A T-cell-engaging B7-H4/CD3 bispecific Fab-scFv antibody targets human breast cancer

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**Translational relevance**

Despite the clinical success of immune checkpoint blockade antibodies against advanced cancers, the response rate is approximately 20-40% even for PD-L1-positive cancers and most cancer patients are unlikely to benefit from treatment. Other therapeutic antibodies have been developed and evaluated in clinical trials in combination with anti-PD-1 antibodies.

In this study, we manufactured anti-B7-H4/CD3 bispecific antibodies (BsAbs) based on the Fab and scFv structure. We found that the B7-H4/CD3 BsAb had potent cytotoxic activity against B7-H4-positive breast cancer cell lines *in vitro*, and *in vivo* using a MHC-dKO NOG mouse model. Because B7-H4 is highly expressed independently of HER2 or PD-L1 expression in breast cancers obtained by the High-tech Omics-based Patient Evaluation for Cancer Therapy (HOPE) project, the B7-H4/CD3 bispecific antibody may be a good therapeutic tool for immune checkpoint blockade or anti-HER2 antibody-unresponsive cancer patients.

**ABSTRACT**
**Purpose:** The B7 homolog 4 (B7-H4, *VTCN1*) is an immune checkpoint molecule that negatively regulates immune responses and is known to be overexpressed in many human cancers. Previously, we generated a mouse anti-human B7-H4 monoclonal antibody that did not have a significant antitumor effect *in vivo* probably because of molecule instability. In this study, we designed a B7-H4/CD3 bispecific antibody (BsAb) and investigated its antitumor activity *in vitro* and *in vivo* using a humanized mouse model.

**Experimental Design:** Complementary DNAs of the antibody-binding fragment (Fab)-single-chain variable fragment (scFv) and scFv-scFv of the anti-B7-H4/CD3 BsAb were synthesized, and the BsAb antibodies were produced in HEK293 cells. The anti-tumor activity against human breast cancer cells by human peripheral blood mononuclear cells (hPBMC) with BsAb was measured by lactate dehydrogenase (LDH) release *in vitro*, and *in vivo* using hPBMC transplanted major histocompatibility (MHC) class I and class II-deficient NOG mice.

**Results:** hPBMCs with anti-B7-H4/CD3 BsAbs successfully lysed the human breast cancer cell line MDA-MB-468 (EC50: 0.2 ng/ml) and other B7-H4-positive cell lines *in vitro*. When BsAb was injected in a humanized mouse model, there was an immediate and strong antitumor activity against MDA-MB-468, HCC-1954 and HCC-1569 tumors and CD8$^+$ and granzyme B$^+$ CTL infiltration into the tumor, and there were no adverse effects after long-term observation. CD8$^+$ T cell depletion by an anti-CD8 antibody mostly reduced the antitumor effect of BsAb *in vivo*.

**Conclusions:** An anti-B7-H4/CD3 bispecific antibody may be a good therapeutic tool for patients with B7-H4-positive breast cancers.

**INTRODUCTION**
Because of the recent success of immune checkpoint blocking antibodies, clinical trials are underway to evaluate their efficacy in various cancers (1-4). However, a majority of cancer patients are unlikely to benefit from anti-programmed death-1 (PD-1)/PD-ligand 1 (PD-L1) antibody treatment because the response rate is approximately 20-40% even in PD-L1-positive cancers.

In addition to these immunomodulatory receptor blockade therapies, other modulating technologies have been developed (5,6). Major histocompatibility complex (MHC) and T cell receptor (TCR) bypassed T cell cytotoxicity was first reported in 1985 (7), and over the past 3 decades, CD3 bispecific antibodies (BsAbs) and chimeric antigen receptor (CAR) T cells have been developed (8, 9, 10).

Recently, two BsAbs catumaxomab (11) and blinatumomab (12, 13) were approved by the FDA, and more BsAbs directly engaging immune cells against tumor cells are now in clinical studies. Improvements in protein engineering technology have enabled the creation of various types of artificial antibodies with greater flexibility in design, size, specificity, half-life and distribution, and dozens of BsAb formats have been proposed, (6, 14).

B7 homolog 4 (B7-H4, VTCN1) is considered to be a negative regulator of immune responses and is overexpressed in many human cancers, which indicates that B7-H4 might be a potential target for cancer therapy. B7-H4 expression is reported to be affected by the tumor microenvironment (15, 16). Previously, we generated an anti-human B7-H4 monoclonal antibody that induced T cell cytotoxicity to a B7-H4 positive breast cancer cell line in an in vitro indirect ADCC system, but the antibody did not suppress the tumor growth in a mouse model (17).
In the present study, we manufactured B7-H4/CD3 bispecific antibodies based on the Fab and single-chain variable fragment (scFv) structure of anti-B7-H4 and anti-CD3 monoclonal antibodies using a human cell-based protein expression system. We found that the anti-B7-H4/CD3 Fab-scFv antibody had potent cytotoxic activity against a B7-H4-positive breast cancer cell line \textit{in vitro}, and \textit{in vivo} using a MHC-double knockout (dKO) NOG mouse model (18). Here, we revealed that the anti-B7-H4/CD3 BsAb might contribute to the development of novel therapeutic antibodies against solid tumors.

**MATERIALS AND METHODS**

**Gene expression profile analysis**

A comprehensive gene expression analysis was performed as previously described in the HOPE (High-tech Omics-based Patient Evaluation) project at the Shizuoka Cancer Center (19). Briefly, the tumor tissues samples were dissected from fresh surgical specimens, and the RNA samples with an RNA integrity number (RIN) $\geq 6.0$ were used for the microarray analysis. The RNA was amplified, labeled, and hybridized to the Sure Print G3 Human Gene Expression 8 $\times$ 60 K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA) and Microsoft Excel software. The data analysis was performed using GeneSpring GX software (Agilent Technologies). Ethical approval for the study was obtained from the institutional review board at the Shizuoka Cancer Center. Written informed consent was obtained from all the enrolled patients. All the experiments using clinical samples were carried out in accordance with the approved guidelines.
Cell lines

Human breast cancer cell lines (MDA-MB-468, MDA-MB-231, ZR75, SKBR3, HCC-1954 and HCC-1569) and the lung adenocarcinoma cell line (NCI-H2170) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were maintained in RPMI1640 (SIGMA, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA). Human mammary epithelial cells (HMEC) were purchased from Lonza Ltd. (Basel, Switzerland), and were cultured in the growth medium MEBM-CC3150 (Lonza Ltd.).

Flow cytometry and antibodies

The mouse anti-human B7-H4 antibody (clone #25) was established in-house as previously described (17). Briefly, the human B7-H4 isoform 1 extracellular domain was constructed and produced in the Expi293 expression system (Life Technologies Corp.) and was immunized in BALB/cA mice. An antibody secreting hybridoma was generated and was screened by a common method using the mouse myeloma cell line P3X63ag8.653 (ATCC). The flow cytometric analyses were carried out on a FACS Canto (BD Biosciences). The human mammary epithelial cells (HMECs) and cancer cell lines were incubated with the anti-B7-H4 monoclonal Ab (clone #25) and were later incubated with a PE-labeled polyclonal anti-mouse Ig Ab (BD Biosciences) on ice. The following antibodies were used for flow cytometric analysis of the in vivo experiments using humanized mice. For the human cell labeling, the anti-CD3-PerCP (HIT3a), anti-CD4-PE or anti-CD4-PE-Cy5 (RPA-T4), anti-CD8-PE-Cy5 (HIT8a), anti-CD11b-PE-Cy7 (ICRF44), anti-CD14-PerCP (MφP9), anti-CD19-APC (HIB19), anti-CD25-FITC (M-A251), anti-CD33-PE (WM53), anti-CD45-FITC (2D1),...
anti-CD45RA-FITC (HI100), anti-CD45RO-APC (UCHL1), anti-CD56-PE (B159), and
anti-CD127-PE-Cy7 (A019D5) were purchased from BD Pharmingen (San Jose, CA,
USA). The anti-FoxP3-PE (hFOXY) antibody was purchased from eBioscience, Inc.
(San Diego, CA, USA). The anti-TIM3-PE (F38-2E2) and anti-LAG3-FITC (17B4)
antibodies were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany) and
Adipogen (San Diego, CA). The anti-mouse CD45 antibody used to label the mouse
cells was purchased from BD Pharmingen. The anti-PD-1-APC (EH12.2H7) and
anti-Ki67-PE-Cy7 (Ki-67) antibodies were purchased from BioLegend Inc., San Diego,
CA. The splenocyte and peripheral blood cells were isolated using ACK lysis buffer.
Tumor-infiltrating lymphocytes (TILs) were also separated from the control or
antibody-treated tumors by anti-human CD45-microbeads (Miltenyi Biotec) using the
autoMACS system (Miltenyi Biotec). The staining method was previously described
(16). The human cells were identified by gating human CD45^+ fractions.

**Production of the B7-H4/CD3 BsAb**

The mouse anti-human B7H4 monoclonal antibody clone #25-derived VH and VL
genomes were cloned, and construct containing Fab (B7-H4)-scFv (CD3) and scFv
(B7-H4)-scFv (CD3) linked by a (Gly4Ser)3 linker and 6× histidine-tag was designed
(Fig. 2A). These cDNAs were chemically synthesized and cloned into the expression
vector pcDNA3.3. The B7-H4/CD3 BsAbs were produced using the Expi293
expression system (Gibco, Thermo Fisher Scientific) at a ratio of 3:7 (VH-containing
long fragment:VL-containing short fragment) in the Fab-scFv format, purified with a
histidine-tag affinity column and used for experiments.
**In vitro BsAb/hPBMC cytotoxicity assay**

Human peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy volunteers or glioma patients as effector cells using Ficoll-Paque PLUS (GE Healthcare UK Ltd). The effector cells were incubated with cancer cell lines or HMEC at an effector/target (E/T) ratio ranging from 1.25 to 40 in the presence of various concentrations of Fab-scFv or scFv-scFv B7H4/CD3 BsAb at 37°C for 16 h or 24 h in a 5% CO₂ atmosphere. The supernatant from the cultures were collected and measured using a lactate dehydrogenase (LDH) cytotoxicity assay kit (Takara Bio Inc., Seta, Shiga, Japan). The percentage of specific lysis was determined by the following formula: percentage of specific lysis = [(effector cells and target cells and agent release – effector cells release) - spontaneous target cell release]/(maximal target cells release – spontaneous target cells release) × 100. The 50% effective concentration (EC₅₀) was calculated using a 4-parameter logistic curve fitting using ImageJ (ver. 1.51J8, National Institutes of Health, USA). The effector T cell subsets were isolated or depleted from the healthy volunteer PBMCs by an autoMACS magnetic cell isolator (Miltenyi Biotec), and secreted Granzyme B and IFN-γ in culture supernatants were measured by an ELISA kit (MABTECH AB, Sweden, BioLegend, San Diego, USA).

**Animal experiments**

The MHC-dKO NOG mice were kindly supplied by Dr. Mamoru Ito at the Central Institute for Experimental Animals (Kawasaki, Japan). All the animals were cared for and treated according to the Guidelines for the welfare and use of animals in cancer research, and the experimental procedures were approved by the Animal Care and Use.
Committee of Shizuoka Cancer Center Research Institute. The clinical experiments using the PBMCs derived from glioma patients and healthy volunteers were approved by the Institutional Review Board of Shizuoka Cancer Center, Shizuoka, Japan. All the patients provided written informed consent.

**In vivo imaging in the tumor-transplanted NOG-MHC dKO mice**

For *in vivo* imaging, all the tumor-transplanted MHC-dKO NOG mice were supplied with a low-fluorescence feed for more than one week. Cy5.5 labeling of the Fab anti-B7-H4/CD3 antibody was performed using a Cy5.5 labeling kit (GE Healthcare UK Ltd, Buckinghamshire England). Cy5.5-labeled Fab-scFv B7-H4/CD3 BsAb localization was performed using the Optix MX2 laser scanner system (ART, Advanced Research and Technologies) with an excitation at 670 nm and an emission at ≥700 nm. The Cy5.5-labeled B7-H4/CD3 antibody was injected intravenously and imaging was performed at sequential time points ranging from 24 h to 28 days.

**BsAb pharmacokinetics in the BALB/cA mice**

In the pharmacokinetic study of anti-B7-H4/CD3 BsAb, 5 nine-week-old BALB/cA mice were injected with 100 μg of Cy5.5-labeled Fab-scFv anti-B7-H4/CD3 BsAb via tail vein, and then blood was drawn at time points ranging from 2 min to 48 h after the antibody injection. The Serum samples were stored at -80°C until the BsAb concentrations were measured by a sandwich ELISA or fluorescence intensity. The sandwich ELISA was performed using the recombinant B7-H4 extracellular region and an HRP labeled polyclonal anti-human Ig antibody (GE health care). The serum BsAb concentration was also determined by the fluorescence intensity levels using the Optix...
MX2 imager and was performed using ART Optix Optiview software (ART, Advanced Research and Technologies).

**In vivo study using the humanized mouse model**

The humanized MHC-dKO NOG mice production method was reported previously (20). Briefly, eight-week-old MHC-dKO NOG mice were irradiated with X-rays, and 1 × 10⁷ human PBMCs from glioma patients were intravenously administered to each mouse via the tail vein. The study design for the experiment evaluating the mice treated with the Fab-scFv B7H4/CD3 BsAb is shown in Fig. 5, Fig. 6 and supplementary Fig. S5. Four in vivo experiments were performed (dose-response, short- and long-term antitumor effect evaluation, and T-cell subset depletion). Specifically, we set the starting day of the antibody injection as day 0. As shown in Fig. 5A (the short-term antitumor effect experiment), on day -14, 1 × 10⁶ MDA-MB-468 human breast cancer cells (B7-H4 positive) or NCI-H2170 lung adenocarcinoma cells (B7-H4 negative) were subcutaneously injected into the fat pad or flank region of the mice. Starting on day 0, each antibody was administered intravenously. The antitumor activity was evaluated by measuring the tumor volume. The tumor volume was calculated based on the National Cancer Institute formula as follows: tumor volume (mm³) = length (mm) × [width (mm)]² × 1/2.

One week after the antibody injection, tumors, spleens, and blood were harvested from the groups. The tumors from one set of 3 mice were used for tumor-infiltrating lymphocyte (TIL) flow cytometry, immunohistochemistry (IHC) analysis, and real-time qPCR of the immune response-associated genes. The schema for the long-term antitumor effect evaluation and T cell subset depletion experiment are shown in Figs.
5B and 5C. For the T cell subset depletion in vivo, anti-CD4 and anti-CD8 monoclonal antibodies were purchased from Bio X Cell (West Lebanon, NH, USA).

In the dose-response experiment, as shown in Fig. 6, humanized MDA-MB-468 tumor-transplanted mice were administered BsAb sequentially in a dose escalating manner (0.2-200 µg). Two weeks after the start of Ab injection, the tumors were resected and used for IHC analysis.

Additional in vivo experiment targeting B7-H4-positive breast cancer cell lines, such as HCC-1954 and HCC-1569, using the humanized MHC-dKO NOG mice was performed in the same method as shown in supplementary Fig. S5C.

**Immunohistochemistry**

The xenografts were harvested one or two weeks after the injection of the anti-B7-H4/CD3 BsAb. Formalin-fixed paraffin-embedded (FFPE) tissue blocks and sections were made. The anti-B7-H4 antibody (clone #25 in-house made) (17), anti-CD8 (C8/144B) and anti-CD4 (4B12) antibodies (Thermo Fisher Scientific, Waltham, MA, USA), anti-granzyme B antibody (GrB-7, DAKO, Glostrup, Denmark), anti-FoxP3 antibody (236A/E7, Abcam, Cambridge, MA, USA), anti-CD204 antibody (SRA-C6, TransGenic Inc., Kobe, Japan), anti-PD-L1 antibody (28-8, Abcam) were purchased and used for immunohistochemistry analysis. The positively stained cell frequency was counted using the image-analyzing software, ImageJ (National Institutes of Health, USA) in randomly selected 1/3 areas of a tumor section whole image at 200× magnification. The necrotic area was excluded.

Human breast cancer paraffin embedded tissue arrays were purchased from US Biomax, Inc., Rockville, MD, USA (Cat#.BR1503f) and human normal and tumor paraffin tissue
arrays were purchased from BioChain Institute Inc., Newark, CA, USA and were used for IHC study using anti-B7-H4 monoclonal antibody #25.

**Statistical analysis**

For *in vivo* studies, the intergroup differences were assessed by a two-way ANOVA with a Shirley-Williams test. Significant difference in the positive cell frequency by IHC was assessed using a two-tailed unpaired Student’s *t*-test. The correlation between different gene expression levels was analyzed using a Spearman co-efficiency test. *P* values of < 0.05 were considered significant.

**RESULTS**

**B7-H4 expression in cancer tissues or cell lines**

A high expression of B7-H4 was frequently observed in breast and ovarian cancer and was partially observed in lung cancers from the HOPE project using 2527 surgically resected tumor tissues (Fig. 1A, Supplementary Fig. S1A). The positive rate of B7-H4 mRNA expression in breast cancers (more than 2-fold upregulation in tumors compared with normal tissues) was 56%. In contrast, PD-L1 expression was low in breast and ovarian cancer tissues. B7-H4 mRNA expression was detected in various types of cell lines, especially in breast cancer cell lines (Supplementary Fig. S2A) by qPCR, and B7-H4 protein expression was detected in 43.5% of breast cancer tissues by IHC using breast tumor tissue array (Supplementary Fig. S7). B7-H4 cell surface expression was observed on breast cancer cells and not on HMEC (Fig. 1B, Supplementary Fig. S2C and D).
Unstable B7-H4 surface expression is suggested in some reports (21, 22). For example, breast cancer cell line SKBR3 changed B7-H4 surface expression under confluent culture conditions (Supplementary Fig. S2B).

**Generation of anti-B7-H4/anti-CD3 bispecific antibodies**

Two types of recombinant bispecific antibodies were constructed from the novel anti-human B7-H4 monoclonal antibody clone #25 (17) and classical anti-CD3 antibody (clone: OKT3). The anti-B7-H4/CD3 Fab-scFv format that connected the antigen-binding fragment (Fab), including the human IgG4 consensus region 1, with the single chain antigen binding fragment variable (scFv) was constructed by a single short chain and a single long chain (Fig. 2A, B). Therefore, the 90 kDa molecular size was larger than albumin, which contributes to the prevention of renal leakage and to the stabilization of anti-B7-H4 affinity. The anti-B7-H4/CD3 scFv-scFv single chain bispecific format was constructed in a manner similar to BiTE (24). The Fab-scFv anti-B7-H4/CD3 BsAb labeled with Cy5.5 showed positive staining for B7-H4 in positive breast cancer cell lines by flow cytometry (Fig. 2C).

**PBMCs with anti-B7-H4/CD3 BsAb show cytotoxic activity against a B7-H4 positive breast cancer cell line in vitro**

BsAb-mediated crosslinking of B7-H4 on the target cell surface with CD3 on T cell causes effector T cell-dependent lysis of the target. The cytotoxic activity of the B7-H4/CD3 BsAbs with human PBMCs against MDA-MB-468 cells for scFv-scFv was 67 ng/ml (EC50). For Fab-scFv, it was 12 ng/ml after 16 h, and for Fab-scFv it was 0.23 ng/ml after 24 h in vitro (Fig. 3A). The Fab-scFv anti-B7-H4/CD3 antibody showed
antibody-dependent cytotoxicity on B7-H4-positive cells, whereas no cytotoxic activity was seen against B7-H4 negative cancer cell lines (Fig. 3B, supplementary Fig. 5B). There was no significant difference in cytotoxic activity (EC50 value) against MDA-MB-468 cells between healthy volunteer-derived PBMCs and glioma patient-derived PBMCs (data not shown). The anti-B7-H4/CD3 BsAb and volunteer PBMCs showed no cytotoxic effect on a normal mammary epithelial cell line (HMEC) (Fig. 3C). The BsAb did not kill target cells without the PBMCs (Fig. 3D, E). The effector cell killing activity was elicited by BsAb at a dose above 1 ng/ml against MDA-MB-468 cells after 16 h in vitro, and a decrease in the killing activity was observed under high concentrations of BsAb (Fig. 3C, D, E), which might be caused by a decrease in crosslinking of the target molecules because of BsAb saturation. Unexpectedly, positively isolated CD4+ as well as CD8+ T cell subsets showed strong cytotoxicity against B7-H4 positive tumor cell lines with granzyme B and IFN-γ secretion by the stimulation of BsAb. Interestingly, even CD4 and CD8 double negative T cells showed a weak cytotoxicity. However, granzyme B and IFN-γ were not produced (Fig. 3F, Supplementary Fig. S3A, B).

**Anti-B7-H4/CD3 BsAb pharmacokinetics in vivo**

BsAb accumulation at the MDA-MB-468 tumor site occurred within 24 h after the antibody injection, and the signal was detected even 28 days after the antibody injection. In contrast, specific antibody accumulation was not recognized in B7-H4-negative NCI-H2170 tumor (Fig. 4A and 4B, Supplementary Fig. S4). The BsAb concentration in the serum was determined by a recombinant B7-H4 and an anti-human-IgG-Ab sandwich ELISA, and the T1/2-beta was 8.5 h. Twenty-four hours after the 100 µg/body
BsAb injection, the serum concentration was estimated at 0.1 µg/ml (Fig. 4C), but the serum concentration by fluorescent imaging of the Cy5.5-labeled BsAb was approximately 1 µg/ml after 24 h (Supplementary Fig. S4), which was higher than the antibody level obtained by the sandwich ELISA method. The BsAb in the sera may partially exist in complex with other proteins.

**Antitumor effect of the anti-B7-H4/CD3 BsAb against MDA-MB-468 and other breast tumors**

A single injection of Fab-scFv B7-H4/CD3 BsAb, triple-negative breast cancer MDA-MB468 xenograft tumors decreased in size by about 70% in hPBMC-transplanted humanized NOG mouse model in a week (Fig. 5A). In the long-term experiment, the reduction in tumor size was maintained until 2 weeks after the BsAb injection (Fig. 5B). In contrast, the growth of the tumors treated with the full-body anti-B7-H4 antibody (clone #25) was not inhibited. Additionally, no antitumor effect was observed in B7-H4-negative NCI-H2170 tumors treated with BsAb (Fig. 5A). The BsAb also induced T cell cytotoxicity to HER2 positive and B7-H4 positive breast cancer cell lines, HCC-1954 and HCC-1569 *in vitro* (Supplementary Fig. S5A, B), and BsAb injection suppressed HCC-1954 and HCC-1956 tumor growth by more than 50% in the humanized mouse model (Supplementary Fig. S5C).

A relatively high dose of anti-CD3 antibody (clone: OKT3), formerly used in the clinic as an immunosuppressive agent, resulted in no inhibitory effect and obvious weight loss. The BsAb-treated mice showed no adverse effects such as weight loss. An escalating BsAb dose (0.2-200 µg/body) was administered without harm (Fig. 6A).

In the T cell subset depletion *in vivo* study, CD8+ T cell depletion blocked the growth
inhibition induced by B7-H4 BsAb, and CD4⁺ T cell depletion showed only a weak blocking effect on growth inhibition (Fig. 5C). These results demonstrated that the antitumor effect of anti-B7-H4 BsAb was mediated partially by CD4⁺ T cells and mostly by CD8⁺ T cells.

**Effector immune cells analysis of MDA-MB468 tumor-bearing mice treated with the anti-B7-H4/CD3 BsAb**

From the flow cytometry analysis of TIL and splenocytes from the antibody-treated mice, the total cell number and CD3⁺CD45⁺ T cell number from spleens showed a tendency to increase in the BsAb-treated mice compared with the controls (Supplementary Table 1), but there were no significant differences in the TIL T cell subset (data not shown).

In the Fab-scFv anti-B7-H4/CD3 BsAb-administered group, hematoxylin-eosin (HE)-stained tumor specimens showed remarkable infiltration of lymphoid cells inside the tumor cores and resulted in almost no viable cancer cells at the tumor site (Fig. 6B). Immunostaining revealed that CD8⁺ and granzyme B⁺ lymphocytes were more frequently observed in the BsAb-treated group (Fig. 6B, C), but CD4⁺ and FoxP3⁺ lymphocytes and CD204⁺ immune cells did not significantly differ (data not shown).

**IHC study of B7-H4 in human normal and tumor tissue arrays**

In 28 human normal and tumor tissues array analysis, seven B7-H4-positive tumors (pharynx, esophagus, stomach, lung, kidney, fallopian tube and kidney) were identified (Supplementary Fig. S6). In the normal tissues, only the tonsil (epithelial cell) was positively stained with the anti-B7-H4 antibody clone #25. The B7-H4 IHC study of
human breast cancer tissue array showed that a B7H4-positive rate of 69 breast cancer tissues was 43.5%, and there was a tendency of high B7H4 stain in triple negative tumor compared with PD-L1 (Supplementary Fig. S7). However, it was not definite because of small number of cases.

**DISCUSSION**

Because of the recent success of immune checkpoint blocking antibodies, such as ipilimumab and nivolumab, in patients with metastatic melanoma and other malignancies, clinical trials are underway to evaluate their efficacy in various solid cancers (1-4). These studies show that the modulation of the suppressed immune system is an effective way to combat cancers and that immunotherapy can be used to treat cancer.

In addition to immune checkpoint antibodies, bispecific antibodies that directly engage immune cells (25) are becoming another promising strategy in cancer antibody therapy. Advances in recombinant protein technology allow for the construction of bispecific antibodies in a variety of formats with great flexibility. A number of formats have been proposed including bispecific T cell engager (BiTE) (26, 27), tandem diabody (28), dual-affinity-retargeting (DART) (29), and antibody-TCR format (ImmTac) (30). Catumaxomab targeting EpCAM and Blinatumomab targeting CD19 are approved for clinical use and others are in various stages of clinical development (31-34).

New T cell-engaging bispecific antibodies, including BiTE and DART, work at a much lower dose in xenograft models and clinical use (24, 29) compared to conventional
antibody therapies. Our BsAb, which connects the anti-B7-H4 Ab and anti-CD3 Ab, immediately elicited a strong B7-H4 positive target tumor lysis at very low dose by CD8+ effector T cells or other T cell subsets, bypassing peptide-MHC/TCR recognition (Figs. 3 and 5).

Trastuzumab and other HER2-targeted agents improved patient outcomes in breast cancer therapy, but initially the responsive tumors develop resistance. HER2-targeted BsAb showed an antitumor potency against trastuzumab-resistant tumor cells in \textit{in vitro} and \textit{in vivo} models (33). Its effective concentrations (EC$_{50}$) was inversely correlated with surface HER2 expression and could be effective for tumors that express a lower level of HER2. Given that B7-H4 is frequently expressed on the breast cancer cells irrespective of HER2 expression (Supplementary Fig. S8) and that B7-H4 expression has a tendency to be higher in triple negative breast cancer cells (35), B7-H4 is potentially a novel substitute target for primary and recurrent breast cancers, including triple negative cancers that are nonresponsive to HER2 targeting therapies. Interestingly, our anti-B7-H4 BsAb showed potent anti-tumor effect \textit{in vivo} against B7-H4-positive breast cancer cell lines, HCC-1954 and HCC-1569, which was demonstrated to be basal type-HER2-positive and trastuzumab-resistant by some researchers (36, 37). This observation might suggest that this type of breast cancers with positive B7-H4 expression could be the target for B7-H4 targeting therapy.

Additionally, based on B7-H4 gene expression and the clinical data from the TCGA tumors samples, high B7-H4 gene expression can be a poor prognostic factor in breast cancer patients (Supplementary Fig. S1B).

B7-H4 is widely detected in cancers and normal tissues at the mRNA level, but its cell surface expression is limited and tends to be unstable (21, 22). Our IHC study showed a
limited expression in normal tissues and positive expressions in several tumor tissues other than breast cancer (Supplementary Fig. S6).

The BsAb eliminated breast cancer cells in a short amount of time (Fig. 5A, 6B), and the immediate antitumor response may help overcome cancers with continuous genomic evolution and immune evasions in contrast to the immune checkpoint blockade antibody therapy, which takes several weeks to induce an adequate amount of tumor antigen specific effector T cells at the tumor site.

Tumor specific antigens, such as carcinoembryonic antigens, cancer/testis antigens or differentiation antigens, are also expressed in normal tissues to varying degrees, except for mutation neoantigens. Therefore, the therapeutic approaches targeting these antigens requires antibody dose control for harnessing cytotoxic effector cells. B7-H4-targeted CAR-T cell treatment showed lethal toxicity including a delayed graft-versus-host-disease (GVHD) in a mouse model (23), and a clinical trial using trastuzumab-based CAR-T cells resulted in patient death and was aborted (38). These reported adverse events suggest that the strict regulation of effector function is important for clinical use. A shorter in vivo half-life of the smaller size BsAb is described as a negative property, but this characteristic enables easier control of effector cells for clinical use compared with CAR-T cell therapy.

Immune check point modulators are not effective for the majority of cancer patients, and positive outcome is likely to require a high mutation burden and effector T cell accumulation against mutation-derived neoantigens (39, 40). BsAb therapy may be an alternative anticancer immunotherapeutic strategy to bypass neoantigen-MHC/TCR recognition.
In this study, we demonstrated that the B7-H4-targeted bispecific antibody had potent antitumor activity \textit{in vitro} and \textit{in vivo}, which was specific for B7-H4-positive tumors and was not cytotoxic on a normal mammary duct epithelial cell line. More importantly, \textit{in vivo} imaging showed a long-lasting retention of the injected antibody at the tumor site. These results suggest that the Fab-scFv anti-B7-H4/CD3 bispecific antibody might be a therapeutic agent for PD-L1-negative B7-H4-expressing tumors or anti-HER2 antibody-non-responsive breast tumors.

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\textbf{Abbreviations:} B7-H4, B7 homolog 4; BsAb, bispecific antibody; PBMC, peripheral blood mononuclear cell; MHC, major histocompatibility complex; NOG, NOD/Shi-scid-IL-2R\(^\gamma^{null}\); GVHD, graft versus host disease; PD-1, programmed death-1; PD-L1, programmed death-ligand-1; HLA, human leukocyte antigen; HER2, human EGFR-related 2; IHC, immunohistochemical

\textbf{Author contributions:}

Yasuto Akiyama designed the study, drafted the manuscript, and were responsible for completing the study. Akira Iizuka and Tadashi Ashizawa performed \textit{in vivo} experiments and were responsible for all animal studies. Akira Iizuka, Chizu Nonomura, Keiichi Ohshima and Ryota Kondo participated in the designing the experiments and
performed the biological assays and bioinformatics analysis. Takashi Sugino examined and diagnosed the pathological sections derived from the mice. Koichi Mitsuya, Nakamasa Hayashi and Yoko Nakasu were responsible for supplying the patient-derived materials for the clinical research. Kouji Maruyama was involved in maintaining the NOG-MHC dKO mouse in the animal facility. Ken Yamaguchi, reviewed the manuscript. All the authors read and approved the final draft.

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**Figure legends**

**Figure 1.** Expression of the B7-H4 gene in breast and gynecological cancer tissues. (A) A comprehensive DNA microarray analysis was performed using 2527 surgically resected cancer tissue samples and the major cancer categories are indicated. The box plots of log2-normalized values of VTCN1 (B7-H4) expression are displayed. The data analysis was performed using Microsoft Excel 2016 software. (B) The B7-H4 expression in the human mammary epithelial cells (HMECs) and various cancer cell lines was determined by flow cytometry. An in-house anti-B7-H4 monoclonal Ab (clone #25) was used.

**Figure 2.** Design and production of the anti-B7-H4/CD3 bispecific antibodies in Fab-scFv and scFv-scFv formats. (A) Molecular design of the BsAbs; Fab (anti-B7-H4)-scFv (anti-CD3) construct consisting of mouse anti-human B7H4 monoclonal antibody clone #25-derived VH, and VL genes and human immunoglobulin constant region domain sequences linked by (Gly4Ser)3 linker sequence to the anti-human CD3 antibody VH and VL genes. (B) Coomassie blue-stained SDS-PAGE of purified anti-B7-H4/CD3 bispecific antibodies, Fab-scFv containing a single short chain (30 kDa) and single long chain (60 kDa) and sc-Fv-scFv containing a single chain (60 kDa). (C) Characterization of the Fab-scFv anti-B7-H4/CD3 bispecific antibody. The binding activity of Cy5.5-labeled Fab-scFv anti-B7-H4/CD3 BsAb to the B7-H4 (+) breast cancer cell line was evaluated by flow cytometry. Gray dotted line: no antibody, black thick line: Cy5.5-labeled anti-B7-H4/CD3 BsAb.

**Figure 3.** Cytotoxic activity of the anti-B7-H4/CD3 BsAb against breast cancer cell
lines. (A) The 50% effective concentration (EC50) of each BsAb for cytotoxic activity against breast cancer MDA-MB-468 cells is shown. Open circle: scFv-scFv 67 ng/ml, closed circle: Fab-scFv 13 ng/ml, and closed triangle: Fab-scFv 0.2 ng/ml. Effector human PBMCs were derived from one representative case of three healthy volunteers and were used at an effector/target ratio (E/T) 40. Human PBMCs from a glioma patient, which were also used for in vivo experiment, were used for a cytotoxicity assay (data not shown). The EC50 values were calculated using ImageJ. (B) Cytotoxic activity of anti-B7-H4/CD3 BsAb against B7-H4-positive or negative cancer cell lines. The E/T ratio was set at 40. Square: ZR75, cross: MDA-MB-231, triangle: SKBR3, diamond: NCI-H2170. (C) Cytotoxic effect of the anti-B7-H4/CD3 BsAb against HMECs. PBMCs derived from 3 different healthy volunteers were used as effector cells. Closed marker: MDA-MB-468, open marker: HMEC. Each point shows the average of 2 experiments from one volunteer. The cytotoxic activity of anti-B7/CD3 BsAb in various E/T ratios using (D) Fab-scFv and (E) scFv-scFv against MDA-MB-468. From the top line, E/T ratios of 20, 10, 5, 2.5, 1.25, and target cells alone are shown. (F) Cytotoxicity assay against MDA-MB-468 using positively or negatively MACS-isolated T cell subsets. The data is representative of three independent experiments with each volunteer T cell subsets at an E/T of 10 for 16 h.

**Figure 4.** Cy5.5-labeled anti-B7-H4 BsAb (Fab-scFv) localization in tumor-bearing mice and BsAb half-life in mouse blood. (A) Sequential fluorescence imaging of the MDA-MB-468 tumor-bearing MHC-dKO NOG mouse after Cy5.5-labeled BsAb intravenous injection from 24 h to 28 days. (B) Fluorescence imaging of the NCI-H2170 tumor-bearing MHC-dKO NOG mouse at 24 h after the BsAb injection.
(C) Fluorescence imaging in the resected organs including the MDA-MB-468 tumor on day 28 after Cy5.5-labeled BsAb injection (200 μg/body). (D) Serum BsAb concentration after a 100 μg injection into 5 BALB/cA mice. The blood was sequentially collected from 2 min to 48 h after the injection to measure serum BsAb concentration. Each point represents the mean ± SD of 5 mice.

**Figure 5.** Antitumor effect of the anti-B7-H4/CD3 bispecific antibody *in vivo* using a humanized MHC-dKO NOG mouse model. (A) The short-term antitumor effect of the antibodies on the MDA-MB-468 tumors. Two weeks after the transplantation of PBMCs and cancer cells, each antibody was administered by a 40 μg/body single injection via the tail vein. Diamond: control group, closed square: Fab-scFv anti-B7-H4/CD3 BsAb, triangle: anti-B7-H4 mouse monoclonal antibody (mAb) (clone #25), and cross: anti-human CD3 mAb (OKT3). *P < 0.05. (B) The long-term antitumor effect of the antibodies on the MDA-MB-468 tumors. Twelve days after the transplantation of PBMCs and cancer cells, the BsAb was administered at 40 μg/body via the tail vein two times weekly. Diamond: control group, closed square: Fab-scFv anti-B7-H4/CD3 BsAb. Each point shows the mean ± SD value of 7 mice. *P < 0.05. (C) The effect of T cell depletion on the antitumor effect of anti-B7-H4/CD3 BsAb. Human T cell subsets were depleted by i.p. injection of 100 μg/body/day anti-CD4 or anti-CD8 mAb (Bio X Cell) from day 7 to day 9. Diamond: control group (no antibody), closed square: isotype antibody + BsAb, closed circle: anti-CD4 antibody + BsAb, and closed triangle: anti-CD8 antibody + BsAb. Each point shows the mean ± SE value of 4 mice.
Figure 6. Breast cancer cell eradication and effector T-cell infiltration inside the tumor after BsAb treatment. (A) PBMCs and MDA-MB-468 tumor-transplanted mice were administered BsAb sequentially in a dose escalating manner (0.2-200 µg) with a 3 or 4 day intervals between injections. Two weeks after the initial Ab injection, the resected control tumor specimens and the BsAb treated-tumor specimens with about 70% size reduction were used for IHC analysis. (B) Images of the anti-B7-H4 BsAb treated mouse tumors stained with hematoxylin-eosin (HE) or with anti-B7-H4 (clone #25) and anti-CD8 antibodies. Magnification: 200×. (C) Infiltrating CD8+ or granzyme B+ T cell counts at the tumor site. Each histogram represents the mean ± SD of more than 10 areas of the tumor section. *P < 0.05.
Fig. 1

(A) VTN1 (B7-H4) gene expression (log2) in various cancer types:
- Breast (n: 159)
- Gynecologic (n: 81)
- Lung (n: 439)
- Gastric (n: 303)
- Hepatic (n: 143)
- Neural (n: 48)
- Colorectal (n: 760)

(B) Flow cytometry analysis of anti-B7-H4 Ab in different cell lines:
- MDA-MB-468
- ZR75
- MDA-MB-231
- SKBR3
- HCC1954
- HCC1569
- NCI-H2170 (Lung)
- Human mammary epithelial cell (HMEC)
Fig. 3

A

EC₅₀: ▲ Fab-scFv (24H): 0.23 ng/ml
● Fab-scFv (16H): 12 ng/ml
○ scFv-scFv (16H): 67 ng/ml

B

Lysis (%)

0 10 20 30 40 50

0 10 100

0 10 1000

Fab-scFv (ng/ml)

C

Lysis (%)

0 10 20 30 40 50

0 10 100

0 10 1000

Fab-scFv (ng/ml)

D

Lysis (%)

0 10 20 30 40 50

0 10 100

0 10 1000

Fab-scFv (ng/ml)

E

Lysis (%)

0 10 20 30 40 50

0 10 100

0 10 1000

scFv-scFv (ng/ml)

F

Lysis (%)

0 20 40 60 80

0 10 100

0 10 1000

Whole/no target

Whole

CD4(+)

CD8(+)

CD4(-)CD8(+)

CD4(-)

CD8(-)
Fig. 4

A  Cy5.5-Fab-scFv iv.: 5μg/body

B  Cy5.5-Fab-scFv iv.: 10μg/body

C  Cy5.5-Fab-scFv iv.: 200μg/body

D  (T$_{1/2-\beta}$: 8.5 hours)
Fig. 5

A (Study scheme)

MHC-KO NOG mice
-15d -14d 0d 7d

2.5Gy X-ray
Human PBMC iv.

Cancer cell line sc.

Antibody injection iv.

Sampling
1. Tumor; qPCR, TIL-FCM
2. Spleen; qPCR, FCM
3. Blood; qPCR

-5 0 5 10 15

Tumor volume (mm$^3$)

Days after BsAb injection

B

-13d -12d 0d 7d 14d

X-ray, hPBMC iv.

MDA-MB-468 sc.

BsaB iv.

BsaB iv.

Tumor volume (mm$^3$)

Days after BsaB injection

C

-13d -12d -5d 0d 7d 14d

X-ray, hPBMC iv.

MDA-MB-468 sc.

Dep-Ab ip. 3 days

BsaB iv.

BsaB iv.

Validating depletion

Tumor volume (mm$^3$)

Days after BsaB injection
Fig. 6

A

2.5Gy X-ray hPBMC iv. -16d
\[
\text{Tumor sampling for IHC}
\]

BsAb iv. /body

-15d

Days after BsAb injection

0
500
1000
1500
2000

Control

BsAb

Granzyme B+ cells (/mm$^2$)

B

HE

B7-H4

CD8

Control

BsAb treated

Days after BsAb injection

Cont. BsAb

Granzyme B+ cells (/mm$^2$)

C

2000

1500

1000

500

0

Control

BsAb

('p < 0.005)

Tumor volume (mm$^3$)
Clinical Cancer Research

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Akira Iizuka, Chizu Nonomura, Tadashi Ashizawa, et al.

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