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B7H6-Specific Bispecific T Cell Engagers Lead to Tumor Elimination and Host Antitumor Immunity

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Substantial evidence showed that T cells are the key effectors in immune-mediated tumor eradication; however, most T cells do not exhibit antitumor specificity. In this study, a bispecific T cell engager (BiTE) approach was used to direct T cells to recognize B7H6⁺ tumor cells. B7H6 is a specific ligand for the NK cell–activating receptor NKp30. B7H6 is expressed on various types of primary human tumors, including leukemia, lymphoma, and gastrointestinal stromal tumors, but it is not constitutively expressed on normal tissues. Data from this study showed that B7H6-specific BiTEs direct T cells to mediate cellular cytotoxicity and IFN- γ secretion upon coculturing with B7H6⁺ tumors. Furthermore, B7H6-specific BiTE exhibited no self-reactivity to proinflammatory monocytes. In vivo, B7H6-specific BiTE greatly enhanced the survival benefit of RMA/B7H6 lymphoma-bearing mice through perforin and IFN- γ effector mechanisms. In addition, long-term survivor mice were protected against an RMA lymphoma tumor rechallenge. The B7H6-specific BiTE therapy also decreased tumor burden in murine melanoma and ovarian cancer models. In conclusion, B7H6-specific BiTE activates host T cells and has the potential to treat various B7H6⁺ hematological and solid tumors. *The Journal of Immunology*, 2015, 194: 5305–5311.

Immunotherapy holds the promise of being an effective means to treat cancer. Evidence shows that T cells are the key effectors in tumor cell recognition and destruction (1). There are multiple immunotherapy strategies that aim to harness T cell effector functions, such as chimeric Ag receptor T cell therapy, cancer vaccines, bispecific T cell engagers (BiTEs), and immunological checkpoint inhibitors. BiTE strategies use protein-engineering techniques to create fusion proteins with dual specificity that lack an Fc region. Its common format is a fusion protein consisting of a tumor-recognition single-chain variable fragment (scFv) linked in tandem with another scFv specific to T cell CD3 ϵ (2). T cells redirected by a BiTE can form immunologic synapses with tumor cells, perform serial killing, secrete cytokines, and are activated in the absence of a costimulatory signal (3, 4).

There are several intrinsic advantages of BiTE therapy. BiTE therapy confers antitumor specificity to T cells without having to genetically engineer T cells; therefore, it avoids the cost and time associated with ex vivo T cell manipulation. BiTEs showed potent therapeutic efficacy in treating non-Hodgkin lymphoma and acute lymphoblastic leukemia (5, 6). This strategy can potentially target any tumor cell surface Ag as long as an Ab can be generated. There are several BiTEs in preclinical and clinical trials that can target multiple tumor types (7, 8). However, many

BiTEs target tumor-associated Ags (TAAs), such as epidermal growth factor receptor, human epidermal growth factor receptor 2, epithelial cell adhesion molecule, and carcinoembryonic Ag, which can be expressed by multiple types of normal tissues. This expression pattern may potentially cause on-target off-tumor side effects.

NK cells recognize a panel of ligands specifically expressed on multiple tumor types with activating receptors, such as NKG2D, NKp30, NKp44, and NKp46 (9, 10). This broad tumor-targeting activity of NK cells makes these receptors attractive for generating tumor-recognizing BiTE proteins with the potential to exhibit a very broad tumor-targeting capacity (11). To develop an effective NK cell receptor–based BiTE therapy, we created a novel BiTE based on the specificity of NKp30, which is an NK cell–activating receptor that mediates antitumor immunity in various tumor types (12, 13). One of its ligands, B7H6, is expressed on ~20% of human tumor cell lines and is also expressed on several types of primary human tumors (14). RNA analysis showed that several subsets of human primary lymphoma, leukemia, ovarian cancer, brain tumors, breast cancers, renal cell carcinomas, and various sarcomas potentially express high amounts of B7H6. In addition, B7H6 mRNA is not found on 48 normal tissues under steady-state conditions (14). NKp30 is a pseudogene in *Mus musculus*, and a B7H6 ortholog is missing in mice (14, 15). In this article, we describe a novel B7H6-specific BiTE that recognizes B7H6. We show that a B7H6-specific BiTE directs T cells to mediate cytotoxicity and IFN- γ secretion against B7H6⁺ tumor cells. B7H6-specific BiTE therapy enhanced the survival of lymphoma-bearing mice and decreased the tumor burden of melanoma- and ovarian cancer-bearing mice. These data suggest that B7H6-specific BiTE therapy might be beneficial for treating various tumors.

Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). Mice were used in experiments at 6–12 wk of age. All experiments were conducted according to Dartmouth College's Institutional Animal Care and Use Committee.

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Abbreviations used in this article: B6, C57BL/6; BiTE, bispecific T cell engager; GIST, gastrointestinal stromal tumor; LDH, lactate dehydrogenase; PEI, polyethyleneimine; scFv, single-chain variable fragment; TAA, tumor-associated Ag.

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Cell culture and cell lines

Anti-B7H6 hybridoma was described previously (16). The anti-mouse CD3 ϵ hybridoma 145.2C11, K562 was obtained from the American Type Culture Collection (Manassas, VA). The B3Z T cell hybridoma was obtained from Dr. Nilabh Shastri (University of California at Berkeley). The mouse T cell lymphoma line RMA, melanoma cell line B16F10, and ovarian cancer cell line ID8 were described previously (17–19). The mouse T cell lymphoma line RMA/B7H6, melanoma cell line B16F10/B7H6, and ovarian cancer cell line ID8/B7H6 were generated by retrovirus transduction of their parental line RMA, B16F10, or ID8 cells, respectively, using dualtropic retroviral vectors containing the human *B7H6* gene, according to previously described protocols (17). RMA, RMA/B7H6, B16F10, B16F10/B7H6, and K562 were cultured in RPMI 1640, supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-ME. ID8 and ID8/B7H6 were cultured in DMEM with a high glucose concentration (4.5 g/l) containing the same supplements. 293F cells were cultured in FreeStyle 293 Expression Medium (both from Life Technologies, Carlsbad, CA) on an orbital shaker (120 rpm). Primary human ovarian cancer samples were obtained from Dartmouth-Hitchcock Medical Center after surgery with informed consent. Cancer samples were mechanically disrupted, and RBCs were lysed with ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA [pH 7.4]). Primary ovarian cancer cells were cultured for 2 d before use in a functional assay. To stimulate PBMCs with LPS, TNF- α , or IL-1 β , human cells from cell cones obtained from leukapheresis (Dartmouth-Hitchcock Medical Center Blood Donor Center) were cultured in 24-well plates at a cell density of 3×10^6 cells/well in complete RPMI 1640 at 37°C and 5% CO₂ for 48 h, with or without LPS (1 μ g/ml; Sigma-Aldrich, St. Louis, MO), TNF- α (100 ng/ml), or IL-1 β (1 ng/ml; both from PeproTech, Rocky Hill, NJ) stimulation.

Design and construction of B7H6-specific and MICA-specific BiTEs

The anti-B7H6 scFv was constructed by fusing the V_H (aa 1–134) and V_L (aa 23–129) regions of an anti-B7H6 hybridoma 47.39 (16) with a 15-aa glycine (G)-serine (S) (G₄S)₃ linker (three repeats of GGGGS). Anti-human CD3 ϵ scFv was constructed by fusing the V_H (aa 20–138) and V_L (aa 23–128) regions of an anti-human CD3 ϵ hybridoma OKT3 with the (G₄S)₃ linker. Anti-mouse CD3 ϵ scFv was constructed by fusing the V_H (aa 20–135) and V_L (aa 21–128) regions of an anti-mouse CD3 ϵ hybridoma 145.2C11 with the (G₄S)₃ linker. All of the fragments mentioned above were amplified using PCR and cDNA derived from individual hybridomas with a high-fidelity DNA polymerase Phusion (New England Biolabs, Beverly, MA). All oligonucleotides for PCR were synthesized by Integrated DNA Technologies (Coralville, IA) or Sigma-Genosys (The Woodlands, TX). Human version B7H6-specific BiTE was constructed by fusing anti-B7H6 scFv with OKT3 scFv via a (G₄S)₃ linker. Murine version B7H6-specific BiTE was constructed by fusing anti-B7H6 scFv with 145.2C11 scFv via a G₄S linker. A histidine tag (six repeats of histidine) was added to the C terminus of both constructs to facilitate protein purification. The construct of human B7H6-specific BiTE was further cloned into a CMV promoter-based expression vector. The construct of murine B7H6-specific BiTE was cloned into the expression vector pCEP4 (Life Technologies). The MICA-specific BiTE was generated by fusing an scFv that recognizes MICA with OKT3 scFv via a (G₄S)₃ linker.

Production and purification of B7H6-specific BiTEs

For production of B7H6-specific BiTEs, a suspension of growing 293F cells cultured in FreeStyle 293 Expression Medium (Life Technologies) was transfected with B7H6-specific BiTE DNA constructs by 40-kDa polyethylenimine (PEI; Polysciences, Warrington, PA). Transfection was done by gently mixing 293F cells with DNA and PEI at a final concentration of 2×10^7 cells/ml, 12.5 μ g/ml DNA, and 25 μ g/ml PEI and letting it shake on an orbital shaker (120 rpm) at 37°C for 3 h. Then the entire mixture was diluted with FreeStyle 293 Expression medium (Life Technologies) at a 1:9 ratio for a final cell concentration of 3×10^6 cells/ml. Valproic acid (Sigma-Aldrich) was added to the culture to a final concentration of 2 mM. The culture was maintained at 37°C shaking at 120 rpm for 4 d, and cell-free culture supernatant was harvested. The supernatant was mixed with 4 \times Ni column-binding buffer (1.2 M NaCl, 200 mM NaH₂PO₄, 80 mM imidazole [pH 7.4]) and loaded onto a HisTrap HP column (GE Healthcare, Waukesha, WI). The column was washed with 10 column volumes of Ni column-binding buffer (0.3 M NaCl, 50 mM NaH₂PO₄, 20 mM imidazole [pH 7.4]), and elution was performed with 20 column volumes of elution buffer (0.3 M NaCl, 50 mM NaH₂PO₄ [pH 7.4]) with a linear gradient of imidazole from 250 to 500 mM. Eluted fractions were collected and examined by SDS-PAGE. The fractions

with B7H6-specific BiTEs were further combined and buffer exchanged to PBS using a 30-kDa molecular mass cutoff Amicon ultrafiltration column (EMD Millipore, Billerica, MA). The final protein solution was filtered sterile with a 0.22- μ m syringe filter (EMD Millipore). The quantity and purity of BiTE were assessed by SDS-PAGE, followed by staining with SYPRO Orange (Life Technologies). The concentration of BiTEs was quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and compared to an OVA protein standard curve.

Flow cytometry

To confirm that human B7H6-specific BiTE binds to T cell CD3 ϵ and B7H6 simultaneously, human T cells were stained with various amounts of human B7H6-specific BiTE (1–1000 ng), followed by staining with DyLight 650-conjugated soluble B7H6. All samples were preincubated with human Cohn fractions (Sigma-Aldrich) to reduce nonspecific binding. Samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Data analysis was done with Accuri (BD Biosciences) or FlowJo software (TreeStar, Ashland, OR).

In vitro cytotoxicity assay

In vitro cytotoxicity was measured by a lactate dehydrogenase (LDH)-release assay. OKT3- and IL-2-activated human T cells or Con A-activated murine splenocytes were incubated with tumor cells at an E:T ratio of 5:1 ($10^5:2 \times 10^4$) in triplicate wells in 96-well V-bottom plates at 1 μ g/ml concentration of B7H6-specific BiTEs. Six hours later, cell-free culture supernatant was harvested, and LDH release was determined using a CytoTox 96 NonRadioactive Cytotoxicity Assay Kit (Promega, Madison, WI), following the manufacturer's protocol. Specific lysis was calculated as: percentage of specific lysis = [(experimental OD value – effector spontaneous OD value – target spontaneous OD value)/(target maximum OD value – target spontaneous OD value)] \times 100. Media OD values were subtracted from all samples.

Cytokine production by T cells

Human T cells, which were activated and cultured with OKT3 for 5 to 12 d, were cocultured with tumor cells at an E:T ratio of 1:1 ($10^5:10^5$) in triplicate wells with various concentrations (0.98–1000 ng/ml) of human B7H6-specific BiTE in 96-well V-bottom plates. To test B7H6-specific BiTE reactivity to primary ovarian cancer cells, OKT3-activated human T cells were cocultured with primary ovarian cancer cells at an E:T ratio of 1:1 ($10^5:10^5$) in triplicate wells with 250 ng/ml human B7H6-specific BiTE in 96-well V-bottom plates. To test B7H6-specific BiTE reactivity to proinflammatory monocytes in PBMCs, human T cells were cocultured with LPS-, TNF- α -, or IL-1 β -stimulated PBMCs at an E:T ratio of 1:1 ($10^5:10^5$) in triplicate wells with 250 ng/ml human B7H6-specific BiTE in 96-well V-bottom plates. Cell-free medium was collected after 24 h, and IFN- γ concentration was determined with a DuoSet IFN- γ ELISA Kit (R&D Systems, Minneapolis, MN). To generate murine T cells for in vivo injection in the ovarian tumor model, murine splenocytes were activated with Con A (1 μ g/ml) and cultured with recombinant human IL-2 (25 U/ml) for 4–6 d. For in vitro experiments, Con A-activated murine splenocytes were cocultured with tumor cells at an E:T ratio of 5:1 ($10^5:2 \times 10^4$ for B16F10/B7H6) or 1:1 ($10^5:10^5$ for RMA, RMA/B7H6) with various concentrations (7.8–500 ng/ml) of murine B7H6-specific BiTE. Cell-free medium was collected after 24 h, and IFN- γ concentration was determined by ELISA.

Quantitative RT-PCR

Total RNA of unstimulated or LPS-, TNF- α -, or IL-1 β -stimulated PBMCs was extracted with an RNeasy Mini Kit (QIAGEN, Valencia, CA). cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). A total of 5 ng cDNA was used as the template for quantitative RT-PCR with iQ SYBR Green Supermix (Bio-Rad). The following primer pair sequences were used for PCR: IL-6F: 5'-ACTCACCTCTTCCAGAACGAATTG-3', IL-6R: 5'-CCATCTTTGGAAGGTTACAGTTG-3'; B7H6-F: 5'-GACCCTGGGACTGTCTACCA-3', B7H6-R: 5'-ATAGGCCACCAATGAATGGA-3'; and GAPDH-F: 5'-CTCCTGTTCGACAGTACGCCG-3', GAPDH-R: 5'-TGACCAGGGGCCAA-TACGAC-3'. IL-6 and B7H6 mRNA fold induction was calculated by the $-2^{\Delta\Delta Ct}$ method.

Treatment of tumor-bearing mice with murine B7H6-specific BiTE

RMA/B7H6 lymphoma cells (10^5 cells) were injected i.v. into B6 mice on day 0. Murine IgG or murine B7H6-specific BiTE (10 μ g) was injected i.v. on days 3, 5, and 7. Mice were monitored closely and euthanized when moribund signs were observed. As a melanoma model, B16F10/B7H6 cells (10^6 cells) were

injected s.c. into the shaved right flank of B6 mice on day 0. Mice were treated with murine IgG or murine B7H6-specific BiTE (10 μg) i.v. on days 5, 7, 9, and 11. Tumors were measured every other day using a caliper, and the tumor area was calculated. Mice were sacrificed when the tumor area reached 200 mm². As an ovarian cancer model, ID8/B7H6 cells (5 × 10⁶ cells) were injected i.p. into B6 mice on day 0, and the mice were treated i.p. with murine IgG or murine B7H6-specific BiTE (10 μg) on days 7, 9, and 11. In some groups, additional Con A-activated and expanded murine T cells (5 × 10⁶ cells, i.p.) were injected at the time of the first BiTE administration on day 7. The numbers of solid tumors on the peritoneal wall and free tumor cells in the i.p. wash were quantified on day 50.

Statistical analysis

One-way ANOVA, the Mann–Whitney *U* test, or the Student *t* test was used to analyze differences between groups, as appropriate; *p* values < 0.05 were considered statistically significant. Kaplan–Meier survival curves were plotted and analyzed using GraphPad Prism software (GraphPad, San Diego, CA).

Results

Human B7H6-specific BiTE binds to T cell CD3ε and tumor cell B7H6 simultaneously and triggers T cells effector responses against B7H6⁺ tumors

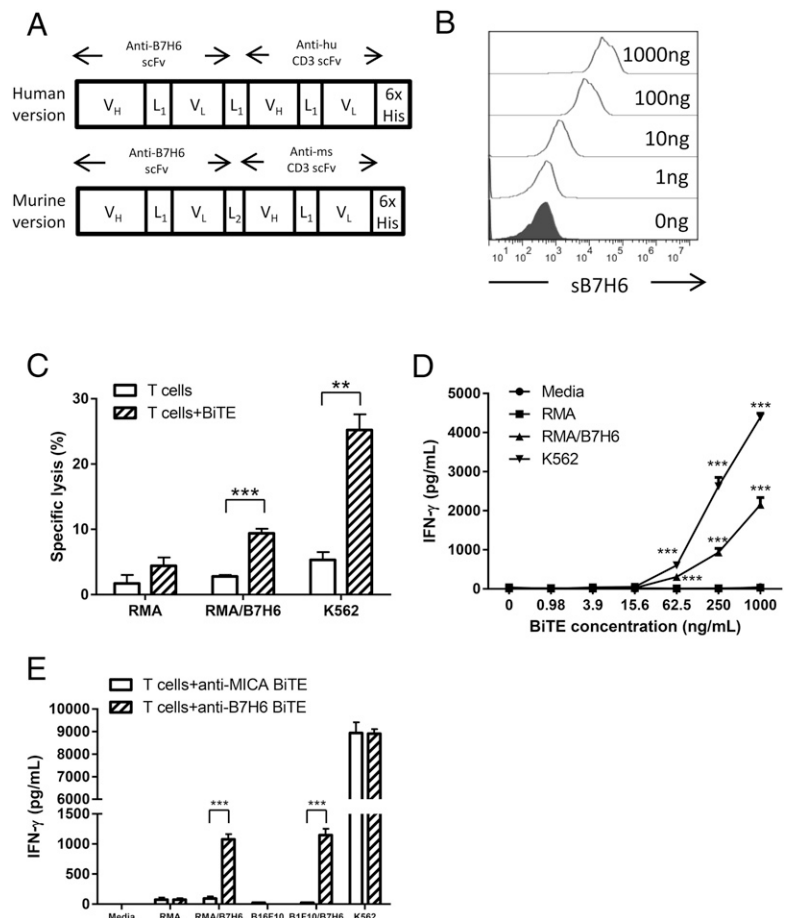
Two B7H6-specific BiTEs, one that binds to human CD3 and another that binds to murine CD3, were constructed by fusing anti-B7H6 scFv with anti-human and anti-murine CD3ε scFv, respectively (Fig. 1A). SDS-PAGE analysis under reducing and nonreducing conditions showed that human and murine B7H6-specific BiTEs were expressed as monomers with the expected molecular mass of 56 and 55 kDa, respectively (data not shown). To confirm that human B7H6-specific BiTE preserved the binding specificity of both parental scFvs, human T cells (CD3ε⁺, B7H6⁻, NKp30⁻) were stained with human B7H6-specific BiTE, followed

by DyLight 650–conjugated soluble B7H6 protein. Only when the B7H6-specific BiTE bound to both human T cell CD3ε and soluble B7H6 simultaneously was there a DyLight 650 signal. We observed a dose-dependent increased staining intensity with increasing amounts of the B7H6-specific BiTE protein (Fig. 1B). Cytotoxicity assays showed that the human B7H6-specific BiTE triggered T cell cytotoxicity against two B7H6⁺ tumors, RMA/B7H6 and K562, but not a B7H6⁻ tumor (Fig. 1C). Human B7H6-specific BiTE also triggered dose-dependent IFN-γ production by T cells when cocultured with RMA/B7H6 or K562 tumor cells (B7H6⁺ tumors) but not RMA tumor cells (B7H6⁻ cells) (Fig. 1D). In contrast, a non-B7H6–specific BiTE (MICA specific) triggered human T cells against MICA⁺ tumor cells (K562) but not against B7H6⁺ tumor cells (Fig. 1E).

Human B7H6-specific BiTE triggers T cells to react to primary human ovarian cancer but not proinflammatory monocytes

B7H6 is known to be expressed on primary human lymphoma, leukemia, and gastrointestinal stromal tumor (GIST) (13, 14). Analysis of the Oncomine gene array data shows that B7H6 mRNA is overexpressed in several other types of primary human tumors, such as ovarian cancer, brain tumors, breast cancers, renal cell carcinomas, and various sarcomas (20). When coculturing human T cells with the BiTE protein and primary ovarian cancer cells, IFN-γ production was observed; this activity could be blocked by preincubating the tumor cells with anti-B7H6 Abs (Fig. 2A). In addition to tumor cells, B7H6 was reported to be expressed on circulating proinflammatory monocytes in a subset of severe sepsis patients or on monocytes stimulated with TLR agonists or proinflammatory cytokines in vitro (21). However, we did not observe B7H6-specific BiTE triggering of T cells to LPS-

FIGURE 1. Design, production, and functionality of B7H6-specific BiTEs. **(A)** Schematic diagram of the human and murine B7H6-specific BiTE proteins. **(B)** Human T cells (CD3ε⁺, NKp30⁻, B7H6⁻) were stained with B7H6-specific BiTE protein (1–1000 ng), followed by soluble B7H6–DyLight 650. Data shown are representative line graphs of staining intensity. **(C)** OKT3-activated human T cells were cocultured with tumor cell lines at an E:T ratio of 5:1 (10⁵:2 × 10⁴), with or without human B7H6-specific BiTE (1 μg/ml). Six hours after coculturing, culture supernatant was harvested, and specific lysis was determined by an LDH-release assay. The data shown are mean + SD of triplicate wells and are representative data from three human donors. **(D)** OKT3-activated human T cells were cocultured with B7H6⁻ (RMA) or B7H6⁺ (RMA/B7H6, K562) tumor cells at an E:T ratio of 1:1 (10⁵:10⁵) at the indicated concentrations of human B7H6-specific BiTE protein. Cell-free medium was collected after 24 h, and IFN-γ concentration was determined by ELISA. The data shown are mean + SD of triplicate wells and are representative of data from three human T cell donors. Statistical analysis compares the IFN-γ value of B7H6⁺ tumor cells (RMA/B7H6 or K562) to B7H6⁻ tumor cells (RMA) at each BiTE concentration. **(E)** OKT3-activated human T cells were cultured with B7H6⁻ (RMA, B16F10), B7H6⁺ (RMA/B7H6, B16F10/B7H6), and B7H6⁺ and MICA⁺ (K562) tumor cell lines at an E:T ratio of 4:1 (10⁵:2.5 × 10⁴) with B7H6-specific BiTE or a MICA-specific BiTE. Cell-free medium was collected after 24 h, and IFN-γ concentration was determined by ELISA. ***p* < 0.005, ****p* < 0.0001.



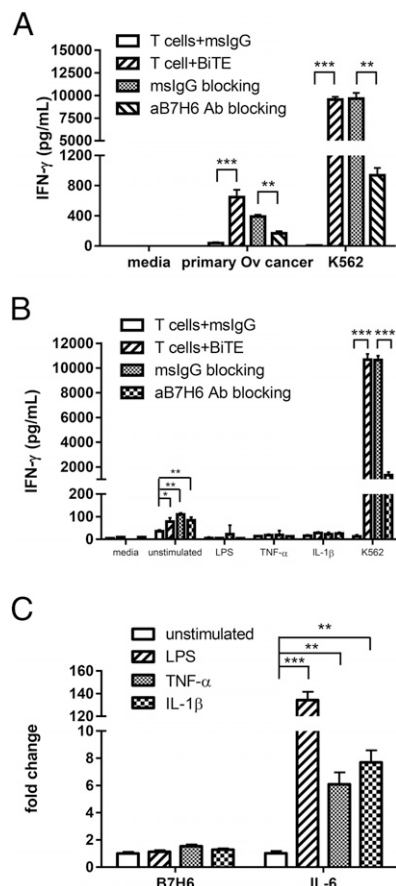


FIGURE 2. B7H6-specific BiTEs trigger T cells to produce IFN- γ to primary human ovarian cancer but not proinflammatory monocytes. **(A)** Activated human T cells were cocultured with primary human ovarian cancer cells or K562 at an E:T ratio of 1:1 in the presence of murine IgG or B7H6-specific BiTE (250 ng/ml). Blocking experiments were done by preincubating tumor samples with 10 μ g of murine IgG or anti-B7H6 mAbs for 45 min before coculturing. Cell-free medium was harvested after 24 h, and IFN- γ concentration was determined by ELISA. Data shown are mean + SD of triplicate wells and are representative of two independent experiments with different T cell donors. **(B)** Activated human T cells were cocultured with unstimulated PBMCs or LPS-, TNF- α -, or IL-1 β -stimulated autologous PBMCs in the presence of murine IgG or B7H6-specific BiTE protein. Blocking experiments were done by preincubating target cells with 5 μ g of murine IgG or anti-B7H6 mAbs for 45 min before coculturing. Cell-free medium was harvested after 24 h, and IFN- γ concentration was determined by ELISA. Data shown are representative of four PBMC donors. **(C)** Unstimulated or LPS-, TNF- α -, or IL-1 β -stimulated PBMCs were harvested after 4 h of stimulation. The amounts of IL-6 and B7H6 mRNA in each sample were measured by quantitative real-time PCR. The amount of mRNA in the unstimulated PBMCs is set to 1. Data shown are representative of two PBMC donors. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

TNF- α -, or IL-1 β -stimulated monocytes in PBMCs (Fig. 2B). In addition, flow cytometry analysis did not show specific binding of the B7H6 Ab on PBMCs or stimulated PBMCs (data not shown). Furthermore, the LPS, TNF- α , and IL-1 β used in the experiments triggered a significant IL-6 mRNA upregulation but did not increase the expression of B7H6 mRNA in these activated PBMCs (Fig. 2C).

A murine B7H6-specific BiTE redirects primary murine T cells to kill B7H6⁺ tumor cells and secrete IFN- γ

To test whether a murine B7H6-specific BiTE triggered effector responses from murine T cells, B7H6⁺ tumor cells were cocultured with murine T cells and increasing amounts of the murine-activating B7H6-specific BiTE protein. Cytotoxicity assays

showed that murine B7H6-specific BiTE triggered murine T cells to specifically kill B7H6⁺ tumors (Fig. 3A). In addition to direct tumor cytotoxicity, cytokines produced by T cells are known to play important roles for in vivo therapeutic efficacy of immunotherapy (22–24). The murine B7H6-specific BiTE elicited a robust IFN- γ production by murine T cells when cocultured with B7H6⁺ tumor cells (B16F10/B7H6) but not B7H6⁻ tumor cells (RMA, B16F10, ID8) (Fig. 3B). The murine B7H6-specific BiTE also elicited a robust dose-dependent IFN- γ production by murine T cells when cocultured with B7H6⁺ tumor cells (RMA/B7H6, B16F10/B7H6) but not a B7H6⁻ tumor cell (RMA) (Fig. 3C).

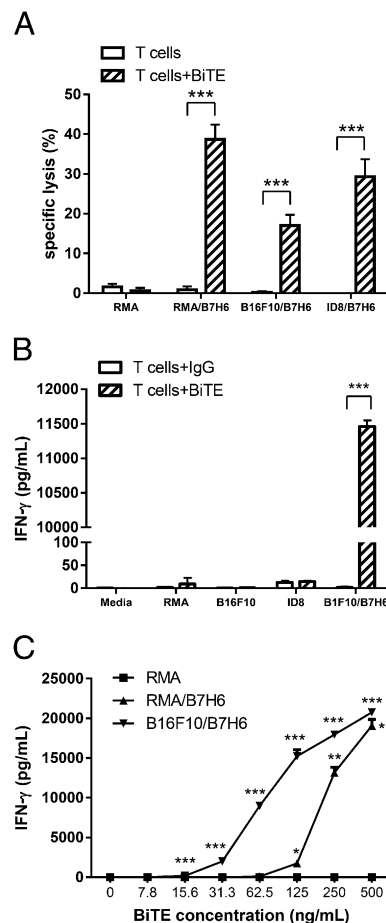


FIGURE 3. Murine B7H6-specific BiTE redirects T cells to specifically kill B7H6⁺ tumor cells and secrete IFN- γ . **(A)** Murine splenocytes were activated with Con A (1 μ g/ml) and cultured with recombinant human IL-2 (25 U/ml) for 4–6 d to generate murine T cells. Murine T cells were cocultured with B7H6⁻ tumor cells (RMA) or B7H6⁺ (RMA/B7H6, B16F10/B7H6, or ID8/B7H6) tumor cells at an E:T ratio of 5:1 for 6 h. Specific lysis was quantified by an LDH-release assay. The data shown are mean + SD of quadruplicate wells and are representative of two independent experiments. **(B)** Murine T cells were cocultured with B7H6⁻ tumor cells (RMA, B16F10, ID8) or B7H6⁺ (B16F10/B7H6) tumor cells at an E:T ratio of 4:1 ($10^5:2.5 \times 10^4$) at a concentration of 500 ng/ml B7H6-specific BiTE. Cell-free medium was harvested after 24 h, and IFN- γ concentration was determined by ELISA. The data shown are mean + SD of triplicate wells and are representative of two independent experiments. **(C)** Murine T cells were cocultured with indicated tumors at an E:T ratio of 5:1 (B16F10/B7H6) or 1:1 (RMA, RMA/B7H6) at various concentrations of murine B7H6-specific BiTE (7.8–500 ng/ml). Cell-free medium was harvested after 24 h, and IFN- γ concentration was determined by ELISA. The data are shown as mean + SD of triplicate wells and are representative of two independent experiments. Statistical analysis compares the IFN- γ value of B7H6⁺ tumor cells (RMA/B7H6 or B16F10/B7H6) to B7H6⁻ tumor cells (RMA) in each BiTE concentration. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

The B7H6-specific BiTE mediates therapeutic efficacy against lymphoma, melanoma, and ovarian cancer in vivo

The therapeutic efficacy of the murine B7H6-specific BiTE in clinically relevant murine tumor models was investigated. In a T cell lymphoma model, B6 mice injected i.v. with 10^5 RMA/B7H6 cells on day 0 were treated i.v. with 10 μ g of mouse IgG or murine B7H6-specific BiTE on days 3, 5, and 7. Data showed that murine B7H6-specific BiTE greatly improved the survival of tumor-bearing mice, and about half (40–60%) of the animals became long-term survivors (Fig. 4A, 4D). When this BiTE therapy was administered to an RMA lymphoma model in which tumor cells did not express B7H6, no therapeutic efficacy was observed. This finding indicated that BiTE must bind to tumor cells to confer therapeutic efficacy (Fig. 4B). Furthermore, the long-term, tumor-free surviving mice were protected against tumor growth when challenged s.c. with RMA tumor cells (B7H6⁻ cells), suggesting that this therapy elicited a broader host immunity against RMA tumor cells (Fig. 4C). To determine the role of host effector molecules in BiTE efficacy, the RMA/B7H6 tumor model was assessed using perforin-deficient or IFN- γ -deficient mice as hosts. The lack of either perforin or IFN- γ in the host resulted in a loss of protection compared with immune intact mice treated with the B7H6-specific BiTE protein. These data indicated that therapeutic efficacy was dependent on host perforin and IFN- γ activity (Fig. 4D), which is consistent with a role for BiTEs in the activation of T cell killing and cytokine production and the inhibition of tumor growth.

To examine the therapeutic efficacy of the B7H6-specific BiTE in a melanoma model, mice were injected s.c. with B16F10/B7H6 on day 0 and treated with murine IgG or B7H6-specific BiTE i.v. on days 5, 7, 9, and 11. These data showed that systemic murine B7H6-specific BiTE administration significantly decreased the rate of tumor growth (Fig. 5A). The same BiTE protein admin-

istered to mice bearing a B16F10 melanoma, which did not express B7H6, did not confer therapeutic efficacy (Fig. 5B). As a model for ovarian cancer, mice were injected with ID8/B7H6 cells i.p. on day 0 and treated with murine IgG or B7H6-specific BiTE i.p. (days 7, 9, and 11), with or without additional T cells (5×10^6 T cells) on day 7. The number of solid tumors on the peritoneal wall and the number of free tumor cells in the i.p. wash were quantified on day 50. The data showed that murine B7H6-specific BiTE therapy significantly decreased overall ovarian cancer tumor burden in these tumor-bearing mice (Fig. 5C). These data demonstrated that B7H6-specific BiTE therapy mediates therapeutic efficacy against both hematological malignancy and solid tumors in vivo.

Discussion

Bispecific Abs (Triomab format: catumaxomab, ertumaxomab; BiTE format: blinatumomab) that trigger T cell effector functions demonstrated promising therapeutic efficacy in several clinical trials (7). Regardless of the format, most bispecific Ab designs focus on targeting TAAs, such as epidermal growth factor receptor, human epidermal growth factor receptor 2, epithelial cell adhesion molecule, carcinoembryonic Ag, CD19, or CD20. These TAAs are upregulated by multiple tumor types, which enables targeting of Ag-expressing tumors. However, these TAAs are also found on many normal tissues and cell types. This nontumor-exclusive expression pattern is likely to compromise the specificity of the treatment and may result in severe on-target off-tumor self-reactivity. Self-reactivity is a major concern for all targeted therapies. As a result of the intrinsic self-amplifying characteristics of the immune response, self-reactivity of BiTE therapy may be severe. Treatment with an anti-CD19 BiTE (blinatumomab) results in elimination of peripheral B cells and B cell progenitors, which gradually recovers after treatment is

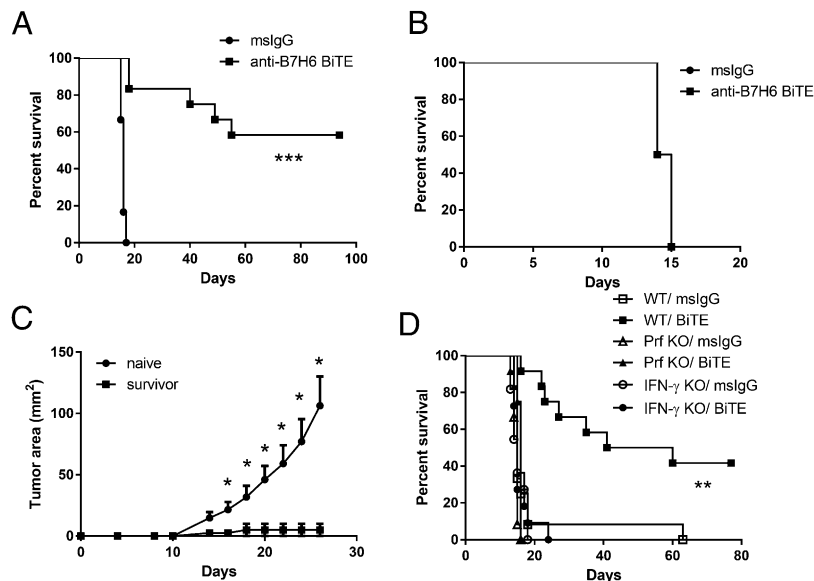
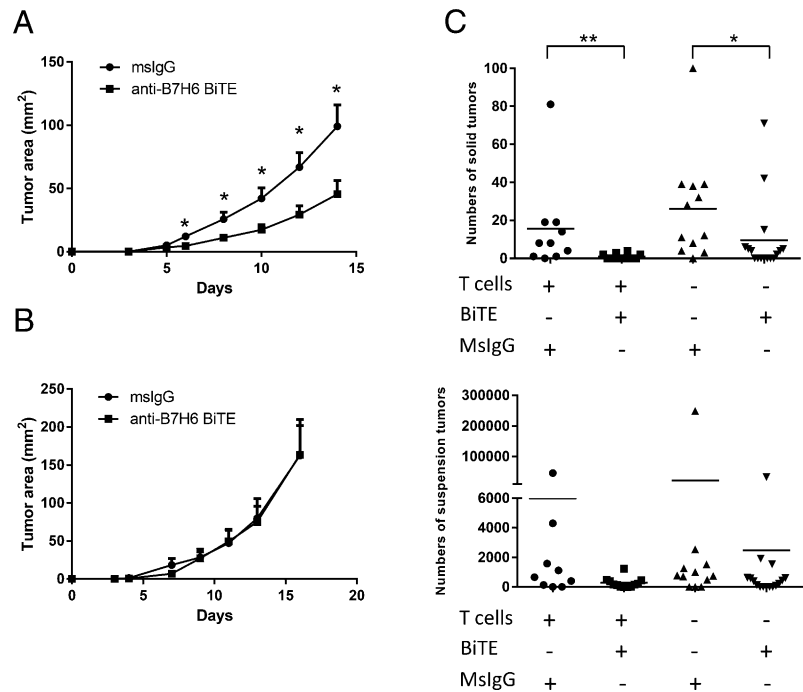


FIGURE 4. Murine B7H6-specific BiTEs mediate therapeutic efficacy against lymphoma in a perforin- and IFN- γ -dependent manner. **(A)** RMA/B7H6 cells (10^5 cells) were injected i.v. into mice on day 0. Murine IgG or B7H6-specific BiTE (10 μ g) was injected i.v. on days 3, 5, and 7. Kaplan–Meier survival curves are shown. Data shown are pooled results from two independent experiments ($n = 12$ for both groups). **(B)** RMA cells (10^5 cells) were injected i.v. into mice on day 0. Murine IgG or anti-B7H6 BiTE (10 μ g) was injected i.v. on days 3, 5, and 7. Kaplan–Meier survival curves are shown ($n = 6$). Note that the survival curves for both treatments are the same. **(C)** Long-term surviving mice from (A) were rechallenged with 2×10^4 RMA (B7H6⁻) s.c. on the right shaved flank, and tumor area (mm²) was measured. Data shown are representative of two independent experiments ($n = 5$). Error bars represent SEM. **(D)** Wild-type B6, *perforin*-deficient, and *Ifn- γ* -deficient mice were injected i.v. with 10^5 RMA/B7H6 cells on day 0. Murine IgG or B7H6-specific BiTE (20 μ g) was injected i.v. on days 3, 5, and 7. Kaplan–Meier survival curves are shown. Data shown are pooled results from two independent experiments ($n = 11$ or 12). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

FIGURE 5. B7H6-specific BiTE protein mediates therapeutic efficacy in melanoma and ovarian cancer. **(A)** B16F10/B7H6 (10^6 cells) were injected s.c. into mice on the shaved right flank on day 0, and the mice were treated with 10 μ g murine IgG or B7H6-specific BiTE i.v. (days 5, 7, 9, and 11). Tumor area was measured. Error bars represent SEM. Data shown are pooled results from two independent experiments ($n = 12$). **(B)** B16F10 (10^6 cells) was injected s.c. into mice on the shaved right flank on day 0, and the mice were treated i.v. with 10 μ g murine IgG or anti-B7H6 BiTE on days 5, 7, 9, and 11. Tumor area was measured. Error bars represent SEM ($n = 6$). **(C)** ID8/B7H6 cells (5×10^6 cells) were injected i.p. into mice on day 0, and the mice were treated i.p. with 10 μ g murine IgG or B7H6-specific BiTE on days 7, 9, and 11, with or without Con A-activated and cultured T cells (5×10^6 cells, i.p. on day 7) as an additional source of effector T cells. The number of solid tumors on the peritoneal wall and suspension tumors in the i.p. wash were quantified on day 50. Data shown are pooled results from three independent experiments. Symbols represent data from individual mice ($n = 9-16$). * $p < 0.05$, ** $p < 0.005$, Mann-Whitney *U*.



completed (5, 6). This treatment also caused reversible symptoms in the CNS (5, 6).

Studies suggest that NKp30 plays an important role in mediating tumor immunosurveillance in several clinical settings. In acute myeloid leukemia, leukemic blasts actively downregulate NKp30 expression on NK cells to evade immune surveillance, and a natural cytotoxicity receptor (NCR)^{dull} phenotype on NK cells correlates with poor prognosis (12). GIST patients expressing immunostimulatory isoforms of NKp30 have a better prognosis than do patients with an immunosuppressive isoform (13). These observations highlighted the importance of NKp30 recognition and control of various tumor types and justify the approach of targeting B7H6, a cell surface NKp30 ligand, with BiTE therapy.

B7H6 is expressed on ~20% of human tumor cell lines, as well as on a subset of primary leukemia, lymphoma (14). In addition, mRNA expression data suggest that B7H6 is overexpressed on some ovarian cancers, breast cancers, brain tumors, renal cell carcinomas, and various sarcomas. B7H6 mRNA is not found on 48 normal tissues under steady-state conditions (14). It was reported that B7H6 can be induced on circulating proinflammatory monocytes in a subgroup of patients suffering severe sepsis or on monocytes after treatment in vitro with TLR agonists (21). However, we did not observe B7H6 expression on proinflammatory monocytes using the 47.39 mAb, which is used as the basis for the B7H6-specific scFv in this BiTE. Furthermore, the B7H6-specific BiTE did not trigger T cells to react to activated proinflammatory monocytes (Fig. 4B). These data suggest that B7H6 is a highly tumor-specific Ag.

The data showed that B7H6-specific BiTEs can be readily produced by a mammalian cell-expression system as monomers, which is consistent with other BiTEs in the literature (11, 25). The B7H6-specific BiTE maintained the specificity of both scFvs and triggered T cells to mediate robust T cell effector mechanisms against B7H6⁺ tumors but not B7H6⁻ tumors. BiTE treatment prolonged survival, and most mice become long-term survivors. Treatment with the B7H6-specific BiTE was able to decrease tumor burden in two models of solid tumors. Solid

tumors are often more difficult to treat than hematopoietic tumors. Solid tumors generally have a lower accessibility for T cells and BiTEs due to high local interstitial fluid pressure (26). BiTEs have a short circulating half-life (27); to maintain optimal BiTE concentration, continuous i.v. pumps or slow-release formulations can be used (5, 6). BiTEs should bind to T cells rapidly, so the absence of free BiTE protein in serum may not reflect the presence of BiTEs on patient T cells. The microenvironment in solid tumors can be immunosuppressive (28). To overcome low accessibility, one could actively enhance vascular and tumor permeability by administering tumor-infiltrating peptides (29) simultaneously with BiTE therapy. One could also use immunological checkpoint blockade strategies, such as anti-CTLA4 or anti-PD1 mAbs, to revert exhausted tumor-infiltrating lymphocytes and to enhance T cell infiltration (30, 31). These strategies and others may enhance BiTE therapeutic efficacy against solid tumors.

In conclusion, the data in this study show that B7H6-specific BiTEs directed T cells to mediate cytotoxicity and IFN- γ secretion against multiple B7H6⁺ tumor cell lines. B7H6-specific BiTE therapy promoted the survival of lymphoma-bearing mice in an IFN- γ - and perforin-dependent manner. To our knowledge, this is the first direct evidence of IFN- γ and perforin mediating BiTE therapeutic efficacy in vivo. Furthermore, BiTE protein decreased tumor burden in melanoma- and ovarian cancer-bearing mice. The findings support the further development of B7H6-specific BiTE therapy for the treatment of lymphoma, melanoma, and ovarian cancer.

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Disclosures

T.Z. and C.L.S. are inventors on a patent application covering the B7H6 BiTE described in this study. This technology has been licensed by Cardio3 Biosciences. This work is managed in compliance with the policies of Dartmouth College.

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