



Strengthening of antitumor effects in breast cancer from a novel B7-H4- and CD3-targeting bispecific antibody by an oncolytic virus

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Background: For programmed cell death protein 1/programmed death-ligand 1 (PD-L1)-positive triple-negative breast cancer (TNBC), the addition of immune checkpoint inhibitors (ICIs) to chemotherapy has been shown to increase the objective response rate (ORR) by only 13.4–16.3%. Thus, examining converting tumor immunogenicity and targeting novel pathways may be needed to improve response to immunotherapy. B7-H4 is an emerging target for breast cancer immunotherapy. However, the antitumor effect of B7-H4 is unstable during cell generation and further research is necessary to strengthen the antitumor efficacy of B7-H4.

Methods: To explore the potential of B7-H4-targeted immunotherapy of breast cancer, current study generated four monoclonal antibodies (mAbs) against B7-H4 through immunization of mice followed by phage display technology. T cell-engaged bispecific antibodies and immunotoxins were created based on these mAbs. To evaluate the anti-tumor activity of B7-H4-targeting bispecific antibody and immunotoxin, the anti-tumor efficacy test *in vitro* and in mice models *in vivo* were performed. Moreover, to boost the efficacy, oncolytic virus herpes simplex virus type 2 (HSV-2) was combined with the bispecific antibody and tested in mice models.

Results: Rigorous *in vitro* cytotoxicity assay showed that both B7-H4 bispecific antibodies and immunotoxins were toxic to B7-H4 positive cells, with the former being more potent. However, *in vivo* studies showed that immunotoxin was slightly more effective in suppressing tumor growth. Further, bispecific antibody combined with oncolytic virus HSV-2 revealed a synergistic effect in suppressing tumor growth without causing obvious adverse effects.

Conclusions: Collectively, our findings uncover a novel strategy of combining oncolytic virus HSV-2 with bispecific antibody reinforce antitumor immune response, which warranted further translational investigation.

Keywords: B7-H4; bispecific antibody; immunotoxin; oncolytic virus; breast cancer

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Introduction

Breast cancer (BC), the most common cancer in women, has three major clinically relevant subtypes (i.e., hormone receptor (HR) positive, human epidermal growth factor receptor 2 (HER2) positive and triple-negative). Compared to triple-negative breast cancer (TNBC) in metastatic setting, the treatment strategies of which used to be limited to chemotherapy, other two subtypes of BC have more treatment options, such as endocrine and anti-HER2-based therapy, other than chemotherapy (1). Over the last decade, Cytotoxic T Lymphocyte-associated antigen-4 (CTLA-4) (2) and programmed cell death protein 1 (PD-1) (3)/programmed death-ligand 1 (PD-L1) (4) inhibitors have become a routine part of treatment for nearly 50 different cancer indications, offering the possibility of long-term clinical response and cure for many patients with metastatic cancers (5,6). In TNBC, the median overall survival (mOS) used to be only 12–18 months in the metastatic setting due to limited treatment options until the treatment landscape started to change and the use of atezolizumab (7) and pembrolizumab (8,9), in combination with other therapies (10), offered new opportunities to extend survival in both early and advanced PD-L1+ TNBC. However, TNBC has long been considered to be a ‘cold’ immune microenvironment (TME) tumor with poor therapeutic effect, due to the relatively low level of T cell infiltration and mutational burden compared to melanoma or non-small cell lung cancer (11,12). Therefore, to permit precision deployment of immunotherapies beyond immune checkpoint inhibitors (ICIs), more effective therapeutic strategies need to be achieved through deeper understanding of other immune coinhibitors, like B7-H4, in addition to the attention that has been focused on co-stimulatory receptors, such as OX40 and 4-1BB/CD137 agonists, to boost overall antitumor immunity (13).

B7-H4, also known as B7x, B7S1, or VTCN1, is a coinhibitory molecule that is overexpressed across cancer cells. B7-H4 negatively regulates inflammatory CD4+ T cells, by which cancer cells evade antitumor immune responses (14–16). It was reported that the expression levels of B7-H4 were high in different subtypes of breast cancer (17). Increased B7-H4 expression levels on breast cancer cells were significantly correlated with one or more clinicopathological factors, including increased TNM malignant tumor score, decreased survival, and decreased number of tumor-infiltrating T cells, which was a significant

independent prognostic indicator (18,19). The strategies to block B7-H4 include the use of classical monoclonal antibodies (mAbs) (20). However, previous data showed that anti-human B7-H4 mAbs alone were not sufficient to suppress the tumor growth in a mouse model (20). The bispecific antibodies targeting B7-H4 and CD3 have recently been discovered to have antitumor effects in TNBC (21).

More recently, the quest to improve the response rate of immunotherapy has prompted the combinations with other established drugs targeting multiple compensatory pathways (22). Chemotherapy and radiation are widely used in clinical practice to treat TNBC but the tolerability is generally poor. Other combination strategies, such as oncolytic viruses (OVs) and immunotoxins, are currently under scientific and clinical investigation and are worthwhile-to-try selections that may potentially open the door to high-order immunotherapy combinations (23–25). Oncolytic viruses are emerging as effective strategy in cancer treatment. A variety of viruses has been explored as potential cancer therapeutic agents (26). A novel oncolytic herpes simplex virus (HSV-2), oHSV2hGM-CSF (oHSV2), which was developed by our group, produces a more potent antitumor response than HSV-1 through 2 major mechanisms of action (27): one of which is the ability of selectively replicating within tumors and directly inducing cell lysis; and another is that OVs improves antigen cross-priming and recruitment of immune cells into TME, eliciting antitumor immunity. Our previous work demonstrated oHSV2 obviously promotes tumor regression and produces almost no side effects in preclinical mice models *in vivo*. Throughout the course of the study, the body weight of mice remained relatively stable and the pathologic changes in the main organs (lung, heart, liver, spleen, and kidney) were not observed (28). Apart from OVs, immunotoxins, which are cytolytic fusion proteins and composed of a targeting moiety, commonly a single-chain variable fragment (scFv) from of an antibody targeting a specific antigen on tumor cells and linked to a toxin, developed for cancer therapy (23,24). To date, two immunotoxins, moxetumomab pasudotox (Lumoxiti) and tagraxofusp (Elzonris), were approved by the Food and Drug Administration (FDA) for the treatment of relapsed or refractory hairy cell leukemia and blastic plasmacytoid dendritic cell neoplasm (29,30), respectively. Based on analysis of the aforementioned mechanism and reasonable speculation, current project aims to explore the efficacy and safety of B7-H4-targeting bispecific antibodies and immunotoxins in breast cancer. Further, it is also

worthwhile to investigate whether oncolytic virus can enhance anti-tumor responses driven by bispecific antibodies. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://atm.amegroupp.com/article/view/10.21037/atm-22-3423/rc>).

Methods

Cell lines

Two human breast cancer cell lines (SKBR3 and ZR75) and a B7-H4-negative A431 cell line (human squamous cell carcinoma cell line) were maintained as adherent monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM, from Invitrogen, Carlsbad, CA) with supplement of 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 100 IU/mL penicillin-streptomycin and incubated in 5% CO₂ at 37 °C. All cell lines (A431, SJBR3 and ZR75) were purchased from the Cell Bank of the Chinese Academy of Science. A431 cells were transfected with a plasmid encoding the full-length *B7-H4* gene (GenBank accession no. NM_024626.2), engineered to overexpress B7-H4. Both the B7-H4-negative A431 and the B7-H4 overexpressed A431 (B7-H4) cells (Feng Lab, Huazhong Agricultural University, Wuhan, China) were maintained in DMEM. All cells were cultured under aseptic conditions, with the media being refreshed every 3 days.

Preparation of recombinant B7-H4 proteins

After fusing the extracellular domain (ECD) of B7-H4 (NM_024626.2) with human IgG1-Fc, we cloned the fusion into mammalian expression vector pFUSE-hIgG1-Fc2 (cat. no. pfuse-hg1fc2; Invitrogen) to generate B7H4-hFc. The expression vectors were then introduced into 293F cells by polyethylenimine (PEI) transfection. The transfected cells were cultured in SMM 293-TII Expression Medium (cat. no. M293TII; Sino Biological Inc., Beijing, China). The expressed protein was secreted into the cell culture medium and purified by protein A affinity chromatography (cat. no. C600952-0501; Sangon Biotech Co., Ltd; Shanghai, China). The purity of the preparations was analyzed by SDS-PAGE (31).

Generation of B7-H4 mAbs

Recombinant B7-H4-hFc was used to immunize 4 mice by subcutaneous injection of 100 µg of protein mixed with

100 µL of Complete Freund's adjuvant (Sigma-Aldrich; Merck KGaA, Burlington, MA, USA). Fourteen days later, mice were boosted with half the amount of the protein mixed with Incomplete Freund's adjuvant. One week later after boosting, the immunized spleens were harvested according to the protocol (32). The total splenic RNAs were isolated using TRIzol (cat. no. 15596018; Invitrogen ThermoFisher Scientific, Inc.) and reverse transcribed into complement cDNA using a PrimeScript kit (Takara Bio, Inc., Shiga, Japan). Polymerase chain reaction (PCR) amplification of the scFv fragments and construction of the phage display library was performed as previously described (33). The resultant library contained about 10⁸ original transformants. Screening of the phage library followed the previous protocol (34).

Production of B7-H4 mAbs

Recombinant scFv of the B7-H4 binders was fused with the rFc tag for purification and detection purposes, inserted into the expression vector, pFUSE-hIgG1-Fc2 (pfuse-hg1fc2; InvivoGen, San Diego, CA, USA); and expressed as a scFv-rFc format in 293F cells according to the aforementioned method. The rFc tag was used in the cell binding assay through flow cytometry.

Enzyme-linked immunosorbent assay

5 µg/mL B7H4-hFc protein was coated on a 96-well MaxiSorp plate and incubated at 37 °C for 30 min. After washing the plate twice with Phosphate buffer saline tween (PBST), blocking buffer [2% bovine serum albumin (BSA) in Phosphate buffered solution (PBS) buffer] was added to the plate and blocked for 30 min with. After removing the blocking buffer, biotinylated B7-H4 mAbs (scFv-rFc format) were incubated within the plate at indicated concentrations starting from 200 µg/mL and followed by 1:2 serial dilutions. Binding of B7-H4 mAbs was detected with horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen; Thermo Fisher Scientific, Inc.; 1:4,000).

Flow cytometry

To test the cell surface expression of B7-H4, cancer cells were coincubated with B7-H4 mAbs (scFv-rFc) of 10 µg/mL for an hour on ice. Cell binding ability of antibodies was detected with phycoerythrin-conjugated goat-anti-rabbit polyclonal antibodies (Jackson ImmunoResearch

Laboratories, Inc., West Grove, PA, USA). We use a FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) to measure the fluorescence intensity.

Production of B7-H4 bispecific antibodies and immunotoxins

To make the B7-H4 bispecific antibody, the coding sequences of B7-H4 scFv were fused with a (G4S)₃ linker and then with OKT3 scFv and hFc knob mutants (N297A/S354C/T366W). The expression cassette was cloned into a pFUSE vector and introduced into Expi293 cells (Gibco, Thermo Fisher Scientific). Purification of the bispecific antibodies was performed by using a protein A column.

Oncolytic viruses

The OV, oHSV2hGM-CSF (HG52/34.5-/47-hGM-CSF), was used in the current project. The virus was produced in a good manufacturing production (GMP) facility by Wuhan Binhui, LLC (www.binhui-bio.com.cn; Wuhan, China) according to standard protocols (batch no. VBT-oHSV2-hGM-CSF-201908A-KZH-NS) (27). The virus was resuspended in preparation buffer and stored at -70°C in aliquots to avoid repeated freezing and thawing.

In vitro cytotoxicity assays

Cytotoxicity of B7-H4 bispecific antibody, immunotoxins, and the OVs was measured through a luciferase reporter assay. To constitutively express the firefly luciferase gene (ffLuc2), target cells were stably transfected with a lentiviral vector. For the assay, target cells were cultured on 96-well tissue-culturing plates at 10,000 cells per well and cytotoxic agent was added to each well at the indicated concentrations. After 3 days of co-incubation in a CO₂ incubator, cell viability was quantified by measuring the intracellular luciferase activity according to previously published method (31).

In vivo efficacy study

NSG mice (5-week-old, female, average weight ~ 21 g; provided by the Animal Facility of Huazhong Agricultural University, Wuhan, China) were assigned to different groups, 5 mice per group. Mice were subcutaneously injected with 2×10^6 A431 (B7H4) cells in a volume of 200 μL serum-free DMEM. When tumor grew to

100–200 mm³, treatments were started. Tumor growth was measured with a caliper every 2 days. The tumor volume (mm³) was calculated using the following formula: $(a \times b^2)/2$, where a is the length of tumor and b is the width of tumor in mm. When the tumor size exceeded 1,600 mm³, mice were sacrificed by anesthesia, and serum and tumor masses were collected for further analysis.

In a dose escalation experiment, the B7H4 bispecific antibody M16-OKT3-hFc was administered intravenously at 0.5, 1.0 and 2.0 mg/kg every three days for a total of five injections. B7H4 immunotoxin M16-PE24 was intravenously administered at 2.5, 5.0 and 10.0 mg/kg on alternate days for a total of 6 injections.

In the bispecific antibody-oncolytic virus combination study, six groups of mice were set up with five mice in each group. The PBS control group received only PBS as solvent. The peripheral blood mononuclear cell (PBMC) group was treated once with 10^7 PBMC by tail vein injection. The OVs group was treated with HSV-2 oncolytic virus at a dose 150 μL by intratumoral administration every other day for three injections. The OVs and PBMC group was treated with PBMC and OVs in the same way as the OVs group and PBMC group. The bispecific antibody group was treated with 10^7 PBMC and 1 mg/kg B7H4-M16-OKT3-hFc via tail vein injection once every three days for a total of five injections. The bispecific/oncolytic combination group was exactly the treatment of bispecific antibody plus that of oncolytic virus (Table 1). All the procedures used in the animal studies were approved by the Animal Care and Use Committee of Huazhong Agricultural University (approval No. HZAUMO-2017-051), in compliance with institutional guidelines for the care and use of animals. Peripheral blood materials used in this study were obtained from healthy donors with informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The use of human blood samples was reviewed and approved by Biomedical Research Ethical Committee, Huazhong Agricultural University (approval No. HZAUHU-2017-002).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism9.3.0 (GraphPad Software, La Jolla, CA, USA). The data are expressed as the mean \pm standard error of mean (SEM). Comparisons between two groups were performed using an unpaired Student's t -test (2-tailed). Comparisons among three and more groups were performed using a one-way analysis of variance (ANOVA) followed by Tukey's

multiple comparison test. P value <0.05 was considered to indicate a significant difference.

Results

Generation and binding properties of B7-H4 mAbs

The extracellular domain (ECD) of B7-H4 was fused with human IgG1 Fc (B7H4-hFc). Recombinant B7H4-hFc was purified via protein A chromatography. SDS-PAGE analysis showed that the protein may be highly glycosylated (Figure 1A), which was consistent with the prediction that the protein has seven predicted N-glycosylation sites. Purified B7H4-hFc was used to immunize mice and to generate the mAbs M2, M16, M37, and M64 via phage display according to published protocol (33). The scFv sequences of the mAbs were fused with rabbit Fc (scFv-rFc) and expressed in 293F cells. SDS-PAGE analysis of the recombinant antibodies revealed high homogeneousness (Figure 1B). ELISA analysis showed

that all mAbs had high affinity binding to recombinant B7-H4, with the calculated EC_{50} values of being 0.1 to 0.4 nM (Figure 1C). Flow cytometry analysis showed that all mAbs bound to B7-H4-overexpressed A431 cells (Figure 1D). Since M37 had the highest affinity to cell surface B7-H4, it was used to screen B7-H4 positive breast cancer cell lines. As shown in Figure 1E, M37 did not bind to A431 cells known to be B7-H4 negative, but efficiently bound to B7-H4 overexpressing A431 cells as well as to the native breast cancer cells ZR75 and SKBR3.

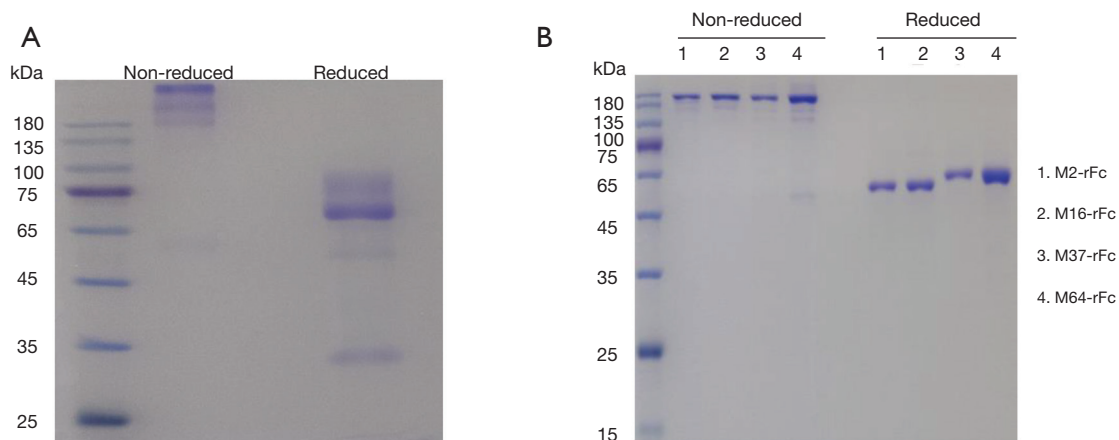
Generation of B7-H4 bispecific antibodies and immunotoxins

B7-H4 bispecific antibody was constructed by fusing the scFv of M2, M16, M37, and M64 with that of CD3-targeted antibody OKT3, followed by hFc knob mutant with N297A mutation (Figure 2A). Immunotoxin was

Table 1 Details of the animal groups and treatments

Groups	Treatments	Schedule (days post inoculation)
Control	PBS	–
PBMC control	PBMC	D10
Bispecific antibody + PBMCs	B7H4-M16-OKT3-hFc and PBMCs	D10 for PBMCs; d10, d13, d16, d19, and d22 for B7H4-M16-OKT3-hFc
OVs	OVs	D10, d12, and d14 for OVs
OVs + PBMCs	OVs and PBMCs	D10 for PBMCs; d10, d12, and d14 for OVs
Bispecific antibody + OVs + PBMCs	B7H4-M16-OKT3-hFc, OVs, and PBMCs	D10 for PBMCs; d10, d13, d16, d19, and d22 for B7H4-M16-OKT3-hFc; d10, d12, and d14 for OVs

PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cell; OVs, oncolytic viruses.



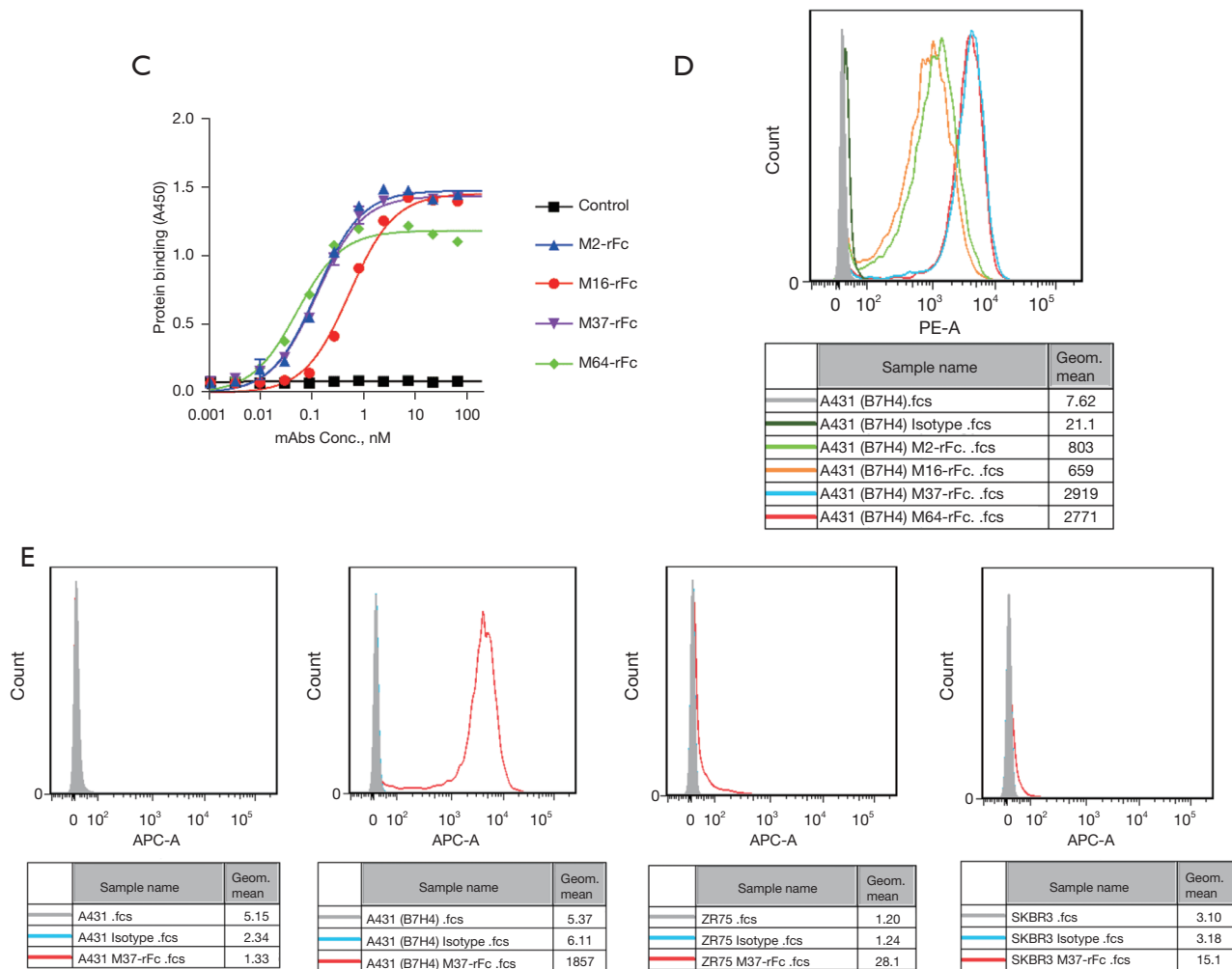


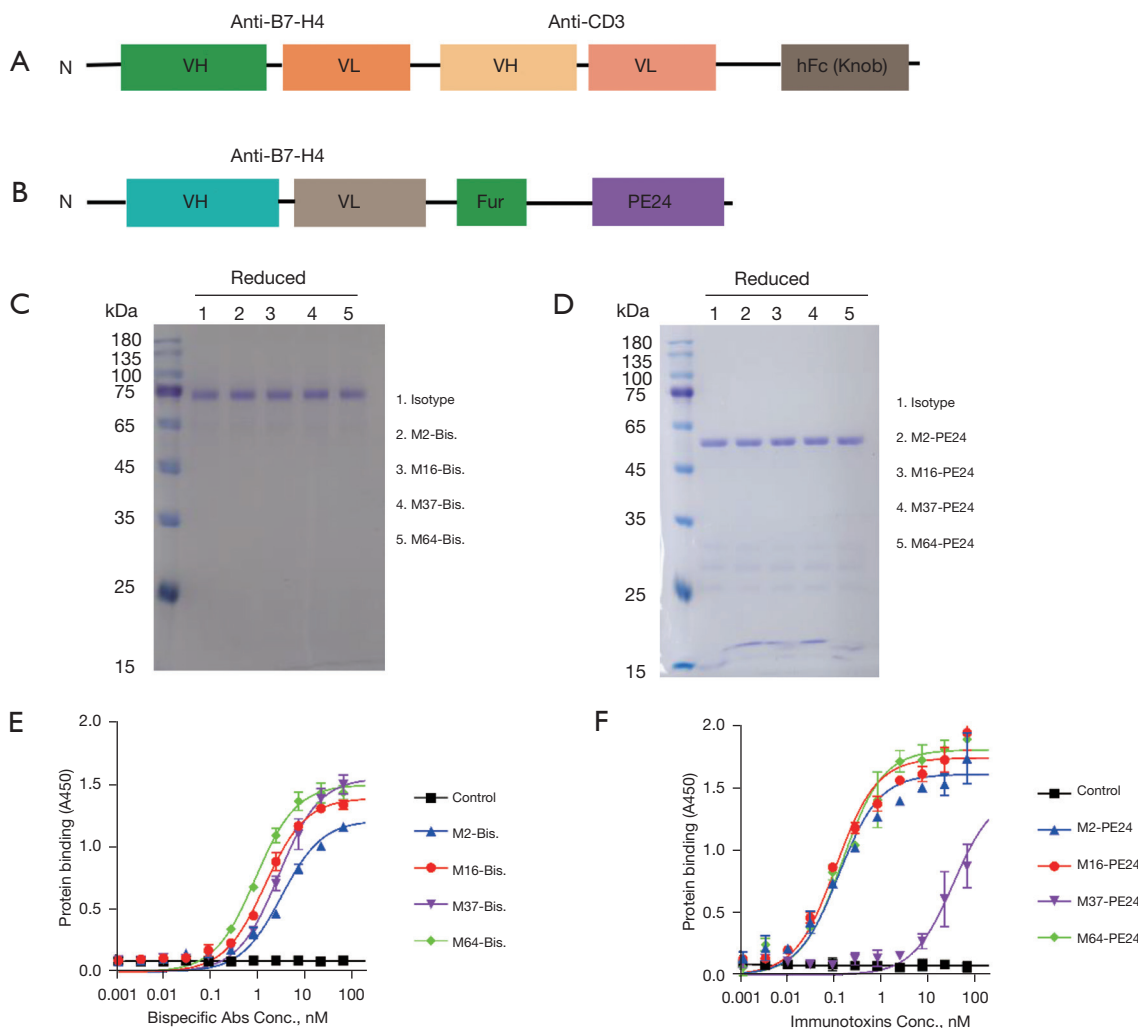
Figure 1 Generation and binding properties of B7-H4 mAbs. (A) SDS-PAGE analysis of purified B7-H4-hFc proteins. Two micrograms of protein were loaded for each lane. Non-reduced, non-reduced condition, showing the dimerized B7-H4-hFc antibody; Reduced, 2-mercaptoethanol reduced condition, showing the reduced monomer of the B7-H4-hFc antibody. Mw: protein marker. (B) SDS-PAGE analysis of recombinant monoclonal antibodies to B7-H4 (scFv-rFc format): M2, M16, M37, and M64. Lane 1: B7H4-M2-rFc; lane 2: B7H4-M16-rFc; lane 3: B7H4-M37-rFc; lane 4: B7H4-M64-rFc. One microgram was loaded for each lane. Non-reduced, the B7-H4 recombinant monoclonal antibodies in the non-reduced condition; Reduced, monomer of the B7-H4 recombinant monoclonal antibodies in the reduced condition, with 2-mercaptoethanol being used as the reduction agent. (C) Protein binding analysis of B7-H4 mAbs by ELISA. Ninety-six well plate was coated with B7-H4-his protein of 5 $\mu\text{g}/\text{mL}$. Antibody binding was detected with HRP-conjugated goat anti-rabbit polyclonal antibodies. (D) Cell binding analysis of B7-H4 mAbs by flow cytometry. One million A431 (B7H4) cells were coincubated with B7H4 mAbs of 5 $\mu\text{g}/\text{mL}$. Antibody binding was detected with PE-conjugated goat anti-rabbit polyclonal antibodies. (E) Expression level of B7-H4 on different cell lines. A431 cell was used as negative control to show the specificity of the antibody, and A431 (B7-H4) was used as positive control. Two breast cancer cell lines SKBR3 and ZR75 were stained with 10 $\mu\text{g}/\text{mL}$ M37 monoclonal antibody, and the binding was detected with Cy5-conjugated goat anti-rabbit polyclonal antibodies. Shaded area, unstained cells; cyanine lines, isotype control staining with an in-house made recombinant anti-HIV monoclonal antibody that had same scFv-rFc structure; red lines, M37 staining.

constructed by fusing afore-mentioned scFv of B7-H4 mAbs with a truncated form of *pseudomonas* exotoxin A (PE24) (Figure 2B). The bispecific antibodies were expressed in 293F cells and purified via protein A column (Figure 2C), and immunotoxins were expressed in *E. coli* and purified via nickel column (Figure 2D). Binding activity of the purified proteins was confirmed by ELISA for both bispecific antibodies (Figure 2E) and immunotoxin (Figure 2F), all of which showed similar affinities except M37 immunotoxin. The calculated EC₅₀ values for bispecific antibodies were about 0.4 nM, while the EC₅₀ values for immunotoxins were about 0.1 nM. Cell surface B7-H4 binding of the bispecific antibodies and immunotoxins was also confirmed by flow cytometry (Figure 2G), where Jurkat cells and PBMC were used as CD3 positive cells, while A431 (B7H4) cells that were forcefully overexpressing B7-H4 were used as B7-H4

positive cells. In the flow cytometry, the bispecific antibody M37 and M64 showed much stronger binding compared to M2 and M16, which was consistent with the binding of monoclonal antibodies.

In vitro cytotoxicity of B7-H4 bispecific antibodies and immunotoxins

Cytotoxicity of the B7-H4 bispecific antibodies and immunotoxins was detected in the B7H4-negative A431 cells and A431 cells that were forcefully overexpressing B7-H4 [named A431 (B7H4)], as well as in B7-H4-positive breast cancer cells SKBR3 and ZR75. A431 cells were used as a control for non-specific toxicity independent of the antigen. As shown in Figure 3, bispecific antibodies had no detectable toxicity to A431 cells (Figure 3A), but potently



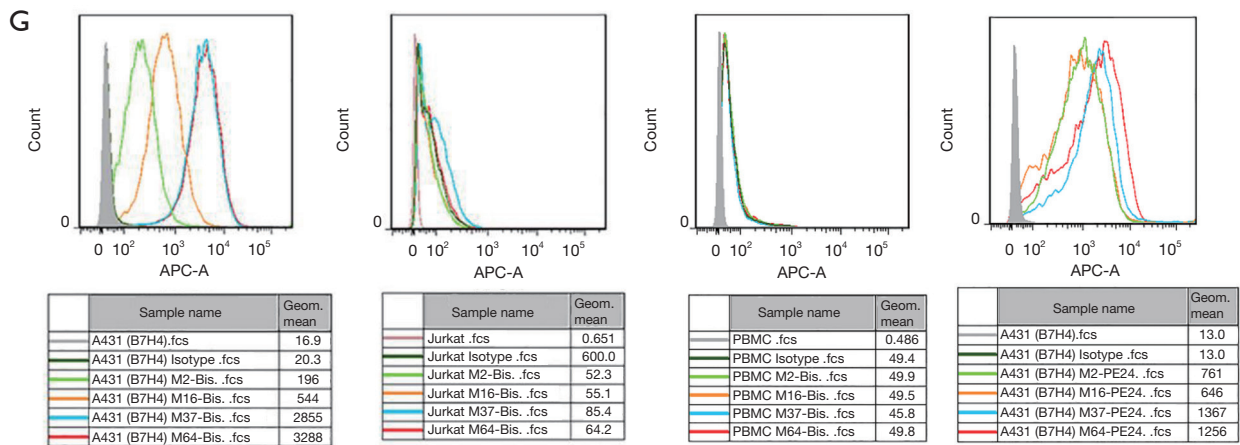
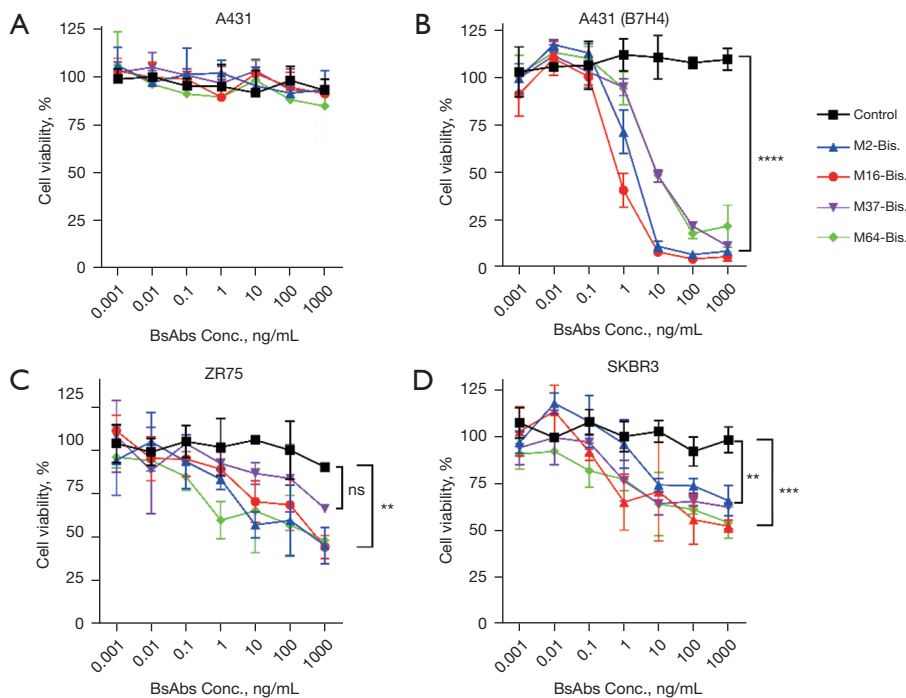


Figure 2 Generation and binding properties of B7-H4 bispecific antibodies and immunotoxins. (A) Schematic diagram of the bispecific antibody that had a scFv-scFv-hFc (knob) structure, where hFc (knob) carried the N297A/S354C/T366W mutations. (B) Schematic diagram of the B7-H4 immunotoxins. Fur, furin cleavage site. (C) SDS-PAGE analysis of the B7-H4/OKT3 bispecific antibodies. Two micrograms of protein were loaded into each lane. Isotype, anti-GPC3 bispecific antibody hYP7 that served as isotype control (35). (D) SDS-PAGE analysis of the B7-H4 immunotoxins. Two micrograms of protein were loaded into each lane. Isotype, anti-GPC3 immunotoxin J80A that served as isotype control (36). (E) Protein binding activity of B7-H4 bispecific antibodies. ELISA plate was coated with 5 µg/mL B7-H4-His, and incubated with increased concentrations of bispecific antibody. Binding of antibody was detected with HRP-conjugated goat anti-human polyclonal antibodies. (F) Protein binding activity of the B7-H4 immunotoxins. ELISA plate was coated with 5 µg/mL B7-H4-His, and incubated with increased concentrations of immunotoxin. Binding of immunotoxin was detected with HRP-conjugated anti-His monoclonal antibody. (G) Cellular binding activities of B7H4 bispecific antibodies and immunotoxins by flow cytometry. One million cells were cultured with 10 µg/mL bispecific antibody or 5 µg/mL immunotoxin. Binding of bispecific antibody and immunotoxin was detected with Cy5-conjugated goat anti-human polyclonal antibodies or Cy5-conjugated anti-his monoclonal antibody.



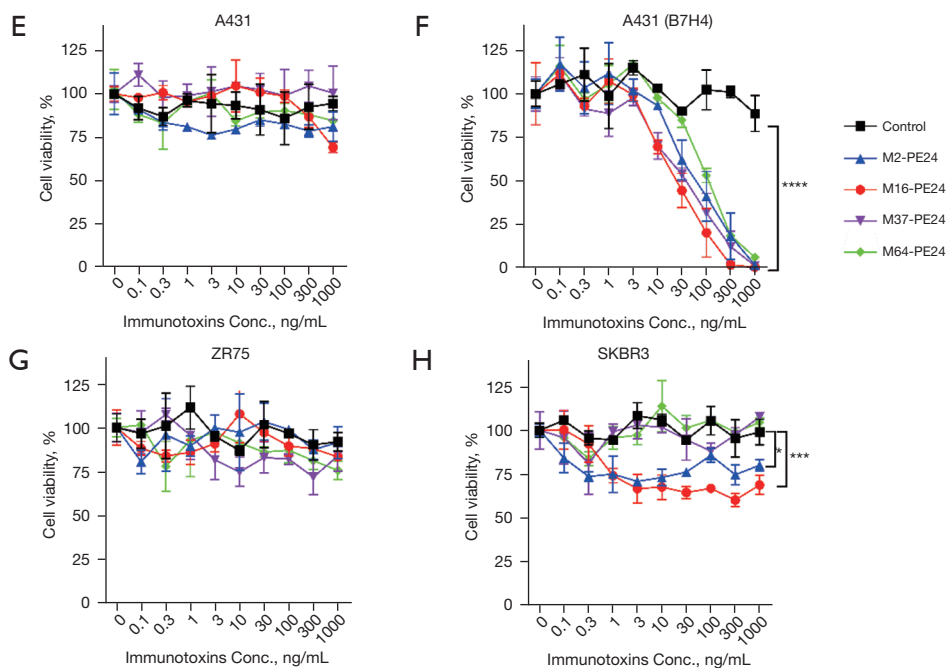


Figure 3 *In vitro* cytotoxicity of B7-H4 bispecific antibodies and immunotoxins. (A-D) *In vitro* cell-killing assay of the B7-H4/OKT3 bispecific antibodies with unstimulated human PBMCs. The cytotoxicity of the B7H4/OKT3 bispecific antibody was tested in the B7H4 negative A431 cells, the artificial B7-H4-overexpressed A431 (B7H4) cells, and the B7-H4-positive breast cell lines, SKBR3 and ZR75. All the tested cell lines were stably transduced with a lentiviral vector to constitutively express the firefly luciferase gene (*ffLuc2*) that was used to measure the cytotoxicity. Ten thousand cells were incubated with increasing concentrations of bispecific antibody in the presence of a hundred thousand unstimulated human PBMC for 72 hours. Cell viability was quantified by measuring the intracellular luciferase activity. B7H4-M16-OKT3-hFc (Knob) N297A, based on B7H4-M16-mAbs, which exhibited the strongest binding activity compared with the other clones, demonstrated the highest ability to kill cells expressing surface recombinant B7H4. (E-H) *In vitro* cell-killing assay of the B7-H4 immunotoxins. Cytotoxicity of the B7-H4 immunotoxins was tested in the same 4 cell lines mentioned above using the same method. Ten thousand cells were seeded on 96-well plated, and incubated with increasing concentrations of immunotoxins for 72 hours. Cell viability was quantified by measuring intracellular luciferase activity. B7H4-M16-PE24, based on B7H4-M16-mAbs, which exhibited the strongest binding activity compared with the others clones, demonstrated the highest ability to kill cells expressing surface recombinant B7-H4. ns, not significant, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. PBMC, peripheral blood mononuclear cell.

killed A431 (B7H4) in the presence of unstimulated PBMC (Figure 3B), and moderately killed native breast cancer cells ZR75 (Figure 3C) and SKRB3 (Figure 3D). Unlike the bispecific antibodies, while the immunotoxin was not toxic to A431 (Figure 3E) and had similar potency against A431 (B7H4) in a dose-dependent manner (Figure 3D), its potency against ZR75 and SKBR3 was almost negligible (Figure 3G, 3H), probably due to the relatively low expression level of B7-H4 on the native cell lines

***In vivo* efficacy of B7-H4 bispecific antibodies and immunotoxins**

M16-based bispecific antibodies and immunotoxins were

selected for *in vivo* studies because of their relatively stronger activity in the *in vitro* cytotoxicity assays. The *in vivo* antitumor efficacy was tested in NSG xenograft mice. As shown in Figure 4A, dose escalating test of bispecific antibody at 0.5–2.0 mg/kg had comparable efficacy that partially suppressed tumor growth, while 1.0 and 2.0 mg/kg caused significant body weight loss (Figure 4B), suggesting a limited efficacy of bispecific antibody in controlling tumor growth. However, immunotoxins at 1.0–10.0 mg/kg dramatically inhibited tumor growth (Figure 4C), though 5.0 and 10.0 mg/kg doses also caused significant body weight loss (Figure 4D). Taken together, immunotoxin was superior to bispecific antibody in terms of efficacy and safety.

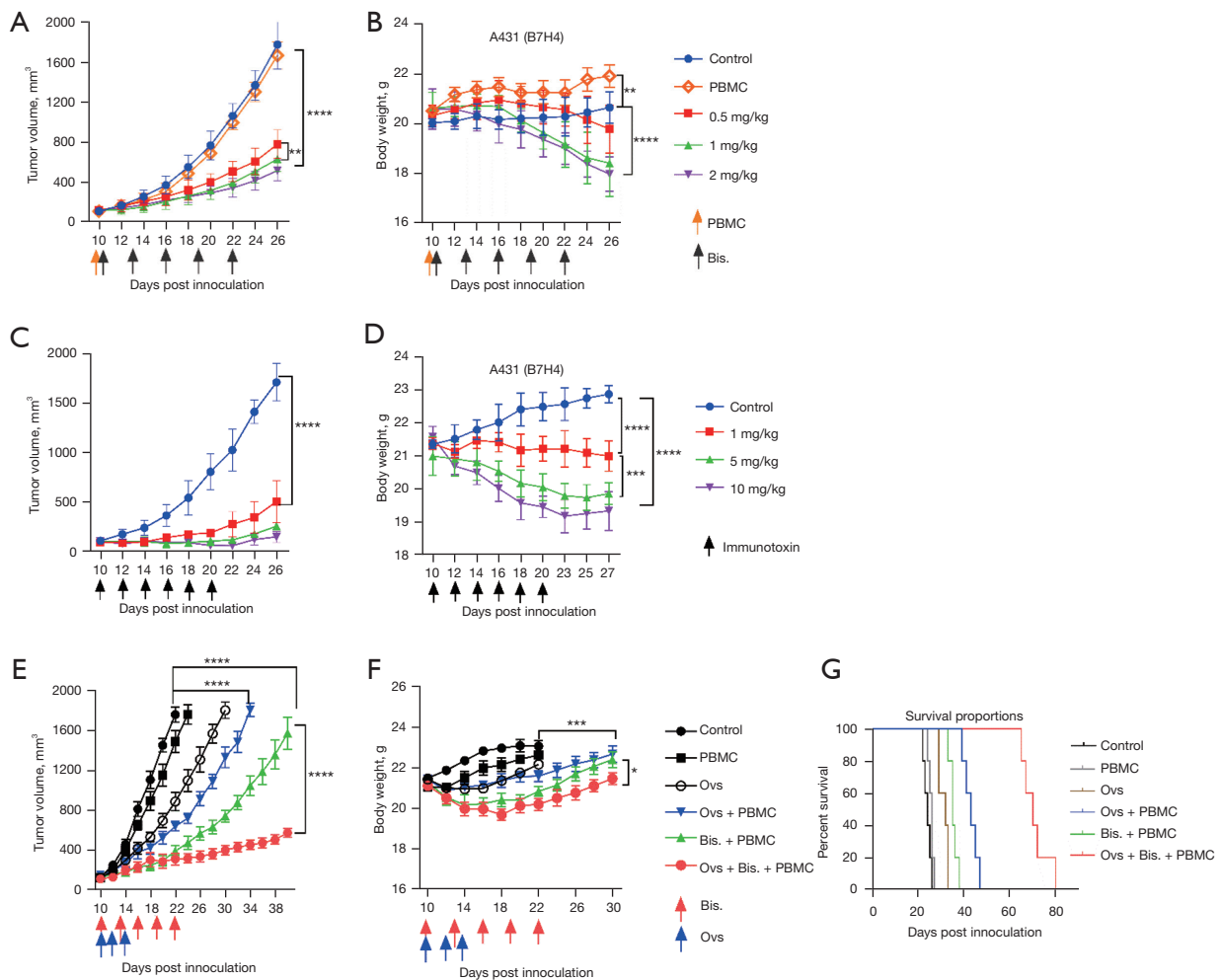
Our previous work had shown that HSV-2 oncolytic

viruses produced strong anti-tumor response through antigen cross-priming and recruitment of immune cells into TME (27), therefore it would be interesting to know whether oncolytic virus could boost the efficacy of bispecific antibody. As expected, combination of oncolytic virus with low dose of bispecific antibody significantly inhibited tumor growth compared to monotherapy (oncolytic virus or bispecific antibody alone) (Figure 4E), and the combination did not cause dramatic body weight loss (Figure 4F), which was in contrast to the treatment with high doses of bispecific antibody. Further, combination significantly prolonged the survival time (Figure 4G). To examine any possible damages in the main organs of mice treated by monotherapy and combination therapy, tissue section and H&E staining of heart, lung, liver, kidney and spleen was performed. The results showed that the immune response caused by bispecific antibodies, oncolytic virus alone or

in combination resulted in a significant and rapid tumor cell clearance and did not cause obvious damage to the organs (40× micrograph) (Figure 4H). In summary, our data supported a more significantly synergistic inhibition of tumor growth by combination of bispecific antibody and oncolytic virus, which was worthy of further clinical investigation.

Discussion

Despite the unprecedented clinical benefits from immunotherapy observed in subsets of breast cancer patients, the majority of TNBC patients show limited responses to single-agent anti-PD-1 or PD-L1 treatment. Therefore, there is still a need to identify new targets and develop optimal immunotherapy strategies to improve the efficacy of breast cancer. B7-H4 is a promising new target



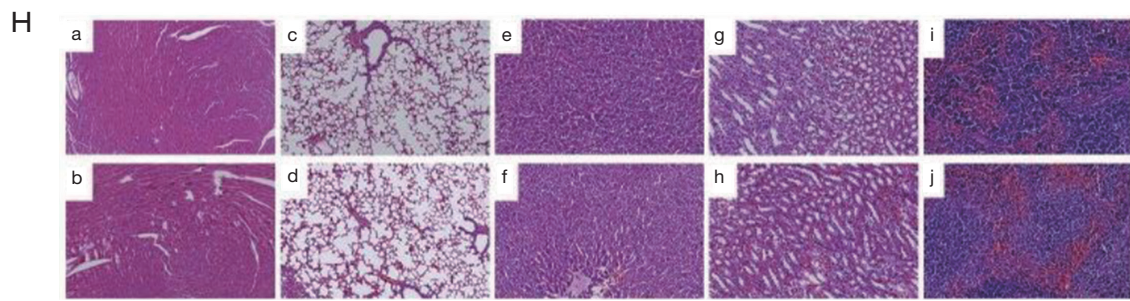


Figure 4 *In vivo* efficacy of B7-H4 bispecific antibodies, B7-H4 immunotoxins, and B7-H4 bispecific antibodies combined with OV in A431 (B7H4) xenograft mice models. (A) Tumor growth curve of the mice treated with different doses of M16 bispecific antibody. NSG mice were subcutaneously inoculated with 2×10^6 A431 (B7-H4) cells. After tumor formed and reached 100 mm^3 , treatment was started by intravenous injection of 1×10^7 PBMC (once, indicated by red arrow) and bispecific antibody (every three days, indicated by black arrow). Control was mice treated with PBS. (B) Body weight of treated mice from (A). (C) Tumor growth curve of mice treated with different doses of M16 immunotoxin. Experimental conditions were same as (A) except that immunotoxins were intravenously injected every other day as indicated by the arrows. (D) Body weight of treated mice from (C). (E) Combination therapy of mice with bispecific antibody and oncolytic virus HSV-2. Bispecific antibody was injected every three days at 1 mg/kg as indicated by the red arrows. The OV was injected every other day at a dose of $150 \mu\text{L}$ (0.15×10^7) as indicated by the blue arrows. PBMC was injected once at day 10. (F) Body weight of the treated mice from (E). (G) Survival curves of treated mice from (E). (H) Representative pathological images from major of mice from (E). HE staining, $40 \times$ micrograph. Images a, c, e, g, and i were the pathological sections of heart, lung, liver, kidney, and spleen in the control group. Images b, d, f, h, and j were the pathological sections of the heart, lung, liver, kidney, and spleen of the combination group. Data represent as mean \pm SEM. Statistical comparisons between the combination of bispecific antibody and oncolytic virus and bispecific antibody or oncolytic virus alone at each group were labeled on the right of the bar. If the difference was not significant, it was not labeled. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. PBMC, peripheral blood mononuclear cell; OVs, oncolytic viruses.

for cancer immunotherapy. It has been reported that B7-H4 was selectively expressed in breast cancer and other cancer types, with minimal expression on normal adult tissues. To explore the potential of B7-H4-targeted immunotherapy in breast cancer, current study generated four mouse monoclonal antibodies, M2, M16, M37, and M64. Among them, M16-based bispecific antibody and immunotoxin had a relatively lower cell binding affinity (Figure 2G), however they exhibited the strongest cancer cell killing activity (Figure 3B-3H), indicating that cell binding affinity of bispecific antibody and immunotoxin does not necessarily correlate with their cytotoxic potency. In some cases, the epitope may be more important than affinity. Similar studies on GPC3-targeted immunotherapy demonstrated that bispecific antibody that targets the C-terminal had stronger potency (35), while immunotoxins target the N-terminal of GPC3 had stronger potency (36).

It has been shown that T cell-engaged bispecific antibody and CAR-T may not need high level of antigen density on cell surface for efficient tumor killing (31). In the current study, we found similar phenomenon. Although ZR75 and SKBR3 had very low level of B7-H4 expression

(Figure 1E), they were sensitive to T cell-engaged bispecific antibody treatment (Figure 3C,3D). However, low antigen density is not sufficient for immunotoxin-based therapy (Figure 3G,3H). Therefore, T cell-engaged immunotherapy may be more suitable for breast cancer that targets B7-H4.

Recently, studies have highlighted the critical role of the immune system in the control of cancer progression/regression, which provides a framework for investigating various immunotherapy approaches, including cancer vaccines, adoptive T cell transfer, and immune checkpoint inhibitors such as anti-PD-1/PD-L1 monoclonal antibodies. Oncolytic virus improves the immunogenic properties of cancer cells by enhancing their necrosis and pyroptosis and their capacity to elicit CD8⁺ interferon- γ -producing T cell responses in tumor microenvironment. Our previous work demonstrated that oHSV2 obviously promotes tumor regression and produces almost no side effects in preclinical mice models *in vivo* (28), and in patients with metastatic esophageal and rectal cancer, intratumoral injection of oHSV2 was well-tolerated, and demonstrated durable antitumor activity (37). For the purpose of improving the efficacy of T cell-engaged bispecific antibody, oHSV2,

which produces a more potent antitumor response than HSV-1 through 2 major mechanisms of action mentioned above (27), are one of the optimal choices. Indeed, our study supported the combination of B7-H4-targeted bispecific antibody with oncolytic virus, which demonstrated a significantly synergistic effect in suppressing tumor growth in xenograft mouse models (Figure 4E). Further, the combination was considerably safe (Figure 4F,4H), and therefore warrants further clinical investigation.

Collectively, our findings uncover a novel strategy that the interrogation of targeting multiple pathways is able to reinforce anti-tumor immune response driven by B7-H4-targeting bispecific antibody in TNBC through high-order immunotherapy combinations. However, there are still some shortcomings in our study, for example, more antibodies with higher B7-H4 affinity and epitope coverage need to be tested thus further research is preferred.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3423/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-3423/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the procedures used in the animal studies were approved by the Animal Care and Use Committee of Huazhong Agricultural University (approval No. HZAUMO-2017-051), in compliance with institutional guidelines for the care and use of animals. Peripheral blood materials used in this study were obtained

from healthy donors with informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The use of human blood samples was reviewed and approved by Biomedical Research Ethical Committee, Huazhong Agricultural University (approval No. HZAUHU-2017-002).

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