Abstract. Bispecific antibodies have been actively studied for cancer therapy due to their potent cytotoxicity against tumor cells. A number of bispecific antibody formats have exhibited strong tumor cytotoxicity in vitro and in vivo. However, effective production of bispecific antibodies remains challenging for the majority of bispecific antibody formats. In the present study, a bispecific antibody was designed that links a conventional antigen‑binding fragment (Fab) against cluster of differentiation 3 antigen (CD3) to a camel single domain antibody (VHH) against human epidermal growth factor receptor 2 (HER2). This bispecific antibody may be secreted and purified efficiently from Escherichia coli culture medium. The purified bispecific antibody is able to trigger T cell‑mediated HER2‑specific cytotoxicity in vitro and in vivo. The data gathered in the present study suggest that this bispecific format may be applied to other tumor antigens to produce bispecific antibodies more efficiently.

Introduction

Human epidermal growth factor receptor 2 (HER2), is overexpressed in ~25% of patients with metastatic breast carcinoma and a number of other human cancer types, including gastric, lung, ovarian, bladder and kidney carcinomas (1-3). As a therapeutic target for HER2-overexpressing cancer (4), monoclonal antibodies have been developed to target HER2-positive tumors (5-7). For example, trastuzumab and pertuzumab have already been approved clinically for HER2-positive breast cancer (8,9). Trastuzumab and pertuzumab are able to directly inhibit HER2 activities and induce antibody-dependent cell-mediated cytotoxicity. The two antibodies may increase the survival time when combined with chemotherapy in patients with HER2-overexpressing breast cancer (10-12). However, for the majority of patients with metastatic breast cancer, the tumors eventually resist trastuzumab, and certain patients do not respond to treatment even with HER2 overexpression (13,14).

In order to improve the therapeutic effect of antibodies, a number of approaches have been studied, including antibody conjugate TDM1 (15). Another approach is to directly engage immune cells to attack tumor cells. As T cells serve an important function in the killing of tumor cells (16-20), bispecific antibody that recruits T cells to kill tumor cells is of interest and has been investigated for cancer therapy (21-23). For example, blinatumomab, a bispecific T cell engager antibody (BiTE), has been approved for the treatment of B-cell leukemia (23). Numerous bispecific antibodies targeting different tumor biomarkers, including HER2, have also been reported (24-27).

The present study reports on a T-cell engaging bispecific antibody, cluster of differentiation (CD)3-S-Fab, which targets HER2 tumor cells. Distinct from previous studies (28-30), CD3-S-Fab was designed by linking a camel anti-HER2 single-domain antibody (VHH) to the C-terminal of a conventional anti-CD3 antigen-binding fragment (Fab). The CD3-S-Fab may be expressed and purified from Escherichia coli. To improve the purification process, different expression and purification schemes were tested, and it was identified that CD3-S-Fab may be secreted and purified directly from E. coli medium with high efficiency. The purified CD3-S-Fab is able to recruit T cells to kill HER2-positive tumor cells specifically. The data gathered in the present study demonstrate that CD3-S-Fab may present a feasible approach to produce bispecific antibodies on a large scale.

Materials and methods

Plasmids. To make the CD3-S-Fab bispecific antibody, the VH-CH1 and VL-CL of anti-CD3 UCHT1 clone (31)
were synthesized (Genscript Biotech., Nanjing, China). The VH-CH1 was cloned into the pET26b plasmid (Addgene, Inc., Cambridge, MA, USA) through restriction enzyme cutting site NcoI and BamHI (Fig. 1A). The VL-CL of anti-CD3 UCHT1 was linked with the single domain anti-HER2 VHH (8), and then cloned into the pET21a (Addgene, Cambridge, MA, USA) through restriction enzyme cutting site NcoI and Xhol (Fig. 1A). The plB signal sequence (5'-ATGAAATACCTGTGCAGG ACCGCTGCTGTGGTCTGCCTCTGCTGCCCA GCGGCCAATGGCATTGG-3') was synthesized (Genscript Biotech., Nanjing, China) and added to the N-terminals of the two constructs for periplasmic expression (32,33). A Flag-tag or His-tag (Genscript, Nanjing, Jiangsu, China) was added to the C-terminals for easy detection.

**Bispecific antibody expression and purification.** In order to purify the CD3-S-Fab protein, *E. coli* BL21(DE3) competent cells were transformed with the two plasmids encoding VH-CH1 and VL-CL-HER2VHH. Briefly, competent cells and plasmids were mixed and incubated at 42˚C for 45 sec, then cooled on ice for 2 min. After incubating cells for 1 h (37˚C), cells were spread on lysozyme broth (LB) plates and incubated at 37˚C for 12 h. For periplasmic expression, the bacteria were cultured in (LB) medium (10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl; Sangon Biotech; Shanghai, China) with antibiotics (0.1 g/l Ampicillin plus 0.05 g/l Kanamycin) at 37˚C until the optical density at a wavelength of 600 nm (OD600) (measured by NanoDrop2000; Thermo Fisher Scientific, Inc.) Next, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce protein expression, and cell growth was continued for an additional 20 h at 16˚C or 4 h at 37˚C using constant rotary incubator (Zhicheng Inc; Shanghai, China) at 180 rpm. Periplasmic protein purification was performed as described previously (34). Briefly, cells were harvested with centrifugation at 4,000 x g for 30 min at 4˚C and the cell pellet was resuspended in a chilled sucrose solution (20 mM Tris-HCl pH 8.0; 25% (w/v) sucrose; 1 mM EDTA). Following incubation on ice for 15 min with occasional agitation, the suspension was then centrifuged at 8,500 x g for 20 min at 4˚C. The supernatant was collected as the sucrose fraction. The cells were resuspended again and incubated in chilled periplasmic solution (5 mM MgCl2) for an additional 30 min. Following centrifugation (20,000 x g, 4˚C for 30 min), the supernatant was collected as the periplasmic fraction.

To test the secreted expression, M9 minimal medium (Sangon Biotech Co., Ltd., Shanghai, China) was used as described previously (32,35). Briefly, the bacteria transformed with the two plasmids were cultured in LB medium with antibiotics at 37˚C. The culture was then transferred to M9 minimal medium (12.8 g/l NaHPO4, 3.0 g/l KH2PO4, 0.5 g/l NaCl, 2.0 g/l NH4Cl, 20 g/l glucose, 0.1 mM CaCl2, 1.0 mM MgSO4 and 10 µM FeCl3), and incubated at 37˚C and 220 rpm in a rotary shaker. When the cell culture reached an OD600 of 2.7-2.9, IPTG (final concentration, 1 mM) and Tris-HCl (final concentration, 180 mM) were added to induce protein expression and secretion. Following culture for another 24 h at 16˚C and 220 rpm in a rotary shaker, the cells were removed by centrifugation (4,000 x g, 4˚C, 30 min) followed by 20,000 x g, 4˚C, 30 min) and the supernatant was recovered and processed for purification as follows:

CD3-S-Fab was purified from the combined sucrose and periplasmic fractions or protein containing medium using Ni-NTA agarose (cat. no.: NINTA-300; Molecular Cloning Laboratories, San Francisco, CA, USA) via a C-terminal His8-Tag. Purified protein was then further analyzed by SDS-PAGE. Briefly, 10 µg per protein sample was separated on 12% SDS-PAGE gel under reducing conditions by adding 2 µM 2-mercaptoethanol, then the gel was stained by coomassie brilliant blue solution for 1 h at room temperature (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After destaining, the gel with water 3 times for 5 min each, the gel was photographed by ChemiDoc XRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The concentration of purified protein was determined by NanoDrop2000 (Thermo Fisher Scientific, Inc.).

**Cell lines and animals.** All cell lines, namely CHO, SKBR-3 and LS174T (HER2+) cells, and Jurkat T cells, were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SKBR-3 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. LS174T, CHO and Jurkat cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% HI-FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. All cells were incubated at 37˚C in a humidified incubator with 5% CO2.

A total of 10 of non-obese diabetic-severe combined immunodeficiency disease (NOD/SCID) mice (female, ~18 g, 6-week-old) were purchased from Beijing Vital River Laboratory Animal Technology, Co, Ltd. (Beijing, China) and housed in the Animal Experiment Center of Sun Yat-Sen University (Guangzhou, China) under sterile and standardized environmental conditions (20-26˚C room temperature, free access to food and water, 40-70% relative humidity and 12 h light-dark cycle). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University.

**Flow cytometry analysis.** Flow cytometry analysis was performed as described previously (8,29). Briefly, aliquots of 1×106 cells were collected and mixed in ice-cold PBS with 0.2% bovine serum albumin (BioTeK China, Beijing, China) in the absence or presence of CD3-S-Fab (final concentration of 50 µg/ml). The mixture was then incubated on ice for 1 h, followed by washing twice with ice-cold PBS. The cells were then incubated on ice for 1 h with goat-anti-human immunoglobulin (IgG (H+L)-AF488 (1:200, cat. no. A11013; Invitrogen; Thermo Fisher Scientific, Inc.) as the secondary antibody. The cells were also incubated with anti-CD3-FITC antibody (5 µl/test, BioLegend, Inc., San Diego, CA, USA; cat. no. 317306), anti-HER2-PE antibody (5 µl/test, cat. no. 340552; BD Biosciences, Franklin lakes, NJ, USA) or goat-anti-human IgG (H+L)-AF488 (1:200, cat. no. A11013; Invitrogen; Thermo Fisher Scientific, Inc.) on ice for 1 h. After the cells were washed twice by cold PBS, flow cytometry analysis was performed by
Isolation of T cells from peripheral blood mononuclear cells (PBMCs). Human PBMCs were retrospectively obtained from healthy donors from the Guangzhou Blood Centre (Guangzhou, China), which provided informed consent (approval no. SYSU-2015-289) using Ficoll-Paque PLUS (cat. no. 17-1440-03; GE Healthcare, Chicago, IL, USA) density centrifugation, as described previously (36). The use of the cells was approved by the Health and Family Planning Commission of Guangdong Province. In brief, 25 ml two-fold diluted peripheral blood from healthy donors was layered on 15 ml Ficoll-Paque PREMIUM and centrifuged at 400 x g for 30 min at room temperature. PMBCs were collected and washed three times with PBS. T cells were then enriched from PBMCs using an EasySep™ Human CD3 Positive Selection kit (Stemcell Technologies, Inc., Vancouver, BC, Canada) as described previously (37). The isolated T cells were cultured in complete RPMI 1640 with 10% FBS and 1% penicillin/streptomycin at 37˚C prior to usage. 

Cytotoxic assays. Cytotoxicity assays were performed as described previously (29). Briefly, SKBR3, LSI74T and CHO cells were used as target cells. T cells without prior stimulation were used as effector cells. A total of 5,000 cells/well of target cells (100 µl) was plated in 96-well plates in triplicate. Following a 6-24 h incubation period, an equal volume of CD3+ T cells (50,000 cells/well) or complete RPMI 1640 medium were added to each well. The CD3-S-Fab and Trastuzumab (a gift from Alphamab, Suzhou, China), which is an approved monoclonal antibody to treat HER2 positive patients with breast cancer, ranging from 1.56x10^2 to 1.56x10^-5 nM, were then added. After 72 h of incubation, cell viability was quantified using cell counting kit (cat. no. CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The survival rate (%) of target cells was calculated using the following formula: [(live target cells (sample)‑medium)/(live target cells (control)‑medium)] x 100.

In vivo efficacy studies. In vivo efficacy studies were performed as described previously, with modifications (18,38). Briefly, HER2-positive SKOV3 cells were harvested and then mixed with freshly isolated human PBMCs. Cell suspensions were injected subcutaneously into the right flank of NOD/SCID mice in a total volume of 0.2 ml/mouse (mixtures of 2x10^6 SKOV3 cells and 1x10^7 human PBMCs). The mice were grouped into control group (PBS) and treatment group (CD3-S-Fab) randomly, 5 mice per group. The first antibody treatment (1 mg/kg) was at 2 h post-transplantation. The animals were then treated daily (1 mg/kg) over the following 7 days. Tumor volume was measured daily. Mice were sacrificed when the tumor volume reached 1,500 mm^3. All results are presented as the mean ± standard error of the mean unless otherwise noted. 

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analysis was performed using Student's t-test, except for the T cell-mediated cytotoxicity assay, in which two-way ANOVA followed by Dunnett's multiple comparisons test was employed. A non-linear regression analysis was used in Fig. 3B-E. P<0.05 was considered a statistically significant difference. Data are presented as the mean ± standard error of the mean unless otherwise noted.
Results

CD3-S-Fab may be secreted and purified from E. coli culture medium. CD3-S-Fab was designed by genetically linking an anti-HER2 VHH at the c-terminal of anti-CD3 VL-CL (Fig. 1A). Anti-CD3 VH-CH1 and anti-CD3 VL-CL-VHH were cloned into pET26b and pET21a, respectively. The pelB signal peptide was added to the N-terminal of the two constructs for periplasmic expression and secretion in E. coli. The CD3-S-Fab was formed via the heterodimerization of VH-CH1/VL-CL-VHH (Fig. 1A).

Periplasmic purification was tested first by adjusting the IPTG concentrations and culture temperature. The optimal expression with improved solubility of CD3-S-Fab was achieved by lowering the induction temperature (0.1 mM IPTG, 16°C induction for 24 h; data not shown). However, the yield of CD3-S-Fab remained low with a yield of ~0.4 mg per 6 liters LB medium following Ni-NTA affinity purification.

To increase the yield of CD3-S-Fab, extracellular expression of CD3-S-Fab was tested (39,40). Compared with the periplasmic expression, the yield of CD3-S-Fab recovered from the M9 medium was ~0.6 mg per 200 ml medium. The secreted CD3-S-Fab was also able to be purified by Ni-NTA-agarose affinity chromatography as heterodimers (Fig. IC). Thus, CD3-S-Fab may be secreted and purified from E. coli culture medium.

Figure 2. Purified S-Fab can recognize T cells and HER2 positive cells. (A) Flow cytometry analysis with CD3-S-Fab (black line), positive control anti-CD3-FITC (dash line) or staining with only anti-human IgG-AF488 staining (dotted line), on CD3-negative CHO cells (left panel) and CD3-positive Jurkat cells (right panel). (B) Flow cytometry analysis with positive control anti-HER2-PE (A fluorescent protein) (dash line, left panels), CD3-S-Fab (black line, right panels) or staining with only anti-human IgG-AF488 (dotted line), on HER2-negative CHO cells (top panel) and HER2-positive cell lines, LS174T and SKBR3 (bottom panels). HER2, human epidermal growth factor receptor 2; Ig, immunoglobulin; CD, cluster of differentiation.
CD3-S-Fab binds CD3- and HER2-positive cells. In order to confirm whether CD3-S-Fab maintains the ability to bind CD3-positive T cells, flow cytometry analysis was conducted using CD3-positive Jurkat cells and CD3-negative CHO cells. CD3-S-Fab was not able to bind CHO cells based on flow cytometry analysis (Fig. 2A), but was able to bind Jurkat cells (Fig. 2B), suggesting that CD3-S-Fab may bind human T cells.

To confirm the binding of CD3-S-Fab to HER2-positive tumor cells, HER2-positive cell lines, SKBR3 and LS174T, and the HER2-negative cell line CHO, were used for flow cytometry analysis. Flow cytometry analysis revealed that CD3-S-Fab did not bind to CHO cells, but that it did bind to SKBR3 and LS174T cells (Fig. 2B, right panels). These data suggest that CD3-S-Fab is able to specifically bind to HER2-positive cells.

CD3-S-Fab has T-cell-mediated cytotoxicity against HER2-positive cells. In order to evaluate whether CD3-S-Fab has T-cell-mediated cytotoxicity against HER2-positive cells, in vitro experiments were conducted. Tumor cells (5,000 cells/well) alone or with T cells (50,000 cells/well) were incubated together for 72 h in the presence of the indicated concentrations of CD3-S-Fab (1 or 10 µg/ml). Cytotoxic activity was measured as described in the Materials and methods section. Data are presented as the mean ± SEM (n=3, ***P<0.001 vs. tumor cells + T cells, two-way analysis of variance followed by Dunnett’s multiple comparisons test). Dose response measurement of CD3-S-Fab with CHO (circle), LS174T (square) and SKBR-3 (triangle) cells. Dose-response curves were assessed using a non-linear regression, log (inhibitor) vs. response using GraphPad Prism software. (B) in the absence of T cells with different concentrations of CD3-S-Fab, (C) in the presence of T cells with different concentrations of CD3-S-Fab, (D) in the absence of PBMCs with different concentrations of Trastuzumab, and (E) in the presence of PBMCs with different concentrations of Trastuzumab. All measurements were normalized against tumor cells only; data points in the figure represent the mean of three samples and error bars represent the SEM. SEM, standard error of the mean; PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation.

CD3-S-Fab binds CD3- and HER2-positive cells. In order to confirm whether CD3-S-Fab has T-cell-mediated cytotoxicity against HER2-positive cells, in vivo experiments were conducted. Non-obese diabetic-severe combined immunodeficiency disease mice (n=5/group) were engrafted subcutaneously with SKOV3 cells and human PBMCs. The mice were then treated with PBS (solid square, PBMC transplant) or CD3-S-Fab (solid circle, PBMC transplant). The data represent the mean tumor volume of 5 mice. Error bars represent standard error of the mean (**P<0.01 vs. PBS group, paired t-test). PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation.
is able to mediate HER2 tumor cell killing. HER2-positive and HER2-negative cell lines were used. CD3-S-Fab did not lead to cytotoxicity in the HER2-negative cell line CHO (Fig. 3A and B). For the HER2-positive cell lines LS174T and SKBR3, T cells alone or CD3-S-Fab alone have no effects on cell viability (Fig. 3B). However, CD3-S-Fab induced potent cytotoxicity when the LS174T and SKBR3 cells were incubated with CD3-S-Fab and T cells (Fig. 3A). Cell number and morphology observed under microscopy also confirmed the specific killing in the presence of CD3-S-Fab and T cells.

To further evaluate the cytotoxic activity of CD3-S-Fab on tumor cells, the dose responses of different cell lines were measured. No cell killing was observed for CD3-S-Fab in the absence of T cells (Fig. 3B). With T cells present, CD3-S-Fab exhibited active cell killing in HER2-positive LS174T cells and SKBR3 cells (Fig. 3C). This is distinct from Trastuzumab, which demonstrated partial inhibition in the absence of PBMCs (Fig. 3D), and higher cell killing in the presence of PBMCs (Fig. 3E). These results suggest that CD3-S-Fab exhibits potent cytotoxic activity against HER2-positive tumor cells in the presence of T cells.

CD3-S-Fab inhibits tumor growth in vivo. To analyze the in vivo antitumor effect of CD3-S-Fab, SKOV3 cells were mixed with freshly isolated human PBMCs and engrafted subcutaneously into NOD/SCID mice. The mice were then treated with either PBS or CD3-S-Fab. Compared with animals only treated with PBMCs, significant tumor growth inhibition was observed in mice treated with CD3-S-Fab (Fig. 4). Minimal tumor growth was observed in mice treated with CD3-S-Fab, even 5 weeks after treatment ended. These data demonstrated that CD3-S-Fab was able to inhibit HER2-positive tumor growth in xenograft mice.

Discussion

Cancer immunotherapy has demonstrated lasting clinical benefits in patients with cancer (41). Besides checkpoint antibodies, a variety of approaches have been actively studied as cancer immunotherapies. Among them, bispecific antibodies have demonstrated some promise. For example, blinatumomab, a BiTE, has already been approved for the treatment of B-cell leukemia, with excellent efficacy (18-21,23,42).

HER2 is one of the most studied oncogenes. Antibodies or small molecule inhibitors have exhibited clinical efficacy by inhibiting HER2 activity. Besides functioning as an oncogene, HER2 also presents as an excellent tumor antigen, as it is overexpressed in numerous tumors and is rarely expressed in normal tissues (4,8,43-46). Vaccines, bispecific antibodies and other approaches have been studied to further improve the clinical outcomes of current HER2 therapeutics.

Different formats of anti-HER2 bispecific antibodies have been studied previously (15,25,28,45,47,48), including recruitment of T cells in various bispecific antibody formats (17,19,49,50). However, those bispecific antibodies present a number of challenges, including a mixed population during purification, a low yield of production, a tendency to aggregate and a short half-life. Previously, it was demonstrated that an S-Fab bispecific antibody against carcinoembryonic antigens demonstrated several advantages, including excellent efficacy, and reasonable expression and solubility in E. coli (29). In the present study, CD3-S-Fab, the bispecific antibody targeting HER2, is described. The purified bispecific antibody CD3-S-Fab can be used for the redirection of T cells toward HER2-positive tumor cells, and was demonstrated to be efficient in vitro and in vivo at killing HER2-positive cancer cells.

Although CD3-S-Fab may be produced in E. coli, the yield of CD3-S-Fab was very low based on purification from periplasmic fractions. Recombinant antibodies are commonly produced by eukaryotic cells or periplasmic expression of gram-negative bacteria (8,18,21,35,50,51). Due to the easy culture and low cost, E. coli has been widely used as an expression host for recombinant proteins. However, the high yield of correctly folded proteins is a frequent problem, and the purification process of the recombinant proteins is also complicated. In the present study, the defined M9 medium was used to facilitate the secretion of CD3-S-Fab complex into the medium, and then the recombinant antibody was purified directly from this medium. This purification schedule greatly increased the yields of CD3-S-Fab.

Compared with the intracellular expression of recombinant proteins, extracellular expression exhibits several advantages, including the following: i) It is efficient at obtaining correctly folded proteins; ii) the secreted proteins are less likely to be degraded by various proteases in the periplasm; iii) secretion reduces the cellular burden for cell growth when a large amount of recombinant protein is produced; and iv) The process of purification is easier due to the elimination of cellular component contamination (33,40,52,53).

In summary, the novel bispecific antibody CD3-S-Fab can be used for the redirection of T cells toward HER2-positive tumor cells and is efficient at killing HER2-positive cancer cells in vitro and in vivo. The easy purification and high yield of CD3-S-Fab suggests this format may be applied to other bispecific antibodies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL, Lli, CZ, JL, Jliu, RS, and BD, performed the experiments. LL, QL and ZW designed the experiments, and wrote the manuscript.
Ethics approval and consent to participate

The use of animals was approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University (Guangzhou, China). (Approve No. IACUC-DD-18-02-01). The use of human blood was approved by Health and Family Planning Commission of Guangdong Province (approval no. SYSU 2015-289).

Consent for publication

The PBMCs were from provided from Health and Family Planning Commission of Guangdong Province (SYSU 2015-289) with consent from healthy donors.

Competing interests

The authors declare that they have no competing interests.

References


