). Furthermore, in this setting, our results suggest that a bicistronic CAR construct expressing two intact CARs on a single T cell is a promising dual-targeting cell therapy approach (Fig. 4).

Of note, the bicistronic CAR constructs do not result in increased total CARs on the surface of transduced T cells, as, surprisingly, CAR staining intensity for the common spacer was similar between T cells gene-modified with mono-targeted or bicistronic CAR vectors (Fig. 1C). We speculate that either the longer transgene and/or mRNA length of the bicistronic construct may lead to decreased expression, or that there may be a homeostatic mechanism (such as enhanced CAR recycling above a certain density) creating a “ceiling” of CAR expression on the cell surface; in either case, thus approximately equalizing the overall CAR surface density between approaches.

We found that it was critical to assess relative responses against both pure WT disease and disease including BCMA-negative cells. The responses in these two settings are summarized in Fig. 4D. In the presence of BCMA-negative multiple myeloma cells, T cells gene-modified with the dual-targeted single stalk design could not induce as deep or as durable a response as pooled CAR or bicistronic CAR T cells. While the long, flexible linker used here has been proposed to be optimal for single-stalk design in the setting of different scFvs and antigens (18); further modifying this design, including

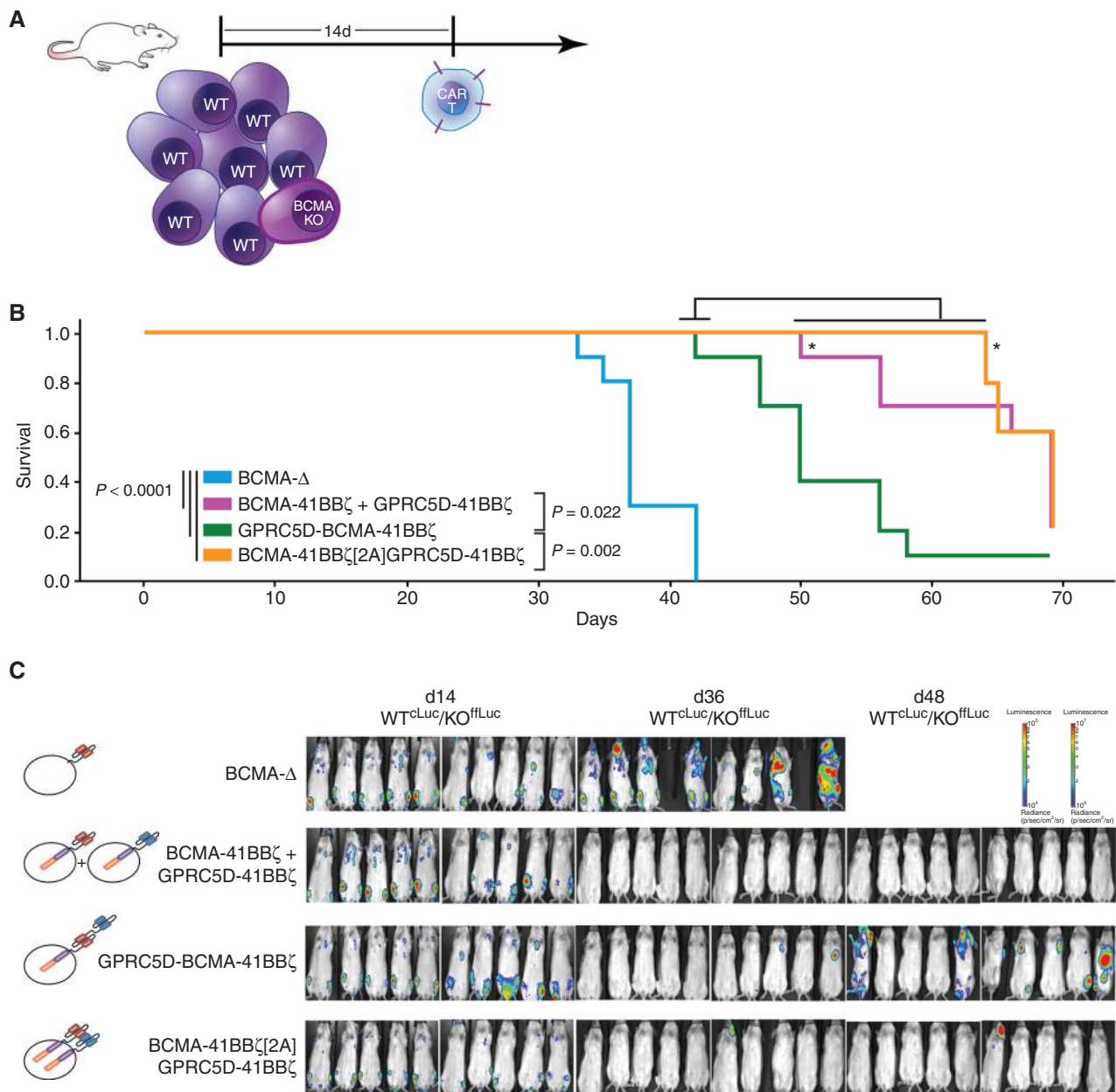


Figure 3. Pooled and bicistronic CAR approaches demonstrate enhanced efficacy over the single-stalk approach at subtherapeutic doses in an established BCMA-escape model. **A**, Experimental scheme, NSG mice were injected intravenously with 2×10^6 OPM2 tumor cells, including a subpopulation (5%–10%) of BCMA-KO cells. Each OPM2 population had been modified to express a distinct luciferase with a nonoverlapping substrate (OPM2-BCMA KO, firefly luciferase; OPM2-WT, membrane-tethered Cypridina luciferase) for *in vivo* imaging from the same animals over time. Day 14, mice were randomized for treatment with 2.5×10^5 gene-modified T cells. **B**, Kaplan–Meier curves for mice treated with the indicated CAR T cells. **C**, Tumor burden by bioluminescent imaging over time of OPM2-WT membrane-tethered Cypridina luciferase⁺ (vargulin substrate) and OPM2-BCMA KO ffLuc⁺ (d-luciferin substrate).

reversing the scFv order with these scFvs for BCMA and GPRC5D might yield enhanced results. Nevertheless, in the experiments presented here, the membrane-distal GPRC5D scFv in the single-stalk approach was less efficacious than when it was in its usual membrane-proximal location, as in the traditional second-generation 4-1BB ζ CAR used in our bicistronic and pooled CAR approaches (Fig. 3). When both antigens are present, however, we demonstrated that the bicistronic CAR approach is superior to the pooled CAR approach.

While these *in vivo* differences between vectors were not predicted by *in vitro* coculture cytotoxicity assays (Fig. 1D; Supplementary Fig. S1A–S1C), the differences did correlate with increased avidity of the bicistronic CAR T cells for target cells (Supplementary Fig. S4). Others, and we, have previously shown that *in vitro* cytotoxicity by CAR T cells does not correlate well with *in vivo* efficacy. For example, *in vitro* cytotoxicity could not distinguish between T cells expressing CD19-targeted CAR constructs \pm coexpression of 41BB-ligand; while

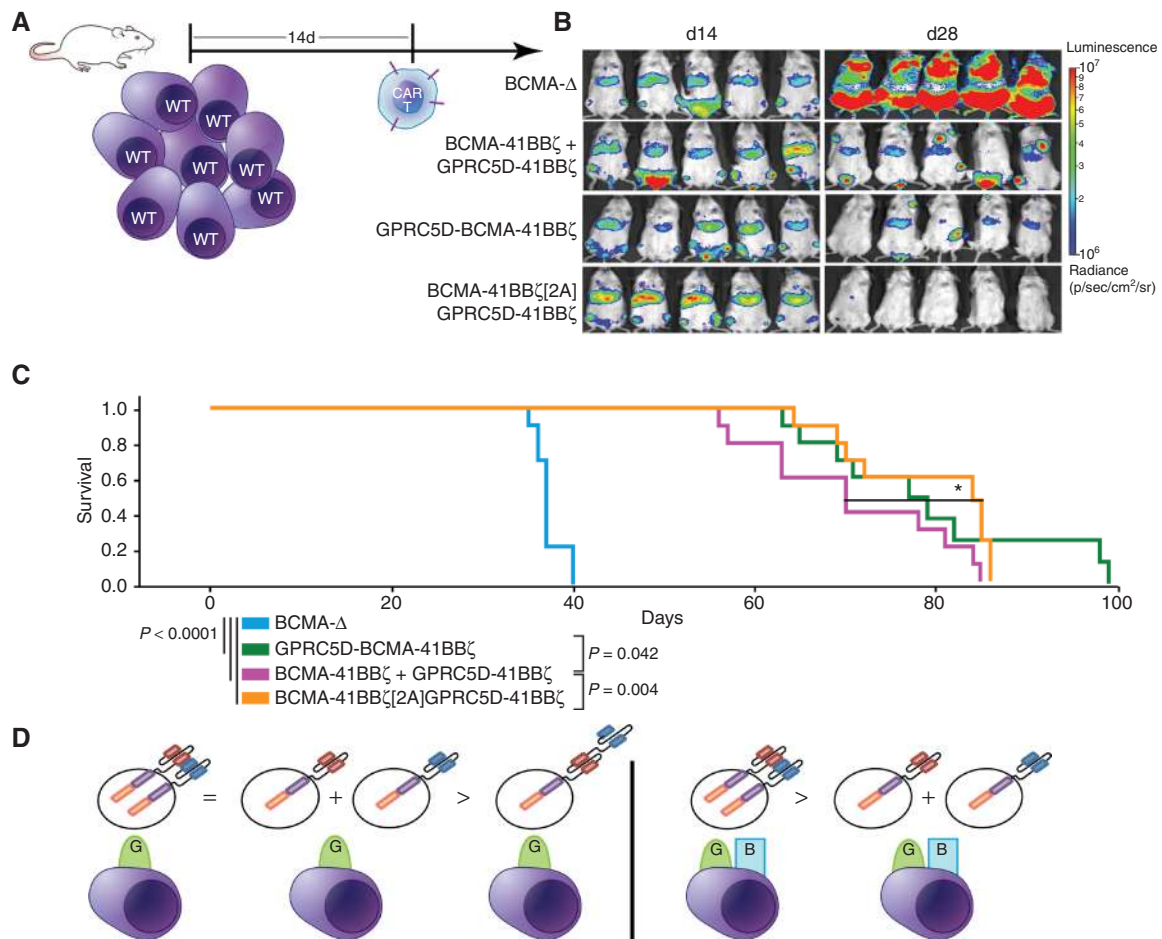


Figure 4. Bicistronic CAR approach demonstrates enhanced efficacy over the pooled approach at subtherapeutic doses in a model when target cells exclusively express both antigens. **A**, Experimental scheme: NSG mice were injected intravenously with a pure population of 2×10^6 OPM2-WT cells (endogenously expressing BCMA and GPRC5D). After a 14-day engraftment/expansion period, mice were randomized to the 4 treatment groups shown in **B**, each receiving a single injection of 2.5×10^5 gene-modified T cells. **B**, Tumor burden imaged at the time of treatment and day 14 posttreatment with gene-modified CAR T cells. **C**, Kaplan-Meier curves of overall survival. **D**, Summary of results. In the presence of BCMA-escape (GPRC5D-only expressing) target cells, the single-stalk CAR approach was less efficacious than pooled or bicistronic strategies, which were similarly efficacious to each other. In contrast, when targeting BCMA/GPRC5D dual-expressing target cells, the bicistronic strategy showed enhanced efficacy compared with the pooled approach.

the addition of 41BB-ligand expression was substantially advantageous in *in vivo* efficacy assays, especially notable when investigating low doses of CAR T cells (25). Similarly, *in vitro* cytotoxicity could not distinguish between 4 of 5 BCMA-targeted CAR constructs; despite these inducing vastly different outcomes in the OPM2 *in vivo* efficacy model (12). We speculate that the enhanced avidity correlating with enhanced *in vivo* efficacy with the bicistronic construct may result from access to a greater number and/or diversity of antigens; although further experimentation to elucidate the exact mechanism is required. In summary, the bicistronic construct best maintains maximal efficacy in the setting of both WT multiple myeloma and disease including BCMA-negative multiple myeloma cells.

Compared with a pooled CAR T-cell approach, bicistronic vectors, in addition to potentially providing superior anti-myeloma efficacy, avoid the required practical challenges of parallel manufacturing separate CAR T-cell populations. If, however, two mono-targeted CARs have been previously clinically validated, in the short term it may be advantageous

to translate dual-targeting with a pooled CAR approach. This approach may provide critical proof-of-concept clinical data while bypassing the need to generate new virus or to conduct extensive investigational new drug-enabling experiments, allowing dual-targeting to reach patients more rapidly. Nevertheless, manufacturing autologous CAR T-cell products is an extremely resource-intensive process. In the long-term, an efficacious bicistronic CAR approach avoids the increased strain on limited good manufacturing practice (GMP) production suites and decreases the need for generation of multiple GMP-grade viruses, thus reducing the demand for the human, physical, and financial resources required to produce two unique CAR T-cell products for each patient.

CAR T-cell therapies have demonstrated high response rates for hematologic malignancies; however, there is room for improvement with respect to the durability of these responses. We believe that multiantigen targeting will be critical to enhance long-term outcomes for patients. These experiments were designed to address the translational question of an optimal

approach for dual-targeting of BCMA and GPRC5D to overcome BCMA-escape mediated relapse. Further studies are required to determine whether the optimal dual-CAR approach should be determined empirically for each malignancy or if these observations can be applied more broadly. The results presented here will inform the prioritization of both designs for future CAR constructs and strategies for clinical investigation of adoptive cellular therapies to treat multiple myeloma.

METHODS

Cell Lines and Transduction of Human T Cells

The human multiple myeloma cell lines MM1S, RPMI8226, and NIH-3T3 were obtained from the ATCC; OPM2 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Multiple myeloma cell lines were maintained in RPMI and 10% FBS; 3T3 cells were maintained in DMEM and 10% FBS (Gibco, Life Technologies). Cells were authenticated by STR DNA Profiling (ATCC) and tested every other week for *Mycoplasma* via MycoAlert Mycoplasma Enzymatic Detection Assay (Lonza). All cell lines are used within 6 weeks of thawing from early passage frozen vials. To generate GFP/luciferase-positive target cells or to express BCMA or GPRC5D antigen in NIH-3T3s, cells were stably transduced with gamma retrovirus expressing the cDNA in a similar manner to T cells (below). To generate the BCMA KO, GFP/luciferase Cas9-expressing OPM2 cells were transduced with a BCMA single guide RNA (sgRNA) lentivirus as described previously (16). In all cases, target cells were sorted into single-cell clones in 96-well plates, and expanded to generate clonal populations, which were confirmed by flow cytometry for use in all experiments.

Primary human T cells were isolated fresh from buffy coats prepared from whole blood collected by the New York Blood Center, in accordance with Memorial Sloan Kettering Cancer Center's (MSKCC) Institutional Review Board (IRB) protocol no. 95-054. T cells were stimulated with phytohemagglutinin (2 mg/mL; Sigma) for 24 hours and grown in the presence of IL2. On days 2 and 3 after stimulation, T cells were spinoculated with gibbon ape leukemia virus pseudotype gamma-retrovirus. HEK293GP-GALV9 retroviral packaging cells have been described previously (12). Transduction efficiency was determined by flow cytometric analysis between day 5 and 8, and confirmed on the day of experiment. Experiments were normalized for CAR⁺, viable cells; after washing, resuspending, and pooling (as appropriate) CAR T-cell populations, we confirmed that all cell counts were within $\pm 10\%$ of the goal cell dose before proceeding with any experiment.

Flow Cytometry

A 10-color Gallios B43618 (Beckman Coulter) was used to acquire data. Analysis was performed with FlowJo software (V10, Tree Star). Expression of CAR was determined by surface staining using either BCMA ECD-Fc (shared by Eureka Therapeutics), GPRC5D-scFv anti-idiotype (generated by Charles River Laboratories and shared by Bristol-Myers Squibb), or anti-human IgG4 antibody to the shared spacer region (clone EP4420; Abcam). Anti-IgG4 primary was conjugated with Lightning-Link Labeling Kits (Innova Biosciences, Novus Biologicals). BCMA antigen detected using clone FAB193A (R&D Systems). Cells were counted with 123count eBeads (Thermo Fisher Scientific). Viability was determined by DAPI exclusion (Thermo Fisher Scientific) and cells were gated for low DAPI before further analysis.

Cytotoxicity

OPM2, RPMI8226, and MM1S human multiple myeloma cell lines and aAPC-3T3 cell lines were stably transduced with luciferase, as described above. 20,000 target cells were plated in 96-well plates in triplicate with CAR⁺ T cells at the indicated effector-to-target (E:T)

ratios; cells were then incubated for 24 hours. Cell viability was determined by an ATP-dependent assay, where % cytotoxicity = $(BLI_{MAX} - BLI_{SAMPLE})/BLI_{MAX}$; BLI_{MAX} = mean target cell alone value of that experiment (12). Bioluminescence was read on a Spark microplate reader (TECAN). Significance determined by two-way ANOVA.

Murine Experiments

All *in vivo* studies were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial Sloan Kettering Cancer Center (MSKCC) [protocol 00-05-065]. Six- to 12-week-old NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory) were injected systemically via tail vein with target cells (22) stably transduced either with firefly luciferase (ffLuc) or membrane-tethered Cypridina luciferase (MT-cLuc plasmid a gift from V. Ponomarev, MSKCC, New York, NY). Injection of D-luciferin substrate (Millipore Sigma) or vargulin (Targeting Systems) allowed for longitudinal *in vivo* bioluminescent imaging of ffLuc or MT-cLuc, respectively. The nonoverlapping substrates allowed the imaging of both luciferases in the same mouse. Tumor engraftment was confirmed by baseline bioluminescent imaging before cellular therapy. A single dose of human T cells genetically modified to express the indicated CAR construct was administered via tail vein at the indicated time point. Studies were planned with the minimum number of animals per treatment group to reproducibly observe statistically significant differences ($n = 5$ to 10 per arm per experiment). All murine experiments were replicated at least twice, using T cells from different donors in each replicate.

Cell-Cell Interaction Assay

Cell-cell interactions between mono-targeted or dual-targeted CAR T cells and 3T3-aAPCs were investigated as described previously (23, 24), in which aAPCs are allowed to adhere to a microfluidics chip overnight, then CAR T cells are flowed over the chip in a benchtop cell avidity analyzer (zMovi, Lumicks). Briefly, chips were first cleaned with water, air, bleach, sodium thiosulfate, 12 mol/L HCl, and 1 mol/L NaOH several times. After adding poly-L-lysine for 15 minutes, we flushed the chip with air and dried on a heat block (45–55°C) for 1 hour, pulling in air every 10 to 20 minutes. Chips were wet with PBS before proceeding to cell immobilization. Trypsinized and washed aAPCs were resuspended and added to the poly-L-lysine-coated chip, then incubated overnight. Chips were placed into the cell avidity analyzer where experiments were performed at 37°C. An initial force ramp was run on immobilized cells to ensure attachment. CAR T cells were resuspended in RPMI at 2×10^6 cells per mL; 50 μ L of cell suspension was flowed into the cell avidity analyzer and allowed to interact with monolayer for 5 minutes. We performed an impedance sweep to set the resonance frequency and to initialize the force ramp (0–1800 pN relative force over 3 minutes). All experiments were performed in triplicate.

Statistical Analysis

All *in vitro* experiments were repeated at least three times, and all *in vivo* experiments at least twice. Statistical analysis was performed using GraphPad Prism (GraphPad Software) or SPSS Statistics v.25 (IBM). All statistical tests were two-tailed. Unless otherwise indicated, a log-rank Mantel-Cox test was used for survival curves, and an unpaired *t* test was used for comparison of experimental groups to controls.

Disclosure of Potential Conflicts of Interest

C. Fernández de Larrea is an employee/paid consultant for Bristol Myers Squibb, Takeda Oncology, Janssen, and Amgen, and reports receiving speaker's bureau honoraria from Bristol Myers Squibb, Janssen and Amgen. Y. Chen is an advisory board member for and reports receiving research commercial grants from Amgen, and reports receiving speaker's bureau honoraria from Bristol Myers Squibb, Janssen, and Takeda Oncology. R.J. Brentjens is an employee/paid consultant

for JUNO Therapeutics, and Gracell Biotherapeutics Inc., and reports receiving research commercial grants from JUNO Therapeutics, and holds ownership interests (including patents) in JUNO Therapeutics. E.L. Smith is an employee/paid consultant for Bristol, Myers Squibb, Fate Therapeutics, and Precision Biosciences, reports receiving research commercial grants from Bristol Myers Squibb, and receiving other remuneration from Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

C. Fernández de Larrea: Formal analysis, investigation, visualization, methodology, writing-original draft. **M. Staehr:** Investigation, methodology. **A.V. Lopez:** Investigation. **K.Y. Ng:** Investigation. **Y. Chen:** Supervision. **W.D. Godfrey:** Investigation. **T.J. Purdon:** Investigation. **V. Ponomarev:** Resources. **H.-G. Wendel:** Resources. **R.J. Brentjens:** Conceptualization, resources, supervision, funding acquisition, writing-review and editing. **E.L. Smith:** Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

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