

B7-H3 Nanobody-Based CAR T Cells Suppress Multiple Myeloma Progression and Dual BCMA/B7-H3 CAR T Cells Prevent Antigen Escape

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Abstract

Although BCMA-targeted CAR T-cell therapy has achieved notable success in treating multiple myeloma (MM), disease recurrence remains common. This is largely driven by T-cell dysfunction and the selection of BCMA-negative tumor variants. Identifying new antigens is essential to counteract antigen escape. We analyzed B7-H3 (CD276) surface expression on primary MM patient specimens. Nanobody-derived CAR T cells (nanoCARs) targeting B7-H3 were constructed and assessed for their ability to kill target cells and secrete cytokines in vitro, including against autologous patient myeloma cells. Therapeutic potential was further examined in two independent MM xenograft mouse models. In addition, dual-specificity BCMA/B7-H3 CAR T cells and a CARpooling approach (combined infusion of separate BCMA- and B7-H3-specific CAR T cells) were evaluated in models simulating antigen heterogeneity and escape. B7-H3 was found on malignant plasma cells in 60% of MM cases examined. The B7-H3 nanoCAR T cells demonstrated robust, antigen-specific killing and effector cytokine production against both MM cell lines and primary tumor cells. In vivo, treatment led to substantial tumor regression and extended overall survival. Dual BCMA/B7-H3 CAR T cells as well as the CARpooling strategy effectively cleared tumor populations displaying mutually exclusive expression of either BCMA or B7-H3. Collectively, these findings highlight the potential of combining BCMA and B7-H3 targeting to overcome antigen loss. B7-H3 emerges as an attractive candidate for immunotherapy in MM. Both single B7-H3-specific and dual-targeted nanobody-based CAR T cells offer a promising route to reduce antigen escape and achieve more durable remissions.

Keywords: B7-H3, CD276, CAR T cells, Multiple myeloma, BCMA, Antigen escape

Introduction

Multiple myeloma (MM) is an incurable plasma cell neoplasm characterized by the progressive accumulation of malignant plasma cells within the bone marrow (BM) [1]. Tumor heterogeneity and evolving resistance mechanisms continue to limit long-term disease control.

However, the introduction of antibody-based agents and CAR T-cell therapies has markedly improved patient outcomes and reshaped the therapeutic landscape for MM [2, 3]. Currently approved BCMA-directed CAR T products include idecabtagene vicleucel (ide-cel), based on a conventional scFv, and ciltacabtagene autoleucel (cilta-cel), which utilizes a bi-paratopic nanobody platform. Both have delivered encouraging clinical responses [4]. Despite these advances, relapses after BCMA CAR T-cell infusion occur frequently, with antigen-negative escape documented in 4%–33% of patients [4–6]. Selective pressure can enrich for BCMA-low/negative clones, while γ -secretase-mediated shedding further reduces surface BCMA availability and generates inhibitory soluble forms [7, 8].

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Given these constraints, complementary target antigens are urgently needed. Here we explore B7-H3 (CD276) as a potential alternative or co-target in MM immunotherapy.

B7-H3, encoded on chromosome 15q24.1, belongs to the B7 family of immune checkpoint molecules. It appears in two isoforms (2Ig and 4Ig) due to gene duplication, with the four-immunoglobulin-domain variant predominating in humans [9]. Although B7-H3 transcripts are widespread in normal tissues, protein expression is tightly regulated and generally low, due to miRNA repression and glycosylation. Among healthy adult tissues, the prostate displays the highest levels, which are still markedly lower than those observed in many cancers. Overexpression of B7-H3 correlates with adverse outcomes in multiple solid malignancies, such as glioblastoma and colorectal carcinoma [10, 11]. It also exerts immunosuppressive activity by dampening CD8⁺ T-cell and NK-cell effector functions [12]. In MM, knowledge is more limited, yet B7-H3 has been implicated in promoting the Warburg effect and STAT3-mediated proliferation, thereby supporting tumor metabolic flexibility, progression, and chemoresistance [13]. These characteristics, together with its differential expression pattern, justify further evaluation of B7-H3 as an immunotherapeutic target in MM [14].

The current work examines B7-H3 as a novel CAR T-cell target in MM. We profiled its expression in patient bone marrow samples, distinguishing tumor cells from normal hematopoietic compartments in the microenvironment. We engineered a second-generation nanobody CAR and confirmed its capacity to lyse MM cell lines and primary patient-derived cells in vitro, with strong anti-tumor effects in vivo. Finally, we showed that B7-H3 targeting can circumvent therapeutic resistance arising from BCMA antigen loss.

Materials and Methods

Gene expression analysis

Publicly available microarray datasets from the Heidelberg-Montpellier cohort and Total Therapy 2 (TT2) studies were examined. TT2 includes data from 345 newly diagnosed MM patients (GSE2658) and 22 healthy donors, 44 MGUS, and 12 SMM samples (GSE5900) generated at the University of Arkansas for Medical Sciences. The Heidelberg-Montpellier dataset (E-MTAB-372) contains expression profiles of CD138-purified plasma cells from untreated MM patients. All

analyses were conducted using Genomicscape [15-17] with MAS5 normalization.

Design and generation of CAR constructs

CAR design followed previously established methods [18]. Binding domains for B7-H3 were sourced from patent WO2020033837 (nanobody format) and enoblituzumab (scFv). The irrelevant R3B23 nanobody served as a non-targeting control [19, 20]. Each CAR incorporated the CD8 α hinge and transmembrane domain linked to 4-1BB and CD3 ζ signaling elements. Constructs were inserted into a pHR' lentiviral backbone carrying a P2A-separated Thy1.1 (CD90.1) reporter gene. Lentiviral particles pseudotyped with VSV-G were produced in HEK293T cells according to standard protocols [21]. Supernatants collected at 48 and 72 hours were filtered (0.2 μ m) and concentrated via ultracentrifugation (22,000 rpm for 1.5 h at 4 °C using an SW28 rotor). Pellets were resuspended in PBS supplemented with 10 μ g/mL protamine sulfate (LeoPharma) and frozen at -80 °C. Protamine sulfate was added to enhance transduction efficiency. Functional viral titers were measured by limiting dilution on HEK293T cells.

Cell culture

Established human MM cell lines (RPMI-8226, LP-1, OPM-2, JJN-3, XG-2, XG-7, U266, MM1S, ANBL-6; ATCC) were grown in RPMI-1640. 2D3 Jurkat cells were maintained in IMDM, and HEK293T cells in high-glucose DMEM. All media contained 10% heat-inactivated FBS (TICO Europe), 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific). Cultures were kept at 37 °C in a 5% CO₂ atmosphere. Cell line authenticity was confirmed by STR profiling, and experiments used cells within 2 months of thawing.

Generation of CRISPR/CAS mediated knock out cell lines

OPM-2 cells expressing doxycycline-inducible Cas9 were generated by lentiviral transduction (Addgene) followed by puromycin selection. B7-H3 knockout lines were created using lentiviral delivery of guide RNAs TTCAGGGACCTGGACCTCCA and AGTGCCACCACTGGGTCTTC. Single-cell clones were isolated by FACS sorting and confirmed for B7-H3 deletion by qPCR (mRNA), flow cytometry, and western blotting (protein). BCMA-knockout OPM-2 cells were kindly supplied by the Bahlis lab (University of Calgary)

using the sgRNA sequence targeting GAAGAACATCGAAGTTGACA.

Generation of CAR-positive reporter cell lines

Reporter 2D3 Jurkat cells were modified to drive GFP expression through NFAT-inducible regulatory sequences. These cells were transduced with lentiviral vectors carrying the different CAR constructs at an MOI of 5. After growing the transduced population, anti-Thy1.1 magnetic beads were used to isolate the positive fraction via MACS enrichment. The sorted Thy1.1-expressing 2D3 cells were then mixed at a 1:1 effector-to-target ratio with various MM cell lines, and functional responses were measured after a 48-hour incubation.

Generation of primary human CAR T cells

PBMCs from healthy volunteers were acquired via the hematology service at UZ Brussels. Apheresis collections performed on the Spectra Optia® device (TerumoBCT) were further processed using the CTS Rotea™ system (Thermo Fisher Scientific) to isolate enriched PBLs, which were subsequently cryopreserved in medium containing 5% DMSO and CryoStor® CS5 (Biolife Solutions). Transduction was performed according to Miltenyi Biotec guidelines. PBLs were plated at 1×10^6 cells/mL in TexMACS medium and stimulated with anti-CD3/CD28 TransAct reagent (Miltenyi Biotec) plus 40 U/mL of both IL-7 and IL-15 (Miltenyi Biotec). Twenty-four hours later, lentiviral particles encoding the CARs were added at an MOI of 2.5. After another 24 hours, the virus and activation reagent were removed by washing. Cell expansion continued in TexMACS with IL-7/IL-15 (40 U/mL each), with fresh medium provided every 2–3 days. Transduction efficiency on day 7 was assessed by Thy1.1 surface staining, a surrogate marker of CAR expression [18]. For dual-specific CAR T cells, co-transduction was performed at an MOI of 2.5 per vector, as detailed in Bachiller *et al.* [22].

Patient samples

This investigation involved bone marrow specimens from 38 individuals with MM, including 25 newly diagnosed (D) and 13 relapsed (R) cases. Mononuclear cells were separated from BM aspirates by Ficoll-Hypaque gradient centrifugation. CD138-positive plasma cells were then positively isolated from the MNCs using anti-CD138 microbeads via the MACS system (Miltenyi Biotec).

Killing assays

Luciferase-expressing versions of OPM-2 and RPMI-8226 cells, generated by lentiviral transduction, served as targets to measure CAR-mediated cytotoxicity. Effector CAR T cells were combined with these Fluc+ MM targets at multiple effector-to-target ratios in RPMI-1640. Remaining luciferase activity was quantified after 24 hours with the Luciferase Assay System (Promega) on a Glomax reader, per manufacturer directions. For continuous monitoring of killing and studies of antigen escape, MM lines were labeled with either Katushka or GFP via lentiviral vectors. These fluorescent MM cells were plated with CAR T cells at a 1:1 ratio in cell-repellent 96-well U-bottom plates (Greiner) using TexMACS medium. Fluorescence intensity and area were recorded over time on the IncuCyte ZOOM platform (EssenBio) and normalized to the starting measurement ($t = 0$). Patient-derived primary MM cells were incubated overnight (18 h) at a 1:1 ratio with primary CAR T cells in RPMI-1640 plus 20% FBS. Tumor cell death was quantified the next day by flow cytometry using a BD Celesta instrument.

Cytokine analysis

Co-cultures of CAR T cells and tumor targets were set up at a 1:1 ratio for 18 hours (with RPMI-8226 or primary MM cells) or 24 hours (with OPM-2). Harvested supernatants were frozen at -80°C until testing. Secreted IL-2, IFN- γ , and TNF- α were measured by specific ELISA kits according to the supplier's protocols (Thermo Fisher Scientific; #88-7316-86, #88-7348-88, #88-7025-88).

Flow cytometry

Data acquisition took place on BD LSRFortessa, BD Symphony A1, or BD FACS Celesta instruments. BD FACSDiva Software (v8.02) handled collection, while FlowJo (Tree Star, Inc.) was used for analysis. Compensation controls utilized BD Comp Beads (cat. 552843) and BM MNCs. Patient samples were prepared in PBS with 5% FCS and stained for 30 min at 4°C in the dark using Brilliant Stain Buffer. Antibodies included: CD19 (BV785, #302239), CD38 (FITC, #356610), CD138 (BV605, #356520), B7-H3 (PE/APC, #351004), BCMA (BV421, #357519), CD14 (BV421, #325627), CD16 (FITC, #302005), CD15 (BV711, #323049), CD33 (BV785, #303428), CD45 (BV510, #368526), CD11b (BV605, #101257), HLA-DR (BV650, #307649), and CD3 (#300316) from

BioLegend; plus CD34 (BV650, #743533), CD45RA (BV711, #563733), and 7-AAD (#559925) from BD Biosciences. CAR detection was performed using anti-Thy1.1 (PE-Cy7/PE, #202523, BioLegend). Stained cells were washed and analyzed promptly in PBS containing 5% FBS.

Western blot

Protein extraction and blotting procedures followed established methods [23]. Cells were disrupted in lysis buffer supplemented with protease and phosphatase inhibitors. Samples were prepared in 2X Laemmli buffer (Bio-Rad), denatured at 95 °C for 5 min, resolved by SDS-PAGE, and transferred onto PVDF membranes (Bio-Rad). Membranes were blocked for 1 hour in 5% non-fat dry milk, incubated overnight with primary antibodies, washed in TBS-T (0.1% Tween), and probed with secondary antibodies for 1 hour. Chemiluminescent detection was performed with West Pico PLUS substrate (Thermo Scientific) on a Li-Cor Odyssey Fc system. Primary antibodies targeted B7-H3 (#14058), α -tubulin (#2144), and HRP-conjugated anti-rabbit secondary (#7074), all sourced from Cell Signaling Technology.

Animal models

Female NSG mice aged 6–8 weeks were sourced from Charles River Laboratories. In the disseminated OPM-2 model, 1×10^6 luciferase-expressing OPM-2 cells suspended in RPMI-1640 were administered intravenously. Weekly bioluminescent imaging was conducted on an IVIS platform following i.p. D-luciferin injection (150 mg/kg), with data processed using M3 Vision software. For the localized RPMI-8226 model, 5×10^6 cells in PBS were implanted subcutaneously in the flank. Tumor size was assessed 2–3 times weekly with a caliper, and volume was computed as $(\text{length} \times \text{width}^2)/2$. On day 14 post-inoculation, mice received 4×10^6 CAR T cells intravenously in 200 μ L plain RPMI-1640. Animals were euthanized once subcutaneous tumors reached 2000 mm³, in line with ethical protocols.

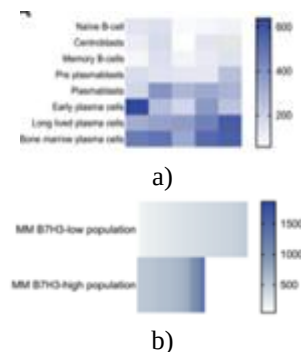
Statistical analysis

Analyses were conducted in GraphPad Prism 9 (GraphPad Software). Multi-group comparisons employed one- or two-way ANOVA with Tukey's multiple-comparison test when appropriate. Statistical significance was defined as $P < 0.05$. Complete details on tests, sample sizes (n), and P-values are specified in the figure legends.

Results and Discussion

B7-H3 is expressed on plasma cells of multiple myeloma patients

To explore B7-H3 (CD276) as a novel surface target in MM, gene expression data from the Heidelberg-Montpellier cohort were reviewed. This dataset comprises Affymetrix profiles from bone marrow of 206 MM patients and 5 healthy controls. CD276 mRNA expression increased along the differentiation pathway from naïve B cells to plasma cells (**Figure 1a**). Applying a threshold set at the mean signal intensity of normal plasma cells (ASU = 481.6), more than one-third of MM cases displayed upregulated CD276 transcripts (**Figure 1b**). Confirmation was sought in the University of Arkansas TT2 cohort. Although not reaching statistical significance, CD276 mRNA tended to be lower in healthy plasma cells relative to MGUS ($P = 0.0532$), SMM ($P = 0.3616$), and MM ($P = 0.2143$), with notable heterogeneity in the malignant samples (**Figure 1c**). Stratification according to the seven molecular subtypes of MM revealed significantly higher CD276 expression in the MMSET-overexpressing group compared to the full cohort ($P < 0.0001$) (**Figure 1d**). DepMap dependency screening indicated that HMCLs exhibited stronger reliance on B7-H3 than on BCMA (**Figure 1e**). Protein confirmation showed B7-H3 expression in 3/5 primary patient specimens and 5/7 MM cell lines by western blotting (**Figure 1f**). The OPM-2 line (MMSET subgroup) was selected for further experiments. A CRISPR/Cas9 B7-H3 knockout variant was established. Flow cytometric analysis of surface B7-H3 and BCMA on patient MM cells and HMCLs demonstrated that B7-H3 was either strongly positive or low/negative, with more consistent expression among positive cases than the variable BCMA pattern (**Figure 1g**). Surface levels of B7-H3 and BCMA on CD138+ cells showed no correlation ($P = 0.9813$) (**Figure 1h**).



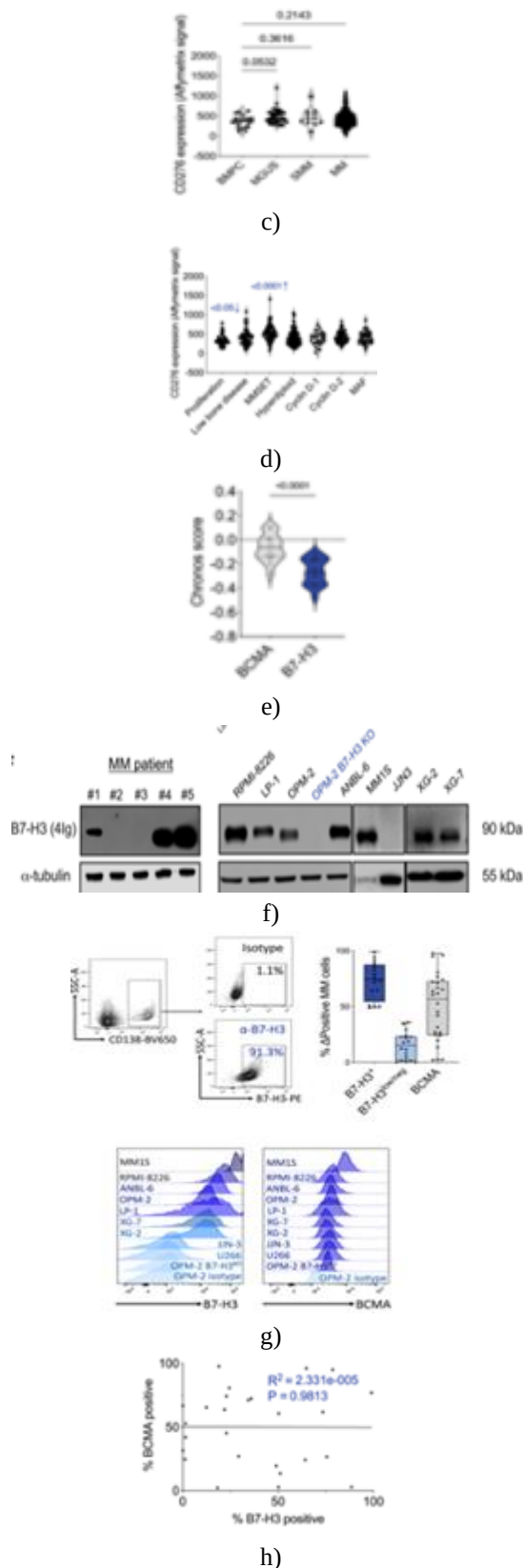


Figure 1. B7-H3 is expressed on MM cells.

(a) CD276 transcript levels during the maturation process from normal B cells into plasma cells, based on data from five healthy individuals. (b) CD276 gene expression profile in plasma cells obtained from MM patients. (c) CD276 mRNA expression across stages of disease evolution. (d) CD276 expression patterns within the established UAMS molecular classifications of MM. Arrows highlight statistically significant increases or decreases relative to the entire patient cohort (Student's t-test). (e) Gene dependency scores for BCMA and B7-H3 across human myeloma cell lines. (f) Western blot analysis of total B7-H3 protein in CD138+ cells from five MM patients (n = 5) (left panel) and in a selection of MM cell lines (right panel). (g) Surface expression of B7-H3 and BCMA on CD138+ cells derived from MM patients (top, n = 38) and MM cell lines (bottom). Delta positivity represents the percentage of positive cells after subtraction of isotype control staining. (h) Relationship between surface BCMA and B7-H3 positivity on CD138+ cells. (i) Surface B7-H3 expression on various BM cell populations. Delta positivity was determined by subtracting isotype control values. Abbreviations: MM = multiple myeloma; BMPC = bone marrow plasma cells; MGUS = monoclonal gammopathy of undetermined significance; SMM = smoldering multiple myeloma; BCMA = B-cell maturation antigen; HSC = hematopoietic stem cell; GMP = granulocyte-monocyte progenitor; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; Mo-MDSC = monocytic myeloid-derived suppressor cell; BM-DC = bone marrow dendritic cell; Gr-MDSC = granulocytic myeloid-derived suppressor cell; NK cell = natural killer cell.

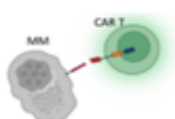
Because B7-H3 functions as an immunomodulatory molecule, its presence on non-malignant cells within the bone marrow microenvironment cannot be ruled out. We therefore examined B7-H3 surface levels on different cell populations in freshly isolated BM mononuclear cells from patients with newly diagnosed MM (NDMM) and

relapsed/refractory MM (RRMM) using flow cytometry (**Figure 1i**). Expression remained minimal on hematopoietic stem and progenitor cells, NK cells, T cells, and B cells. Within the myeloid lineage, variable expression was observed in both normal myeloid cells and immunosuppressive subsets, with considerable patient-to-patient variation. Overall, B7-H3 is present on the surface of MM cells and, when expressed, displays more uniform levels than BCMA, supporting its suitability as an immunotherapeutic target. B7-H3 is a viable target for CAR T-cell therapy in multiple myeloma.

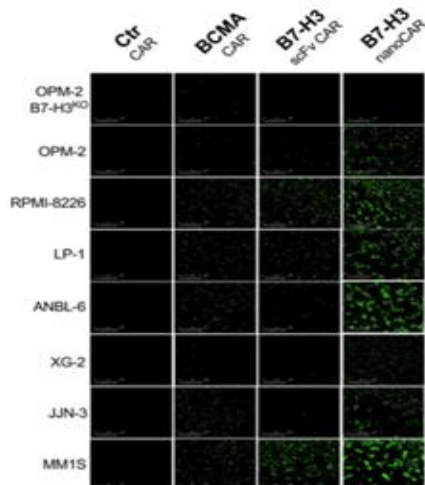
To investigate the therapeutic utility of B7-H3 in MM, we constructed four different second-generation CARs (**Figure 2a**). These included an irrelevant control CAR incorporating a nanobody (VHH) specific for the 5T2 mouse idiotype, a B7-H3-targeted nanobody-based CAR (B7-H3 nanoCAR), and a CAR utilizing the anti-B7-H3 scFv sequence from enoblituzumab. A BCMA-directed CAR based on the idecabtagene vicleucel (Abecma) scFv was also generated for comparison [18, 24, 25].



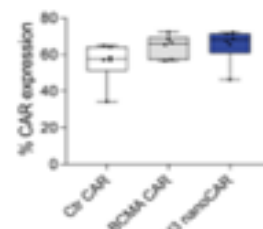
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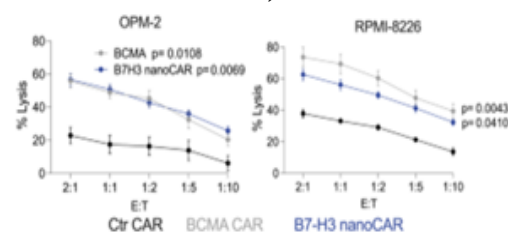
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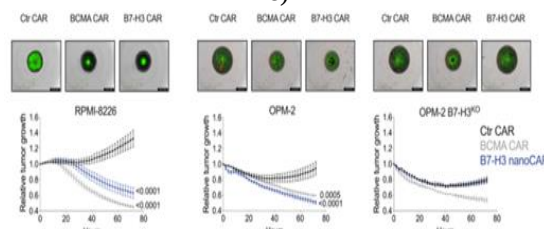
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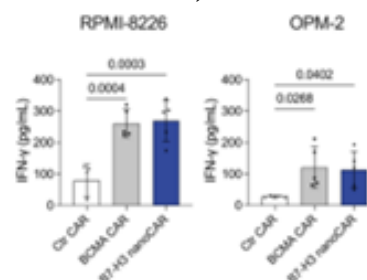
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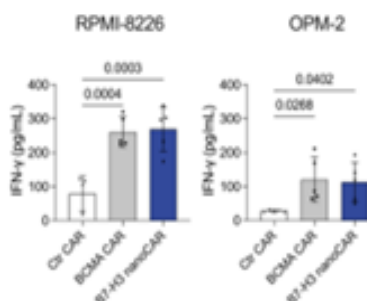
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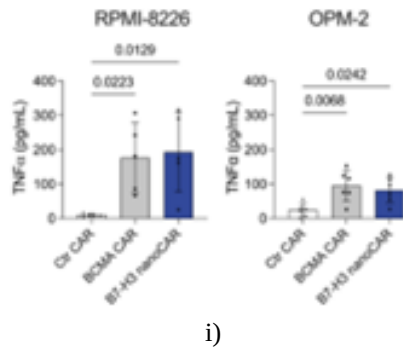


Figure 2. B7-H3 expressed on multiple myeloma cells triggers recognition and destruction by CAR T cells.

(a) Diagram illustrating the different CAR designs included in the experiments. Control constructs (nanobody specific for the irrelevant mouse 5T2 idiotype) were tested in parallel with B7-H3-targeted scFv CARs, B7-H3 nanoCARs, and the BCMA CAR modeled after ide-cel. (b) Principle of the 2D3 reporter cell assay. (c) Activation of CAR-transduced 2D3 cells (visualized as green fluorescence) upon incubation with selected MM cell lines. (d) CAR surface expression on primary T cells from healthy donors, measured by Thy1.1 levels seven days following lentiviral transduction. (e) Cytotoxic function of B7-H3 nanoCAR T cells in vitro. Effector cells were incubated for 24 h with MM targets across a range of effector-to-target ratios. (f) Time-lapse imaging of GFP-labeled MM cell lines co-cultured at a 1:1 ratio with CAR T cells over 72 h, depicting normalized remaining tumor burden. (g–i) Production of IFN- γ (g), IL-2 (h), and TNF- α (i) following 1:1 co-culture with RPMI-8226 or OPM-2 cells. Data in panels D–I represent $n \geq 5$ independent experiments using CAR T cells generated from at least three separate healthy donors. Statistical testing in E–I used one-way ANOVA (two-sided), with p-values displayed directly on the graphs.

The capacity of these CARs to stimulate T-cell signaling was first examined with 2D3 Jurkat cells engineered to express GFP under the control of NFAT promoter elements (**Figure 2b**). When these CAR-positive reporter cells were mixed 1:1 with various human myeloma cell lines, strong GFP induction occurred specifically with the B7-H3-directed CARs (**Figure 2c**). The nanobody-based B7-H3 CAR consistently outperformed the scFv version in driving reporter activation. Based on this superior performance, further work focused solely on the nanoCAR platform.

Primary human T cells isolated from healthy volunteers were subsequently transduced with the second-generation CAR lentiviruses. Transduction efficiency was routinely assessed on day 7 via Thy1.1 detection (**Figure 2d**). Functional cytotoxicity assays against luciferase-expressing HMCLs revealed clear dose-dependent elimination of both OPM-2 and RPMI-8226 cells by B7-H3 nanoCAR T cells (**Figure 2e**). Real-time monitoring using GFP+ target cells and the IncuCyte ZOOM imaging system confirmed comparable kinetics of tumor cell clearance over time (**Figure 2f**). Moreover, exposure to RPMI-8226 or OPM-2 cells elicited markedly higher release of key effector cytokines (IL-2, IFN- γ , and TNF- α) from B7-H3 nanoCAR T cells than from control CAR T cells (**Figures 2g–2i**).

B7-H3 nanoCAR T cells demonstrate activity against primary patient-derived myeloma cells in vitro

To strengthen the translational relevance of these observations, CD138+ plasma cells were magnetically isolated from bone marrow samples of MM patients and co-incubated with allogeneic B7-H3 nanoCAR T cells. After testing several effector-to-target ratios, an 18-hour incubation at a 1:1 ratio was selected because it produced minimal non-specific background lysis (**Figure 3a**). Compiled results across multiple patients showed significantly enhanced killing of primary MM cells by the B7-H3 nanoCAR T cells relative to control nanoCAR T cells (**Figure 3b**). This killing was accompanied by robust IFN- γ secretion, confirming effective T-cell activation (**Figure 3c**).

To investigate possible unintended effects on the surrounding bone marrow milieu — especially considering the variable B7-H3 expression detected on certain myeloid subsets in individual patients — additional experiments were performed using unseparated BM mononuclear cells. In these assays, both the destruction of MM cells and the survival of non-malignant populations, particularly CD34+ hematopoietic progenitors, were tracked (**Figure 3d**). While both BCMA and B7-H3 nanoCAR T cells potentially eliminated the malignant plasma cells, the majority of normal BM compartments, including CD34+ cells, remained largely unaffected. Nevertheless, a reduction in monocytic cells was recorded in the sample from one patient (**Figure 3e**). Increased IFN- γ levels in the supernatant verified CAR T-cell activation within the complex BM environment (**Figure 3f**). Taken together, these findings establish that B7-H3 nanoCAR T cells can effectively target primary MM cells, yet they also

highlight the importance of monitoring for potential on-target, off-tumor effects on specific BM populations.

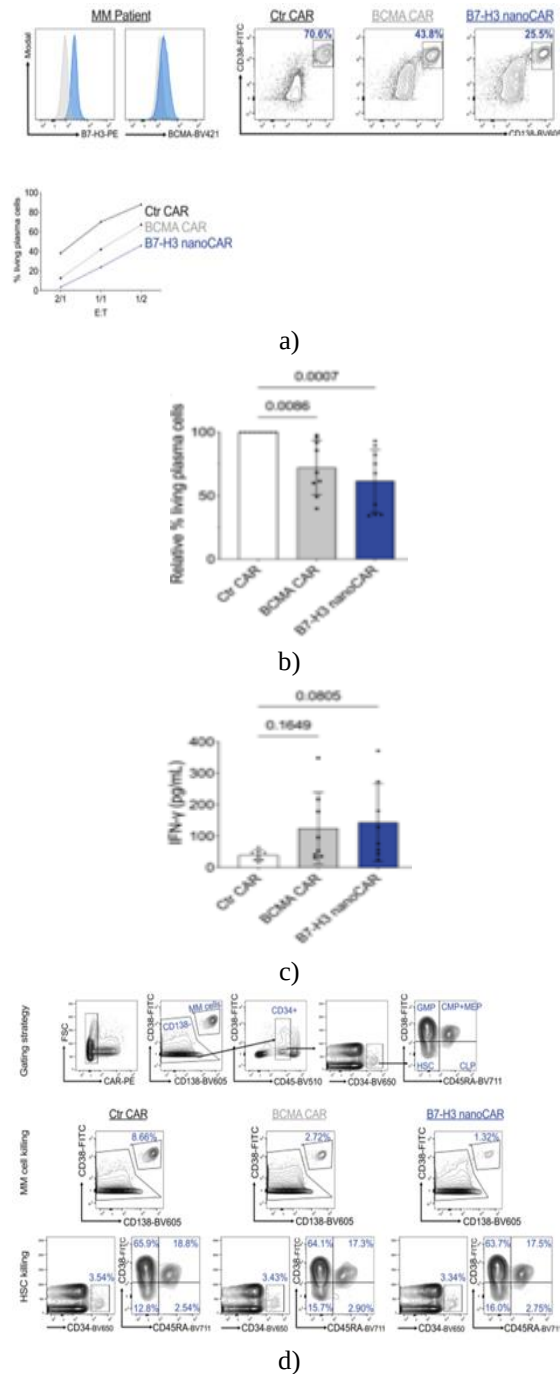


Figure 3. B7-H3 nanoCAR T cells effectively eliminate primary MM cells.

(a) Example data from one MM patient sample. Isolated bone marrow plasma cells were incubated with nanoCAR T cells at several effector-to-target ratios for 18 hours. Remaining viable MM cells were identified by flow cytometry after excluding dead cells and T cells, using the CD38⁺ CD138⁺ phenotype. (b) Combined analysis of relative viability of CD138⁺ plasma cells from nine MM patients after 18-hour co-culture with CAR T cells at a 1:1 ratio. Results are normalized to the control CAR condition. (c) IFN-γ concentrations measured in the supernatants from the experiments shown in panel B. (d) Representative gating approach used to assess survival of hematopoietic stem/progenitor cells during co-culture of nanoCAR T cells with total BM mononuclear cells (top). Demonstration of MM cell depletion in these BM MNC co-cultures (middle) while preserving CD34⁺ progenitor cells (bottom). (e) Summary quantification of viability across different BM cell subsets under these conditions. (f) IFN-γ levels detected in supernatants from the experiments in panel E. All experiments utilized nanoCAR T cells generated from three independent healthy donors. Statistical analysis for panels B–C was conducted by one-way ANOVA (two-sided), with p-values indicated on the graphs.

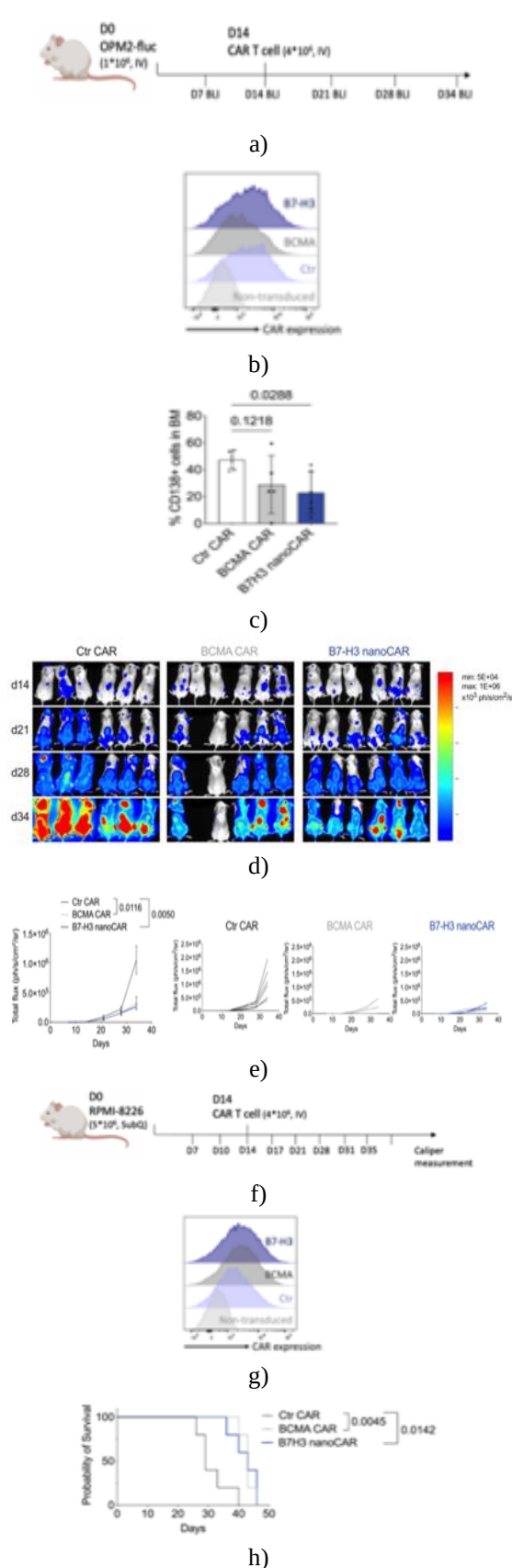


Figure 4. B7-H3 nanoCAR T cells demonstrate in vivo efficacy.

(a) Study design for the orthotopic model in NSG mice that received intravenous OPM-2-fluc tumor cells followed by infusion of 4×10^6 CAR T cells. (b) CAR expression determined through Thy1.1 detection. (c) Frequency of residual MM cells recovered from bone marrow at the experimental endpoint. (d) Serial bioluminescent whole-body images acquired on day 14 (before CAR T-cell administration), day 21, day 28, and day 34. (e) Quantitative analysis of bioluminescence signals ($n = 6$ mice per group for control CAR and B7-H3 nanoCAR; $n = 5$ for BCMA CAR). Day-34 data were used for statistical comparisons. (f) Outline of the subcutaneous xenograft model in NSG mice injected with RPMI-8226 cells and subsequently treated with 4×10^6 CAR T cells. (g) Verification of CAR surface expression by Thy1.1 staining. (h) Survival curves for treated animals (log-rank Mantel–Cox test; p-values reported). (i) Mean tumor volumes pooled from each group ($n = 5$ mice/group) determined by caliper measurements. Statistical testing was performed on day 21. (j) Tumor growth kinetics plotted for every individual animal from panel I. The dashed line denotes the predefined ethical tumor size limit. Each experiment utilized T cells derived from separate healthy donors. One-way ANOVA (two-sided) was applied for statistical evaluation in panels C, E, and I, with corresponding p-values displayed on the graphs.

B7-H3 nanoCAR T cells demonstrate therapeutic activity in two distinct mouse models of multiple myeloma

The therapeutic potential of B7-H3 nanoCAR T cells was assessed in an orthotopic bone marrow-homing model established by intravenous administration of luciferase-tagged OPM-2 cells into NSG mice. Tumor burden was

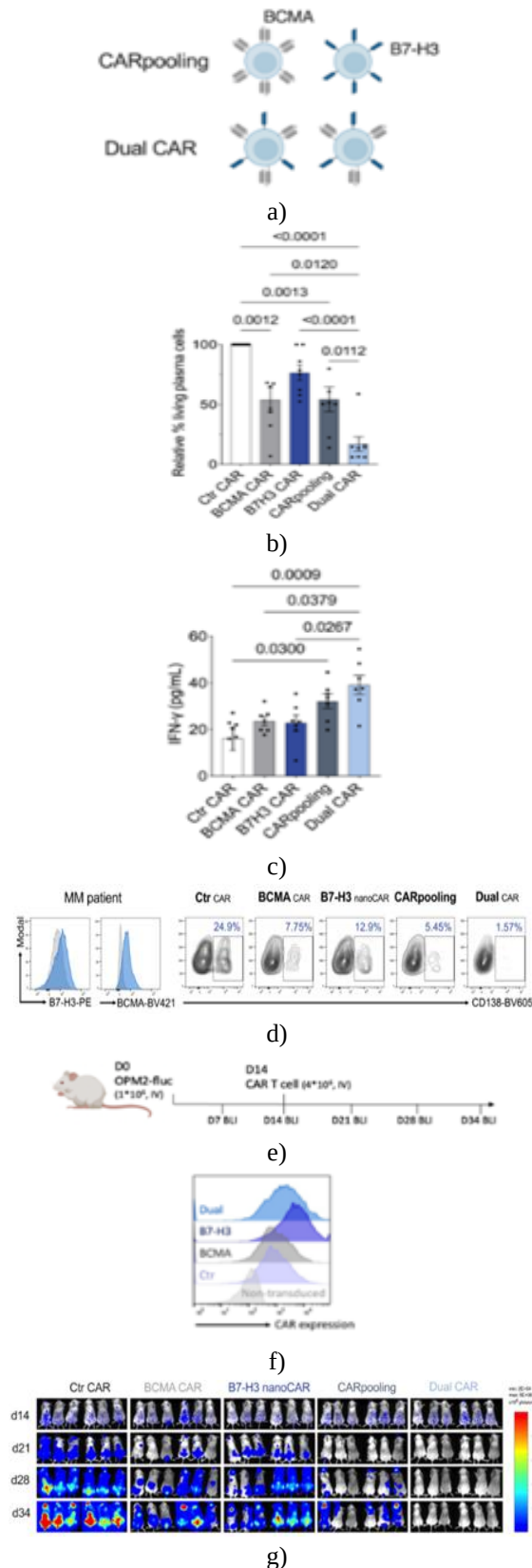
followed longitudinally using non-invasive bioluminescence imaging (**Figure 4a**). CAR T-cell infusion occurred on day 14 after tumor challenge (**Figure 4b**). By day 34, moderate hind-limb paralysis appeared across several cohorts, prompting simultaneous euthanasia of all animals to enable direct comparison. Analysis of bone marrow at this time point revealed a statistically significant decrease in MM cell infiltration exclusively in mice that had received B7-H3 nanoCAR T cells (**Figure 4c**). This finding was consistent with the longitudinal bioluminescence data and corresponding signal quantification (**Figures 4d and 4e**).

A separate, localized tumor model was established by subcutaneous injection of RPMI-8226 cells into NSG mice (**Figure 4f**), followed by CAR T-cell treatment 2 weeks later (**Figure 4g**). In this setting, both BCMA-specific and B7-H3-specific CAR T cells slowed disease progression (**Figures 4h and 4i**), with per-animal tumor volume trajectories presented in **Figure 4j**. Overall, B7-H3 nanoCAR T cells mediated clear anti-tumor responses in vivo across both models. However, complete tumor eradication was not achieved.

Simultaneous targeting of two antigens enhances activity against wild-type multiple myeloma

We postulated that engaging two distinct antigens concurrently could augment treatment efficacy. Earlier studies reported that tandem CAR designs frequently exhibit lower potency than cells co-expressing two independent CAR molecules, termed dual CARs [26, 27]. In contrast, the CARpooling approach, which involves mixing two separate monospecific CAR T-cell populations, had not been examined. We therefore directly compared single-specificity BCMA nanoCAR T cells, single B7-H3 nanoCAR T cells, dual CAR T cells (simultaneous expression of BCMA scFv CAR plus B7-H3 nanoCAR), and CARpool preparations (**Figure 5a**). Proper CAR expression on the engineered cells was validated by staining with specific antigens.

In vitro killing experiments against MM cell lines showed modest gains with the multi-specific strategies, with the strongest average performance seen in the dual CAR and CARpool groups. Dual CAR T cells furthermore produced the most robust cytokine responses when stimulated with OPM-2 or RPMI-8226 targets. When tested on primary MM cells isolated from patients, the superiority of dual targeting became even more apparent, yielding the highest levels of tumor cell lysis and IFN- γ release (**Figures 5b–5d**).



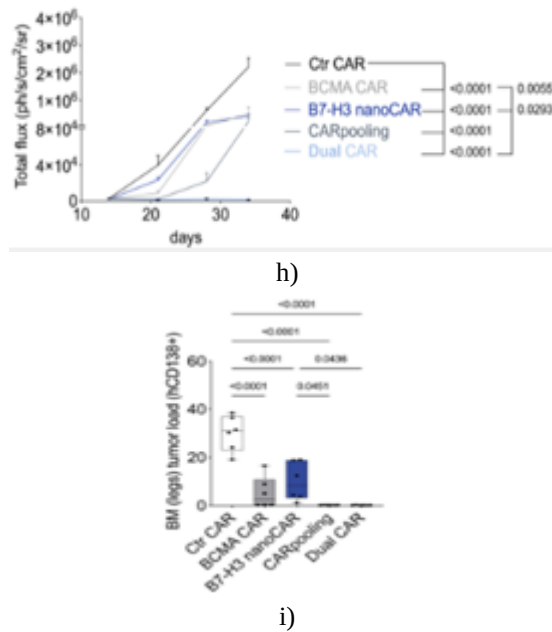


Figure 5. Dual targeting improves CAR-mediated cytotoxicity.

(a) Schematic illustration of CARpool and dual CAR T-cell configurations. (b) Aggregated relative viability of bone marrow plasma cells from 8 MM patients after 18-hour co-culture at a 1:1 ratio with the indicated CAR T-cell populations. Values are normalized to the control CAR condition. (c) IFN- γ concentrations measured in supernatants from the experiments in panel B. (d) Example data from one MM patient: surface levels of B7-H3 and BCMA on plasma cells (left) and corresponding plasma cell viability following 18-hour incubation with different CAR T-cell populations (right). (e) Experimental outline for the orthotopic NSG mouse model involving intravenous injection of OPM-2-fluc cells and subsequent treatment with 4×10^6 CAR T cells. (f) CAR expression levels assessed by Thy1.1 staining. (g) Representative bioluminescent images captured on day 14 (pre-treatment), day 21, day 28, and day 34. (h) Quantification of bioluminescence intensity across groups ($n = 6$ mice per group). (i) At day 34, bone marrow from the legs was harvested, and human CD138⁺ plasma cells were enumerated by flow cytometry ($n = 6$ per group). One-way ANOVA was applied to panels B, C, and I; two-way ANOVA was used for panel H. All tests were two-sided, and the relevant p-values were indicated in the graphs.

The orthotopic OPM-2 model was employed once more to evaluate combination strategies (Figure 5e). Fourteen days after tumor cell injection, animals received

infusions of control CAR T cells, single BCMA CAR T cells, single B7-H3 nanoCAR T cells, CARpool mixtures, or dual CAR T cells (Figure 5f). Tumor development was tracked weekly via bioluminescence imaging (Figures 5g and 5h). At the same time, CAR T-cell persistence was monitored in peripheral blood. The dual CAR T-cell group displayed the most potent suppression of tumor growth, whereas single CAR and CARpool treatments resulted in similar residual tumor signals by day 34 (Figure 5h). Endpoint examination of leg bone marrow revealed detectable residual tumor cells in mice treated with B7-H3 nanoCAR T cells. In contrast, no tumor cells were found in 50% of BCMA CAR T-cell-treated mice and in all animals that received either CARpool or dual CAR T cells (Figure 5i). Circulating CAR T cells were detectable over time in blood and at endpoint in bone marrow across all treatment arms. Analysis of serum at the study endpoint confirmed IFN- γ production, consistent with sustained CAR T-cell activity. IL-1 β concentrations and body weight changes were monitored as surrogates for potential toxicity; no major differences were observed among groups, apart from weight loss in control CAR T-cell-treated mice attributable to progressive disease.

Dual targeting overcomes antigen escape

Antigen loss represents a major cause of relapse following BCMA-directed therapies. Therefore, we investigated whether B7-H3 nanoCAR T cells and dual-targeting approaches could address this issue. CRISPR/Cas9-generated knockout cell lines lacking BCMA or B7-H3 were established (Figures 6a and 6b). To simulate BCMA antigen escape, GFP-labeled wild-type OPM-2 cells (green) were mixed with Katushka-labeled BCMA-knockout OPM-2 cells (red) (Figure 6c). B7-H3 CAR T cells and dual-targeting conditions efficiently cleared both cell populations (Figures 6d and 6e). Some degree of BCMA-knockout cell killing was also noted in the BCMA CAR condition, likely mediated by locally released cytokines. In addition, primary bone marrow plasma cells from a BCMA-negative MM patient proved resistant to BCMA CAR T cells. Still, they remained sensitive to B7-H3 CAR T cells and the CARpool strategy (Figure 6f). To model heterogeneous antigen expression more comprehensively, a mixed population consisting of GFP⁺ B7-H3-knockout OPM-2 cells and Katushka⁺ BCMA-knockout OPM-2 cells was generated (Figure 6g). Dual-targeting strategies again achieved robust elimination of both subpopulations,

whereas single-antigen CAR T cells showed reduced effectiveness (**Figures 6h and 6i**). The strongest anti-tumor effects were consistently observed with the dual CAR T-cell approach. These results indicate that dual targeting enables effective clearance of antigen-heterogeneous tumor populations, addressing a critical obstacle in multiple myeloma treatment.

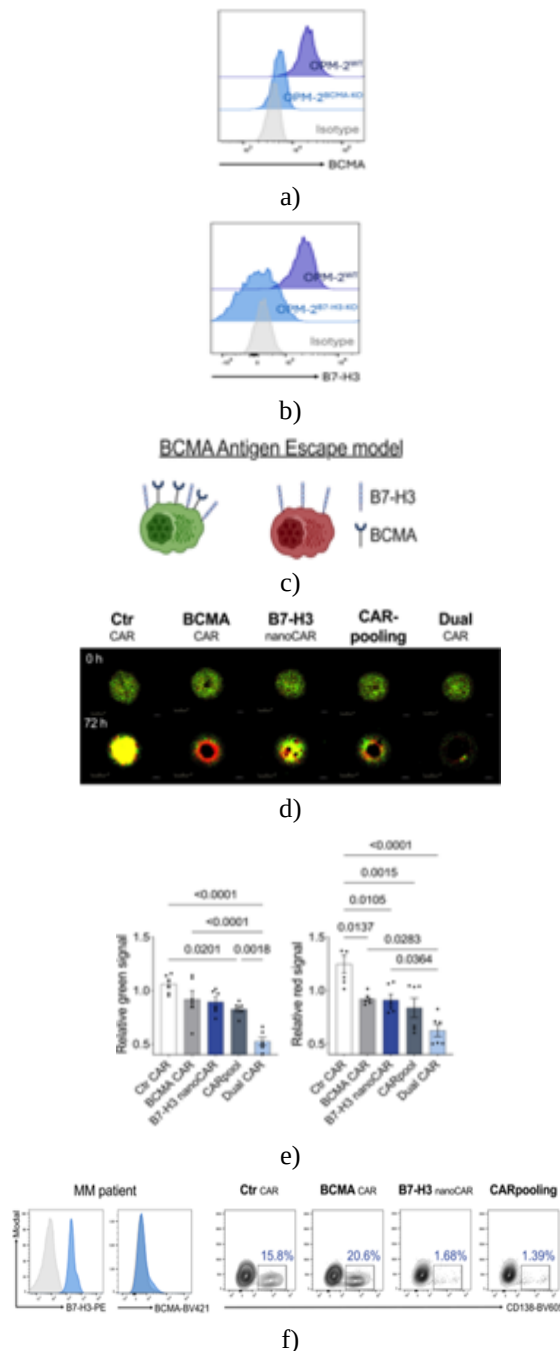


Figure 6. Dual targeting overcomes antigen escape.

(a) BCMA surface expression on OPM-2 wild-type and knockout cells. (b) B7-H3 surface expression on OPM-2 wild-type and knockout cells. (c) Schematic of the in vitro model designed to recapitulate BCMA antigen escape. GFP⁺ OPM-2 wild-type cells and Katushka⁺ BCMA-knockout OPM-2 cells were combined at a 1:1 ratio and (d-e) incubated with the indicated CAR T-cell populations for 72 hours at a 1:1 effector-to-target ratio. (f) CD138⁺ plasma cells purified from a BCMA-negative MM patient were co-cultured for 18 hours at a 1:1 ratio with the indicated CAR T cells. (g) Schematic of the heterogeneous tumor model. GFP⁺ B7-H3-knockout OPM-2 cells and Katushka⁺ BCMA-knockout OPM-2 cells were mixed at equal ratios and (h-i) exposed to the indicated CAR T cells for 72 hours at a 1:1 ratio. One-way ANOVA was performed for statistical analysis; all tests were two-sided, with p-values shown in the graphs. This work establishes that B7-H3 is present on the plasma cell surface in a substantial fraction of MM patient samples and introduces, for the first time, a second-generation CAR T-cell product directed against this antigen for MM immunotherapy. The construct employs a nanobody for antigen recognition and incorporates 4-1BB co-stimulation. Nanobodies offer multiple benefits over traditional scFvs, including

reduced size, which promotes better CAR expression and membrane trafficking; improved stability and folding properties; and potentially decreased immunogenicity owing to their single-domain structure [28]. In addition, nanobody-based CARs are less susceptible to spontaneous antigen-independent clustering, thereby limiting tonic signaling that can accelerate T-cell exhaustion [29, 30]. The overall architecture of this nanoCAR mirrors that of the most clinically successful BCMA-targeted products, which have yielded the best survival data in trials [31], positioning it as a strong candidate for further development in MM.

BCMA has become a cornerstone of modern MM therapy [32-34]. The approval of ciltacabtagene autoleucel in the second-line setting and the increasing use of teclistamab have solidified its importance. However, heightened therapeutic pressure on BCMA also increases the risk of antigen downregulation, mutational escape, and outgrowth of BCMA-negative subpopulations [35, 36]. Although total BCMA loss is observed in only 4–33% of cases, our findings indicate heterogeneous BCMA expression on MM cells, which may serve as a source for future antigen-negative relapses.

To counteract these adaptive mechanisms, alternative approaches including APRIL-based CARs (targeting both BCMA and TACI) and GPRC5D-directed therapies have been developed. Nevertheless, APRIL CAR T cells have shown limited clinical activity [37], and dual antigen loss has already been documented after sequential BCMA and GPRC5D targeting [38]. Our results suggest that incorporating B7-H3 as an additional target could help overcome these challenges, as B7-H3-specific CAR T cells effectively control BCMA-negative disease. At the same time, dual BCMA/B7-H3 targeting provides broader protection against loss of either antigen. We deliberately avoided generating bispecific tandem CARs (a single receptor with two binding domains) because multiple independent studies have shown inferior performance relative to dual CAR T cells that co-express separate receptors [26, 27]. We instead evaluated dual CAR T cells alongside a CARpooling strategy that combines two distinct monospecific CAR T-cell populations, an approach that had not been previously tested. Dual BCMA/B7-H3 CAR T cells consistently outperformed single-specificity constructs, whereas CARpooling produced more variable results. The improved activity of dual CARs may stem from greater signaling strength per cell, since each engineered T cell expresses both receptors simultaneously. In the OPM-2

orthotopic model, dual CAR T cells achieved superior tumor control and a higher complete response rate.

In contrast, single B7-H3 nanoCAR T cells and CARpooling failed to produce durable clearance. This difference might reflect insufficient CAR T-cell potency or suboptimal timing/dosing relative to tumor burden at the time of treatment. Future optimization could involve refined dosing schedules or pharmacological upregulation of B7-H3 on MM cells. For instance, the FDA-approved agent ingenol-3-angelate can increase B7-H3 levels by activating PKC α [39]. Epigenetic modifiers such as decitabine represent another attractive option, as they not only elevate B7-H3 expression [40] but also exert direct anti-MM effects and may enhance CAR T-cell persistence via epigenetic modulation [41-43]. Conversely, certain combinations should be avoided, as B7-H3 protein levels are controlled by mTORC1 signaling in MM [44]. Prior work from our laboratory demonstrated that metabolic targeting in MM cells can suppress mTORC1 activity and consequently reduce B7-H3 expression [23, 45].

Although such combination strategies may modulate target antigen density, the expression of B7-H3 on non-malignant cells requires careful evaluation to define the therapeutic window. We therefore profiled B7-H3 in bone marrow aspirates. In contrast to CD38 targeting with daratumumab, which can impair hematopoietic progenitors, B7-H3 expression on stem and progenitor cells was low to absent, and ex vivo exposure to B7-H3 nanoCAR T cells did not reduce CD34⁺ cell numbers. However, variable expression was detected in the myeloid-monocytic compartment in some patients, leading to partial depletion of monocytes and Mo-MDSCs in one case. While this could theoretically provide an additional benefit by reducing immunosuppressive cells, the potential clinical consequences of monocyte loss, including increased infection risk, warrant further investigation.

Ongoing clinical trials of B7-H3 CAR T cells in solid tumors have generally reported acceptable tolerability, with mainly cytokine-related symptoms [46-48]. A preclinical study noted possible on-target, off-tumor effects following tumor eradication [18], although a recent clinical trial did not observe such toxicities despite modest anti-tumor efficacy [47]. In our in vivo experiments, toxicity was monitored indirectly by measuring body weight and serum IL-1 β [49]; no overt adverse effects were observed in B7-H3 nanoCAR T-cell-treated mice. Nevertheless, more complete tumor

clearance might unmask such toxicities. A limitation of the current study is the use of immunodeficient NSG mice lacking a human immune system [49], which precludes full assessment of potential off-target effects on monocytes or hematopoietic progenitors. Future safety studies would benefit from humanized mouse models or human bone marrow xenograft systems [49-51]. Risk mitigation strategies could include transient mRNA-based CAR expression or incorporation of inducible suicide switches to allow controlled elimination of CAR T cells if necessary [52, 53].

B7-H3 is under active investigation as an immunotherapy target across multiple malignancies [9]. The successful development of a safe, allogeneic “off-the-shelf” B7-H3 CAR T-cell product could offer a broadly applicable therapy for various cancers. Additionally, the clinical success of bispecific T-cell engagers in MM and encouraging preclinical data in other tumor types suggest that B7-H3-targeted bispecific engagers may also hold significant promise [54].

Conclusion

In summary, this study provides strong preclinical evidence that B7-H3 constitutes a promising target for MM immunotherapy. Both monospecific B7-H3 and dual-specific BCMA/B7-H3 CAR T cells exhibit potent cytotoxic activity and can effectively eradicate BCMA-negative tumor clones. These findings support the clinical translation of B7-H3-directed therapies and offer an additional treatment avenue for patients with multiple myeloma.

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