Switchable targeting of solid tumors by BsCAR T cells

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The development of chimeric antigen receptor (CAR) T cell therapy has become a critical milestone in modern oncotherapy. Despite the remarkable in vitro effectiveness, the problem of safety and efficacy of CAR T cell therapy against solid tumors is challenged by the lack of tumor-specific antigens required to avoid on-target off-tumor effects. Spatially separating the cytotoxic function of T cells from tumor antigen recognition provided by protein mediators allows for the precise control of CAR T cell cytotoxicity. Here, the high affinity and capability of the bacterial toxin-antitoxin barnase-barstar system were adopted to guide CAR T cells to solid tumors. The complementary modules based on (1) ankyrin repeat (DARPIn)-barnase proteins and (2) barstar-based CAR (BsCAR) were designed to provide switchable targeting to tumor cells. The alteration of the DARPIn-barnase switches enabled the targeting of different tumor antigens with a single BsCAR. A gradual increase in cytokine release and tunable BsCAR T cell cytoxicity was achieved by varying DARPIn-barnase loads. Switchable BsCAR T cell therapy was able to eradicate the HER2+ ductal carcinoma in vivo. Guiding BsCAR T cells by DARPIn-barnase switches provides a universal approach for a controlled multtargeted adoptive immunotherapy.

Significance

The use of tumor-associated antigens to target solid tumors by chimeric antigen receptor (CAR) T cells remains limited by the lack of tumor-specific antigens required to avoid on-target off-tumor effects. Programmable CAR systems are being developed to achieve more sophisticated tumor recognition and enhance the controllability of CAR T cells. Here, we propose to harness the RNase toxin barnase as a targeting module that provides the cytotoxic effects itself or in combination with CAR T cells. Molecular switches based on the DARPIn-barnase proteins and universal barstar-based CAR (BsCAR) were designed and validated in vitro and in vivo. We expect this strategy may be a promising approach for the generation of safe and efficient CAR T cell therapy against solid tumors.

The authors declare no competing interest.
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The use of tumor-associated antigens to target solid tumors by chimeric antigen receptor (CAR) T cells remains limited by the lack of tumor-specific antigens required to avoid on-target off-tumor effects. Programmable CAR systems are being developed to achieve more sophisticated tumor recognition and enhance the controllability of CAR T cells. Here, we propose to harness the RNase toxin barnase as a targeting module that provides the cytotoxic effects itself or in combination with CAR T cells. Molecular switches based on the DARPIn-barnase proteins and universal barstar-based CAR (BsCAR) were designed and validated in vitro and in vivo. We expect this strategy may be a promising approach for the generation of safe and efficient CAR T cell therapy against solid tumors.
antitoxin, representing an outstanding example of molecular switching based on the extraordinary affinity of the barnase-barstar complex ($K_D \sim 10^{-11}$) (36–39). Here, the barnase–barstar interaction was applied to guide barstar-modified CAR (BsCAR) T cells to tumor cells by barnase-based molecular switches. Barnase was fused with designed ankyrin repeat proteins (DARPins) that are specific to tumor antigens HER2 (human epidermal growth factor receptor 2) and EpCAM. The resulting DARPin–barnase (DARPin-Bn) proteins enabled specific targeting of tumor cells by T cells modified with universal BsCAR. The high affinity of barnase-barstar binding provides a unique regulatory potential of CAR T therapeutics in vivo. This approach can reinforce the CAR technology with therapeutic modes, including the redirection of T cell cytotoxicity toward combinations of multiple tumor antigens.

**Results**

**DARPin-Bn Switching Modules Mediate HER2-Specific Targeting.** Fusion proteins of anti-HER2 DARPin (G3 and 9.29) with barnase (40) were used as molecular switches directing BsCAR T cells against HER2$^+$ tumor cells. DARPin G3 and 9.29 were reported to interact with different domains of the HER2 receptor (Fig. 1A), which is frequently overexpressed in solid tumors. G3 binds to the HER2 membrane-proximal domain IV, whereas 9.29 interacts with the membrane-distal subdomain I, with $K_D = 0.09$ nM (41) and $K_D = 3.8$ nM (40), respectively. Similar to the therapeutic anti-HER2 monoclonal antibody (mAb) trastuzumab, recombinant G3-Bn and 9.29-Bn proteins specifically interact with the HER2$^+$ BT-474 human aggressive ductal breast carcinoma cell line (Fig. 1B). HER2 overexpression resulted in specific targeting of BT-474 cells by G3-Bn and 9.29-Bn, which was not observed in human MDA-MB-231 epithelial breast cancer cells with normal HER2 expression and in HER2$^+$ Chinese hamster ovary (CHO) cells (Fig. 1C). The level of RNase activity of G3-Bn and 9.29-Bn was similar to that of free barnase (SI Appendix, Fig. S1); half-maximal effective concentration ($EC_{50}$) = 6.1 nM, 3.6 nM, and 2.2 nM, respectively. The specific cytotoxicity against HER2$^+$ BT-474 cells mediated by the RNase activity of G3-Bn and 9.29-Bn was observed only after prolonged incubation (>10 d), with half-maximal inhibitory concentration ($IC_{50}$) = 9.4 ± 1.2 nM and 4.2 ± 0.5 nM, respectively (Fig. 1D). To assess apoptosis induction by DARPin-Bn in BT-474 cells, phosphatidyserine exposure to the outer plasma membrane was analyzed by the annexin V assay. The signs of apoptosis were detected after 48 to 72 h after incubation (SI Appendix, Fig. S2). As one can see, the antitumor activity of HER2-specific DARPin-Bn conjugates required both the HER2 targeting moiety and the functional RNase component, because we did not observe similar cytotoxicity in a clonogenic assay with trastuzumab or unconjugated barnase at equimolar concentrations (SI Appendix, Fig. S3).

**BsCAR T Cells Provide DARPin-Bn-Guided Cytotoxicity.** A panel of barstar mutants was created to optimize the extracellular barnase-binding module of CAR. The barstar amino acid sequence contains unpaired cysteines (C40 and C82) and isoleucine 87, which may lead to homodimerization (33, 36, 42) (Fig. 2A). If barstar dimerizes, it could block the barnase interaction site on CAR. Moreover, the interaction of DARPin-Bns with barstar in the context of CAR requires a certain flexibility, which could be achieved by inserting peptide linkers (44–46). To address these potential issues, a panel of BsCARs was designed. These BsCARs were based on wild-type barstar and barstar point mutants having short (G4S) and long 3X(G4S) linkers (short linkers: BsCARv1, wild type; BsCARv2, C40A and C82A substitutions and long linkers: BsCARv3, wild type; BsCARv4, C40A and C82A, BsCARv5, I87E, BsCARv6, C40A, C82A, and I87E) (Fig. 2A and B and SI Appendix, Fig. S4). All CAR variants had a mutated immunoglobulin G4 (IgG4) hinge with two point mutations (L235E and N297Q) to suppress interaction with FcyR and promote the persistence of CAR T cells (47). CAR comprised a membrane and an intracellular part of CD28, intracellular cytoplasmic activation domain of 4-1BB (CD137), and CD3ζ (Fig. 2B). All of the variants of BsCAR were detected on the T cell surface at similar levels, except for BsCARv5 (Fig. 2 C, Left). BsCAR T cells were stained with DARPin 9.29-Bn-FITC to determine which BsCAR variant retained the ability to interact with barnase. Only four out of six BsCAR variants (v2, v3, v4, and v6) efficiently interacted with barnase (Fig. 2 C, Right), and BsCARv4 demonstrated the strongest binding.

The cytotoxicity of the BsCAR variants toward BT-474 cells was evaluated in the presence of 1 nM 9.29-Bn or G3-Bn (Fig. 2D). The BsCAR variants v2, v3, v4 ($P < 0.0001$), and v6 ($P < 0.05$) exerted cytotoxic effects mediated by 9.29-Bn or G3-Bn switches. The BsCARv4 T cells induced the death of tumor cells more efficiently than other BsCAR variants ($P < 0.0001$). BsCARv4 was chosen for the following in vitro and in vivo experiments based on its surface expression (Fig. 2 C, Left), barnase binding (Fig. 2 C, Right), and high cytotoxicity (Fig. 2D). The signal transduction efficiency of BsCARv4 was confirmed by the reporter T cell activation assay ($EC_{50} = 0.1 ± 0.02$ nM for 9.29-Bn) (SI Appendix, Fig. S5).

**Tunable Targeting of Different Tumor Antigens by BsCAR T Cells.** Switchable BsCAR T cells enable the targeting of multiple tumor antigens (Fig. 3A). The extracellular domain of the HER2 receptor has four subdomains. The DARPin 9.29-Bn and G3-Bn fusions are specific to subdomains I and IV of HER2, respectively, that allow for targeting different sites of the tumor antigen by the same BsCAR and tuning the CAR T cell activity. Incubating BT-474 target cells with BsCAR T cells with increasing concentrations of the 9.29-Bn or G3-Bn proteins resulted in dose-dependent cytotoxicity.

A dose-response effect was observed by varying the 9.29-Bn and G3-Bn concentration and cell ratio (effector to target ratio [E:T]) in the BsCAR T cell functional assays. BsCAR T cells efficiently induced the death of BT-474 cells in the presence of 9.29-Bn or G3-Bn switches; $IC_{50} = 0.1 ± 0.06$ and 0.2 ± 0.1 nM, respectively (Fig. 3B). Cytotoxicity exceeding 50% was observed in the presence of 1 nM switches starting from an E:T ratio of 10 (Fig. 3C). A specific proinflammatory cytokine release by BsCAR T cells was detected in the presence of switches: $EC_{50} = 0.2 ± 0.1$ nM for interferon-γ (IFN-γ) (Fig. 3D) and $EC_{50} = 0.6 ± 0.1$ nM for interleukin-2 (IL-2) (Fig. 3E). The cytotoxicity of the switchable BsCAR T cells was comparable to T cells modified with the scFv4D5 HER2-CAR construct (48) (SI Appendix, Fig. S6).

The modular approach of the switchable CAR T cells allows redirecting of the BsCAR T cells to other cancer antigens (Fig. 3A). EpCAM-specific fusion based on DARPin EC1 (49) linked with barnase was designed to demonstrate antigen switching (SI Appendix, Fig. S7). BsCAR T cells effectively lysed EpCAM$^+$ BT-474 cells in the presence of EC1-Bn fusion protein ($IC_{50} = 0.3 ± 0.2$ nM) (SI Appendix, Fig. S8).

**BsCAR T Cell Therapy Eradicates HER2$^+$ Xenogenic Ductal Carcinoma In Vivo.** The therapeutic efficacy of the BsCAR T cells was analyzed in the presence of DARPin 9.29-Bn and...
Fig. 1. Design and functional activity of DARPin-Bn switches. (A) Structure model illustrating the interaction between the HER2 extracellular domain (ECD) and DARPin-Bn switches G3-Bn and 9.29-Bn. HER2 ECD (solid surface, PDB ID: 1N8Z), G3-Bn (yellow ribbon, PDB ID: 4HRN), and 9.29-Bn (red ribbon, PDB ID: 4HRL) were aligned by HER2 subdomains (colored individually). DARPins-Bn were constructed de novo, and the free energy was minimized in the elongated conformation, barnase (cyan ribbon, PDB ID: 1BRS). (B and C) DARPins-Bn specifically bind to the surface of HER2+ cells. (B) Confocal scanning microscopy. Bright field, Top Panels: FITC (λex = 488 nm, λem = 492 to 550 nm) and Hoechst 33342 (λex = 405 nm, λem = 410 to 520 nm) overlay, Bottom Panels. (C) Flow cytometry. The histograms show the fluorescence distribution of unlabeled (autofluorescence) and labeled cells. HER2+ BT-474 cells, MDA-MB-231 cells with a low level of HER2 expression, and control HER2−/C0 CHO cells were stained with 9.29-Bn and G3-Bn labeled with FITC. Trastuzumab was used as a positive control. (D) DARPin-Bn switches mediate specific cytotoxicity after a prolonged incubation with HER2+ cells. Resazurin cell viability assay of BT-474, MDA-MB-231, and CHO cells treated with 9.29-Bn, G3-Bn, and free barnase after 10 d of incubation. Data are represented as means ± SDs.
G3-Bn in vivo. SCID mice underwent subcutaneous transplantation with luciferase-expressing BT-474 Fluc cells, followed by the intravenous administration of BsCAR T cells (Fig. 4 A). Subsequently, varied doses of the 9.29-Bn and G3-Bn were injected. The dose-escalation protocol was applied to protect against CRS (50) and improve the persistence of CAR T cells, as well as to delay the exhaustion of the modified T cells (51).

In vivo imaging of the animals demonstrated the promising therapeutic potential of the DARPin-Bn-driven BsCAR T cells against ductal breast carcinoma in vivo (Fig. 4 B–D). Tumor growth was inhibited by the combination of DARPin-Bn and BsCAR T cells (Fig. 4D). The tumor was eliminated after the second cycle of 9.29-Bn administration (Fig. 4 A–D). The DARPin G3-Bn that binds to the membrane-proximal domain of HER2 did not completely suppress tumor growth (Fig. 4 B–D). Monotherapy with the dose-escalation cycles of 9.29-Bn or infusion of BsCAR T cells alone did not suppress tumor growth in vivo (SI Appendix, Fig. S9). Recruitment of CD8 T cells to the tumor was observed only in animals that received the combination of BsCAR T cells and 9.29-Bn or G3-Bn (Fig. 4E). Tumor-infiltrating BsCAR T cells represented ∼18% of total cells in the tumors from the 9.29-Bn-treated group (SI Appendix, Fig. S10).

Discussion

CAR T therapy has revolutionized cancer treatment; however, the major challenge is to control its efficacy and thus prevent adverse effects. A conventional CAR cannot undergo simple switching between different tumor antigens, which critically restricts the clinical applications of CAR T cell therapy. Once the tumor-associated antigen is directly recognized by CAR, the modified T cells are stimulated, leading to the hyperactivation and uncontrollable proliferation of the CAR T cells, accompanied by CRS. Modular CAR approaches are based on universal CARs that allow separating antigen recognition and CAR T activation into two distinct and controllable steps. This enables activating and redirecting universal CAR T cells by molecular switches that target different tumor antigens. This therapeutic strategy is particularly promising against highly heterogeneous and immune-evasive solid tumors. Moreover, the dose of the switches determines therapy intensity. Universal CAR platforms are highly versatile and therefore have the potential for a broad range of clinical applications and a substantial reduction in CAR T cell therapy cost. A variety of switchable CARs have been designed so far. The switchable modular designs include dimerizing platforms that use leucine zippers, biotin-avidin system, sortases, conjugates of single-chain mAbs with small molecules, and others (16–20, 52–54). Three switchable CAR systems are undergoing clinical trials: hmCD16, UniCAR, and peptide neo-epitope (PNE)-based switch CARs. The UniCAR and anti-PNE CAR use an adapter with the CAR epitope linked to a single-chain variable fragment directed against CD123 (55) or CD19 (19). The antibody-coupled T cell receptor adaptor CD16 CAR differs and uses clinically approved mAbs as a switcher (rituximab, trastuzumab, SEA-BCMA the antibody that targets B cell maturation antigen). Only mAbs switches have therapeutic potential themselves; other CAR switches play only a pretargeting role in CAR T cells. Small-molecule control over the functionality of switchable CAR-T cells makes therapy more like the usual pharmaceutical intervention (22–27). Small-molecule drugs have a known average short half-life and some of them were approved by the FDA (28, 29).

Here, we propose harnessing the RNase toxin barnase as a targeting module that provides the cytotoxic effects by itself.

Fig. 2. Design and characterization of switchable barnase CAR T cells. (A) Model of the 3D structure of barnase (Bs) [PDB ID: 1BRS (43)], where mutations introduced for the generation of BsCAR variants are indicated. The yellow surface represents the interface for interaction with barnase. Amino acids at the mutation sites are represented with balls and sticks. (B) A panel of BsCAR variants. G3S, Gly-Ser linker; CD28 TM/cyt, transmembrane and cytoplasmic domains of CD28; 4-1BB: cytoplasmic activation domain from CD137; CD3ζ, the cytoplasmic activation domain of CD3 zeta. (C) BsCAR level on T cell surface was measured by anti-IgG4 antibody (Left) and barnase-FITC staining (Right). The numbers indicate the proportions of CAR/Bs± cells. (D) BsCAR-mediated specific versus nonspecific lysis of BT-474 cells induced by adding 1 nM of 9.29-Bn, G3-Bn, or no switcher. The ratio of BsCAR T/BT-474 was 10:1 (n = 4). The data were analyzed using the two-way ANOVA with Dunnett’s correction for multiple comparisons and represented as means ± SDs. P values between BsCAR variants were determined by the unpaired t test. Statistical significance: *P < 0.05, ***P < 0.001, ****P < 0.0001. ns, not significant.
Barnase and its antitoxin barstar represent an outstanding example of molecular recognition, interacting with an exceptionally high rate and affinity (36). The designed G3-Bn and 9.29-Bn switches demonstrate specific binding to HER2 receptor on the surface of tumor cells. The DARPin-Bn fusions display prominent RNase activity, resulting in a dose-dependent death of HER2+ tumor cells. In contrast to the existing modular systems based on noncovalent interactions, barnase and barstar have no endogenous inhibitors or nonspecific targets in mammals. The barnase protein can be easily genetically fused with any artificial scaffold that can recognize tumor antigens, making it a versatile receptor platform module (37).

A panel of BsCAR constructs was designed to optimize their interaction with the DARPin-Bn switches. BsCARv4 harboring C40A and C82A substitutions and a longer Gly-Ser linker was specifically chosen because it had high surface expression, maximum interaction with barnase, and high cytotoxicity. DARPins-Bn specifically directed the human primary BsCAR T cells, causing the death of HER2+ tumor cells in a dose-dependent manner. Hence, the administration of free barnase or barstar can rapidly abolish BsCAR T cell therapy that is essential to prevent severe side effects. Another option of the switchable BsCAR T cell therapy was demonstrated in the retargeting of BsCAR T cells from HER2 to EpCAM tumor antigen by replacing 9.29-Bn by EC1-Bn. It is important to acknowledge the potential immunogenicity limitations of many universal and programmable CARs. The bacterial origin of barnase and barstar means that they are potentially immunogenic and therefore may be suppressed by the immune system of the patient. For some bacterial RNases (binase, RNase Sa), low immunogenicity has been shown (56–58); however, in a series of studies, barnase and barstar are used in DNA vaccines aimed at obtaining an immune response (59, 60). Further clinical use of this approach requires detailed pharmacokinetics and immunogenicity studies of the DARPin-Bn switches in a BsCAR T cell therapy context. Based on these findings, we believe that tunable BsCAR T cells directed by the personalized sets of DARPin-Bn switches can provide promising options for the controlled eradication of solid tumors by simultaneous targeting of multiple tumor antigens.

**Materials and Methods**

**Cell Culture.** Cell lines were maintained in media (Dulbecco’s modified Eagle’s medium [DMEM] or RPMI 1640) supplemented with 10% fetal bovine serum (FBS) (Gibco), 10 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM GlutaMAX (Gibco). Cell lines of human ductal carcinoma BT-474 (HTB-2; ATCC), MDA-MB-231 (HTB-26; ATCC), HEK293T lentiviral packaging cell line (Clontech), and CHO (Russian Cell Culture Collection) were incubated in a humidified atmosphere with 5% CO2 at 37°C. The BT-474 cell line was transduced with lentiviruses to generate a stable line expressing Fluc (pCDH-CMV-LUC-EF1 Hygro, no. 129437, Addgene), and Fluc-positive cells were enriched by hygromycin B selection. The cell lines were repeatedly tested for mycoplasma contamination (MycoReport Mycoplasma Detection Kit, Evrogen).

**FITC Conjugation.** FITC-labeled Bn, DARPins 9.29-Bn, G3-Bn, and trastuzumab (Roche) were prepared as follows: 100 μg of the protein in 90 μL of
phosphate-buffered saline (PBS) buffer was rapidly mixed with 10 μL FITC in dimethyl sulfoxide (DMSO) at concentrations of 1 g/L, 1 g/L, 1 g/L, and 0.3 g/L, respectively. The proteins were incubated overnight at room temperature and purified from the unreacted FITC molecules using Zeba Spin Desalting Columns, 7K molecular weight cutoff (Pierce) according to the manufacturer’s recommendations.

**Flow Cytometry.** The harvested cells were washed with PBS, resuspended in 300 μL PBS with 1% bovine serum albumin (BSA) at a concentration of 10^6 cells per milliliter, labeled with Bn-FITC, 9.29-Bn-FITC, and G3-Bn-FITC at a final concentration of 1 μg/mL and anti-IgG4 goat anti-human, DyLight 650 (SA510137, Invitrogen) according to the manufacturer’s recommendations, washed, and analyzed using NovoCyte 3000 WYB or NovoCyte 2060 flow cytometer (ACEA Biosciences) in BL1 (excitation laser 488 nm, emission filter 530/30 nm) and BL3 channels (excitation laser 635 nm, emission filter 660/20 nm). Data were analyzed with NovoExpress Software (ACEA Biosciences) and FlowJo ×10.

**Fig. 4.** BsCAR T cells targeted by DARPin-Bn suppress HER2^+^ tumors in vivo. (A) A treatment regimen based on the combination of BsCAR T cell and DARPin-Bn. NSG mice were implanted subcutaneously with 2 x 10^6 BT-474 Fluc cells. After 10 d of tumor inoculation 10 x 10^6 BsCAR T cells were infused intravenously (i.v.). Mice were then randomized into groups and i.v. injected with either PBS or increasing doses of 9.29-Bn and G3-Bn (5, 50, and 500 nmol/kg on days 10, 12, and 14, respectively) (n = 6 in 3 groups). This course of DARPin-Bn injections was repeated 3× with a 1-wk intermission. (B) Representative IVIS images of the mice treated with the BsCAR T cells alone or in combination with 9.29-Bn and G3-Bn switches. The bioluminescence of tumors from individual mice in each group is presented. (C) Quantified tumor burden estimated as the average radiance from luciferase activity per mouse for days 10 to 50. The data were analyzed using the Mann-Whitney test and presented as means ± ranges. Statistical significance: *P < 0.05. (D) Tumor growth dynamics in control and DARPin-Bn-treated groups. The data were analyzed using the Mann-Whitney test and presented as means ± SDs. Statistical significance: *P < 0.05, **P < 0.01. (E) Representative immunohistochemical images of tumor sections stained with anti-human-CD8 antibodies (red), and Hoechst 33342 (blue). Scale bar, 20 μm. The percentage of CD8^+^ cells was determined in 5 stained sections of 3 biological repeats in each group and analyzed by 1-way ANOVA with Turkey's multiple comparison test (n = 3). Statistical significance: *P < 0.033, **P < 0.002, ***P < 0.001. Data are represented as means ± SDs.
Confocal Laser Scanning Microscopy. Protein binding was visualized by confocal laser scanning microscopy. Cells were incubated with 2 μg/mL proteins and Hoechst 33342 (1 μg/mL) in PBS with 1% BSA on ice for 30 min, washed from unbound proteins, and imaged by confocal laser scanning microscopy using an LSM 980 (Zeiss) confocal microscope under the following conditions: excitation 488 nm, emission 492 to 550 nm for FITC detection and excitation 405 nm, emission 410 to 520 nm for Hoechst 33342 detection.

Resazurin Toxicity Assay. Protein cytotoxicity was tested using a resazurin-based assay. Cells were seeded on a 96-well plate at 5 × 10^3 cells per well in 100 μL DMEM supplemented with 10% FBS and cultured overnight. The proteins were added to wells in 100 μL DMEM growth medium, and cells were incubated for 10 d. The medium was then removed and 100 μL resazurin solution (0.13 g/L in PBS) was added to the cells. The samples were incubated for 3 h at 37 °C, and the optical density of each well was measured using an Infinite 1000 Pro (Tecan) microplate reader at an excitation wavelength of λ = 530 nm and emission of λ = 590 nm. The IC50 value was determined by GraphPad Prism software.

Cytotoxicity Assays. The cytotoxicity of engineered T cells was evaluated using the standard lactate dehydrogenase release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) following the manufacturer’s recommendations. The 5 × 10^3 BT-474 cells were cocultured with Mock T or BsCAR T cells for 12 to 16 h and in the presence of 10 to 0.016 nM or 1 nM in case of E:T titration of DARPin-Bn in RPMI (Gibco) media supplemented with 40 U/mL human IL-2 (R&D Systems). To assess the basal levels of BsCAR T and mock T cell cytotoxicity, the T cells were incubated in the absence of drug conjugates. The maximum cell lysis was determined by target cell lysis using 10% (vol/vol) cell lysis solution. The following formula was used to calculate the release of lactate dehydrogenase: cytotoxicity = 100 × ([IC50 value of T cell + target cell + DARPin-Bn – (CAR T cell + target cell)]/max target cell lysis – target cells alone). The IC50 value was determined by GraphPad Prism software.

CAR T Cell Cytokine Detection. For the cytokine release assays, 5 × 10^4 BT-474 cells were mixed with 5 × 10^3 mock T cells or BsCAR T cells in a 96-well plate for 24 h in the presence of different concentrations of DARPin-Bns in RPMI without IL-2. Basal levels of IFN-γ and IL-2 were detected in nonstimulated CAR T samples. The supernatant was separated from cells by centrifugation (4 °C, 300 × g, 5 min), transferred to a new 96-well plate, and stored at −20 °C. IL-2 and IFN-γ secretion by human CAR T cells were analyzed by cytokine-specific ELISA kits (Vector-Kit) according to the manufacturer’s instructions. The IC50 values were determined by GraphPad Prism software.

In Vivo Study. Animals were housed under specific pathogen-free conditions in the Puschnic Animal Breeding Facility IBCh RAS (Bioresource collection “Collection of Laboratory Rodents SPF Category for Basic, Biomedical, and Pharmacological Research”). The experiments were conducted on 6- to 8-week-old female and male NSG (NOD/SCID/IL2rgnull) mice with an average weight of 16 to 20 g. All of the procedures were approved by the IBCh RAS Institutional Animal Care and Use Committee. The mice were inoculated subcutaneously with 2 × 10^4 BT-474 Fluc HER2-overexpressing cancer cells in 30% Matrigel in 100 μL complete culture medium. Once the tumors became detectable, the mice were randomly assigned to experimental and control groups. Tumor-bearing mice from all of the groups were injected intravenously with 10 × 10^6 BsCAR T cells. Four hours after BsCAR T cell injection, different doses of the DARPin-Bn and G3-Bn switches were administered to the mice. The doses of DARPin-Bn were escalated according to the established protocol. Every other day, animals were injected with increasing doses of switches (5, 50, and 500 nmol/kg, respectively). The mice were then rested for 7 to 8 d, and the treatment cycle was repeated two times. Tumors were monitored every 10 d using the IVIS Spectrum In Vivo Imaging System (IVIS; PerkinElmer) after intraperitoneal injection of 5-doxorubicin (GoldBio).

Histology. Formalin-fixed paraffin-embedded 10-μm sections were stained with anti-CbD antibodies (1:300, clone RPA-TB) conjugated with phycoerythrin. Anti- gen was retrieved using a heated 10 mM Tris-ethylenediaminetetraacetate buffer (pH 9.0) before staining. For immunostaining, sections washed with PBS buffer (pH 7.4) were incubated in PBS containing 10% (vol/vol) FBS, 0.3 mM glycine, and 0.5% (vol/vol) Triton X-100 for 1 h, and stained with an antibody at the appropriate dilution overnight at room temperature. Finally, sections were washed with PBS containing 0.1% Tween and mounted in a medium containing Hoechst 33342. The images were acquired using an Eclipse Ti-E microscope with confocal module A1, CFI Plan Apo VC 20 × 0.75, and Apo TIRF Plan Fluor 20 × 1.49 (Nikon Corporation).

Statistical Analysis. Statistical processing of the experimental results was performed using the Prism 9 software package (GraphPad software).

Ethics Approval. All of the experiments on human subjects were approved by the Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology, and Immunology local ethics committee (decision on January 18, 2018) and were carried out following the approved protocols. All of the donors were introduced to the final goal of their blood collection and signed an informed consent form.

Data, Materials, and Software Availability. All of the data study are included in the article and/or the SI Appendix.

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