Defucosylated mouse-dog chimeric anti-HER2 monoclonal antibody exerts antitumor activities in mouse xenograft models of canine tumors

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Abstract. Human epidermal growth factor receptor 2 (HER2) overexpression has been reported in various types of cancer, including breast, gastric, lung, colorectal and pancreatic cancer. A humanized anti-HER2 monoclonal antibody (mAb), trastuzumab, has been shown to improve survival of patients in HER2-positive breast and gastric cancer. An anti-HER2 mAb, H_2Mab-77 (mouse IgG$_1$, kappa) was previously developed. In the present study, a defucosylated version of mouse-dog chimeric anti-HER2 mAb (H77Bf) was generated. H77Bf possesses a high binding-affinity [a dissociation constant (K_D): 7.5x10^{-10} M, as determined by flow cytometric analysis] for dog HER2-overexpressed CHO-K1 (CHO/dHER2) cells. H77Bf highly exerted antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) for CHO/dHER2 cells by canine mononuclear cells and complement, respectively. Moreover, administration of H77Bf significantly suppressed the development of CHO/dHER2 xenograft tumor in mice compared with the control dog IgG. H77Bf also possesses a high binding-affinity $(K_D$: 7.2x10^{-10} M) for a canine mammary gland tumor cell line (SNP), and showed high ADCC and CDC activities for SNP cells. Intraperitoneal administration of H77Bf in mouse xenograft models of SNP significantly suppressed the development of SNP xenograft tumors compared with the control dog IgG. These results indicated that H77Bf exerts antitumor activities against dHER2-positive canine cancers, and could be valuable treatment regimen for canine cancers.

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Abbreviations: HER2, human epidermal growth factor receptor 2; mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; FDA, Food and Drug Administration; PI3K, phosphatidylinositol-3 kinase; TKI, tyrosine kinase inhibitor; FcγR, Fcγ receptor; NK, natural killer; CMT, canine mammary tumor; RPMI, Roswell Park Memorial Institute; PBS, phosphate-buffered saline; K_D, dissociation constant; DAPI, 4',6-diamidino-2-phenylindole; MNC, mononuclear cell; SEM, standard error of the mean; IHC, immunohistochemistry; T-DXd, Trastuzumab deruxtecan

Key words: HER2, monoclonal antibody, ADCC, CDC, antitumor activity

Introduction

Human epidermal growth factor receptor 2 (HER2, also known as ERBB2) is a cell surface type I transmembrane glycoprotein that is highly expressed on various solid tumors and enable a broad repertoire of oncogenic signaling upon homo- and heterodimerization with HER/ERBB families. HER2 overexpression is observed in ~20-30% of human breast cancers, which are associated with poor prognosis and higher rates of recurrence (1). In 1998, trastuzumab became the first monoclonal antibody (mAb), which U.S. Food and Drug Administration (FDA) approved for treatment of HER2-positive breast cancers (2) and later in HER2-positive gastric cancers (3).

Trastuzumab was initially considered to inhibit HER2 signaling (4,5). Numerous studies have confirmed the inhibition of downstream phosphatidylinositol-3 kinase (PI3K)/Akt pathway, and the suppression of tumor cell proliferation (6-8). Concurrently, the HER2-selective tyrosine kinase inhibitors (TKIs) such as lapatinib, neratinib and tucatinib, were developed and exhibited a superior activity to suppress HER2 signaling (6,9,10). However, regardless of a weaker inhibitory

activity to HER2 signaling, trastuzumab has exhibited greater clinical efficacy than TKIs. Trastuzumab has been the most effective therapy for HER2-positive breast cancer for more than 20 years (11). Clinically, this difference in efficacy suggests the involvement of immunologic engagement of antibody therapy, hardly observed in TKIs (12).

Trastuzumab possesses an Fc domain which allows for the direct engagement with Fcγ receptors (FcγRs) on various types of immune cells. The FcγR engagement allows for phagocytic engulfment of antibody-bound pathogens or cells, termed antibody-dependent cellular phagocytosis. The FcγR-mediated signaling activates dendritic cells, macrophages and neutrophils, which can alter adaptive immune responses through antigen presentation, cytokine production and chemotaxis. Furthermore, the FcγR engagement can stimulate natural killer (NK) cells which attack and lyse the target cells, termed antibody-dependent cellular cytotoxicity (ADCC) (13). Margetuximab contains several optimization mutations and exhibits improved FcγRIIIA engagement and ADCC activity compared with the parental Ab trastuzumab (14). Margetuximab was recently approved by FDA in heavily pretreated patients based on modest but significant improvement in progression-free survival (15,16). Moreover, the Fc domain can trigger the activation of complement family, and exert the complement-dependent cytotoxicity (CDC) (17,18).

With the increase in lifespan of both humans and dogs, the increased cancer incidence has been observed as well. Mammary neoplasia is the most frequently observed in dog tumors (19). Among them, ~50% are malignant. These spontaneous canine mammary tumors (CMT) share biological and histological characteristics with human breast carcinoma (20). Compared with murine model, CMT models have advantages as a naturally occurring models of human cancers (21). In canine tumors, the overexpression of dog HER2 (dHER2) has been reported not only in mammary carcinoma (22) but also osteosarcoma (23), bladder cancer (24), and anal sac gland carcinoma (25). Furthermore, in accordance with the American Society of Clinical Oncology and the College of American Pathologists guidelines for HER2 immunostaining, dHER2 has been revealed to be overexpressed in 32% of CMT (26), 81% of intestinal tumor, 42% of rectal carcinomas, and 28% of cutaneous squamous cell carcinomas (27). Additionally, a HER2-expressed recombinant Listeria vaccine administration resulted in the induction of anti-dHER2 immunity, which resulted in the reduced incidences of metastasis, and prolonged survival in a phase I study for canine osteosarcoma (28). These clinical outcomes promoted the evaluation of anti-dHER2 mAbs as a therapeutic modality for canine cancers.

Previously, an anti-HER2 mAb, H_2Mab-77 (mouse IgG$_1$, kappa), was developed (29). In the present study, a defucosylated mouse-dog chimeric anti-HER2 mAb (H77Bf) was produced. The present study aimed to investigate the ability of H77Bf to induce ADCC, CDC and antitumor efficacy in dHER2-expressing cells.

Materials and methods

*Cell lines.* A canine mammary gland tumor cell line, SNP, was purchased from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University (Miyagi, Japan) (30). CHO-K1 cells were purchased from the American Type Culture Collection. Dog HER2 (accession no. NM_001003217)-overexpressed CHO-K1 (CHO/dHER2) was established by transfection of pCAG/3xRIEDL-dHER2 into CHO-K1 cells as previously described (31). 3xRIEDL sequence represented three repeat of RIEDL amino acid sequence (32). RIEDL tag is an affinity tag that is used for the one-step membrane protein purification (32-36). CHO-K1, CHO/dHER2, and SNP were cultured in RPMI-1640 medium (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin, 100 units/ml of penicillin, and 0.25 μg/ml amphotericin B (Nacalai Tesque, Inc.). The cell lines were maintained at 37°C in a humidified atmosphere under 5% CO_2.

Animals. Animal experiments were performed following regulations and guidelines to minimize animal distress and suffering in the laboratory. Animal experiments for antitumor activity of H77Bf were approved (approval no. 2021-056) by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan). Mice were maintained on an 11 h light/13 h dark cycle with food and water supplied *ad libitum* in a specific pathogen-free environment across the experimental period. Mice were monitored for weight and health every 2-5 days during the experiments. The loss of original body weight was determined to a point >25% and/or a maximum tumor size >3,000 mm^3 as humane endpoints for euthanasia.

Antibodies. Anti-HER2 mAb H_2Mab-77 was established as previously described (29). To generate H77B, we subcloned V_H cDNA of H_2Mab-77 and C_H of dog IgGB into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). V_L cDNA of H_2Mab-77 and C_L cDNA of dog kappa light chain were also subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation). The vector of H77B was transduced into BINDS-09 (FUT8-deficient ExpiCHO-S) cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.) (37-41). H77Bf was purified using Ab-Capcher (ProteNova Co., Ltd.). Dog IgG was purchased from Jackson ImmunoResearch Laboratories, Inc.

Flow cytometry. CHO-K1, CHO/dHER2, and SNP were harvested by 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.) treatment. After washing with blocking buffer [0.1% bovine serum albumin (BSA; Nacalai Tesque, Inc.) in phosphate-buffered saline (PBS)], cells were treated with H77Bf, or blocking buffer (control) for 30 min at 4°C. Then, cells were incubated in FITC-conjugated anti-dog IgG (cat. no. A18764; 1:1,000; Thermo Fisher Scientific, Inc.) for 30 min at 4°C. Fluorescence data were collected by the Cell Analyzer EC800 and analyzed by EC800 software ver. 1.3.6 (Sony Corp.).

Determination of binding affinity. CHO/dHER2 and SNP were suspended in serially diluted H77Bf (0.006-25 μg/ml) followed by FITC-conjugated anti-dog IgG (1:200). Fluorescence data
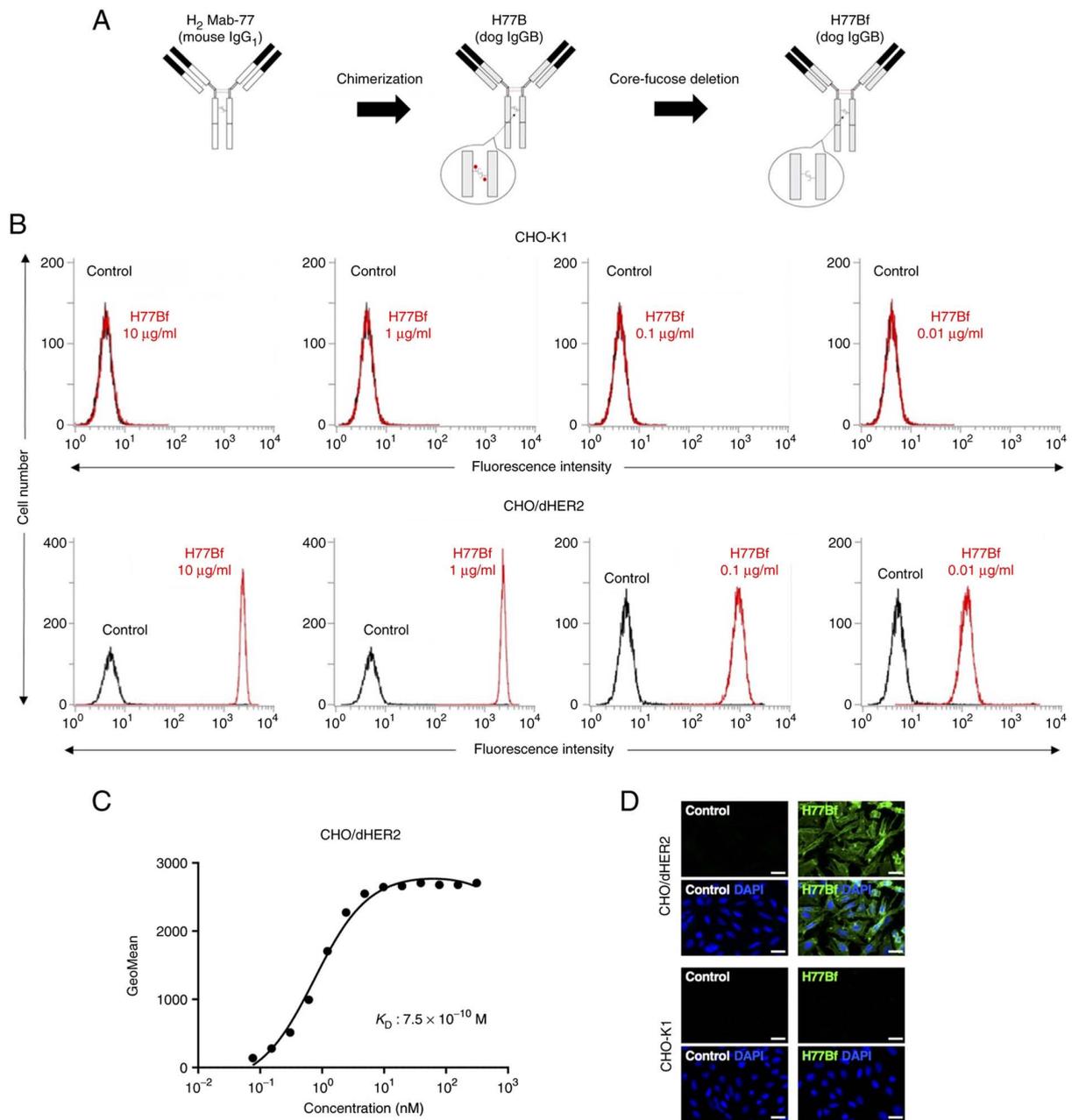
Figure 1. Flow cytometry using H77Bf. (A) Production of H77Bf (core-fucose-deficient dog IgGB) from H_2Mab-77 (mouse IgG_1). (B) CHO-K1 and CHO/dHER2 cells were treated with H77Bf or buffer control, followed by FITC-conjugated anti-dog IgG. (C) Determination of the binding affinity of H77Bf using flow cytometry for CHO/dHER2 cells. CHO/dHER2 cells were suspended in serially diluted H77Bf, followed by the addition of FITC-conjugated anti-dog IgG. Fluorescence data were analyzed using the EC800 Cell Analyzer. (D) Immunocytochemistry using H77Bf. CHO-K1 and CHO/dHER2 cells were incubated with buffer control or 10 µg/ml H77Bf for 1 h, followed by the incubation with Alexa Fluor 488-conjugated anti-dog IgG and DAPI for 45 min. Fluorescent images were acquired using a fluorescent microscope BZ-X800. Scale bars, 20 µm.

were collected using the Cell Analyzer EC800. The dissociation constant (K_D) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc.).

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde-PBS for 10 min and quenched with 50 mM NH_4Cl in PBS with 0.2 mM Ca^{2+} and 2 mM Mg^{2+}. The cells were blocked with blocking buffer (PBS containing 0.2 mM Ca^{2+}, 2 mM Mg^{2+} and 0.5% BSA) for 30 min and incubated with 10 µg/ml of H77Bf or blocking buffer for 1 h. The

cells were further incubated with Alexa Fluor 488-conjugated anti-dog IgG (1:400; Jackson ImmunoResearch Laboratories, Inc.) and 0.3 µM of 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Inc.) for 45 min. The whole processes were performed at room temperature. Fluorescence images were acquired with a 40x objective on a BZ-X800 digital fluorescence microscope (Keyence Corporation).

ADCC of H77Bf. Canine mononuclear cells (MNCs) obtained from Yamaguchi University were resuspended in DMEM
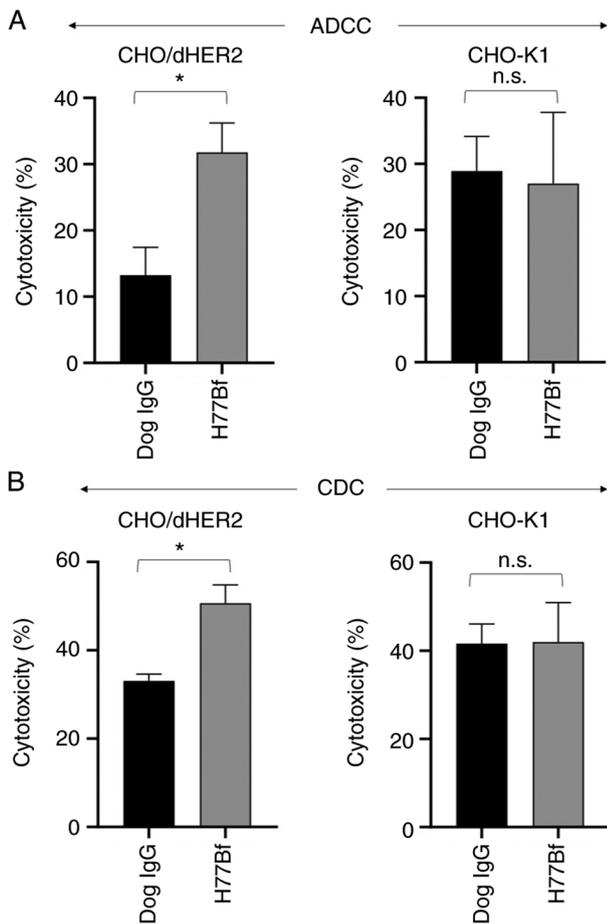
Figure 2. Evaluation of ADCC and CDC elicited by H77Bf. (A) ADCC elicited by H77Bf and control dog IgG targeting CHO/dHER2 and CHO-K1 cells. (B) CDC elicited by H77Bf and control dog IgG targeting CHO/dHER2 and CHO-K1 cells Values are presented as the mean ± SEM. (*P<0.05; Welch's t- test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; n.s., not significant.

(Nacalai Tesque, Inc.) with 10% FBS and were used as effector cells (37,38,42). Target cells (CHO-K1, CHO/dHER2, and SNP) were labeled with 10 μg/ml Calcein AM (Thermo Fisher Scientific, Inc.) (31,39-41,43-53). The target cells (2×10^4 cells) were plated in 96-well plates and mixed with effector canine MNCs (effector/target cells ratio, 50), 100 μg/ml of H77Bf or control dog IgG. Following incubation for 4.5 h at 37˚C, the Calcein release into the medium was analyzed using a microplate reader (Power Scan HT; BioTek Instruments, Inc.,) with an excitation wavelength (485 nm) and an emission wavelength (538 nm).

Cytolyticity (% lysis) was calculated as follows: % lysis=(E-S)/(M-S) x100, where 'E' is the fluorescence in cultures of both effector and target cells, 'S' is the spontaneous fluorescence of only target cells, and 'M' is the maximum fluorescence following the treatment with a lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM of EDTA, and 0.5% Triton X-100).

CDC of H77Bf. Target cells (CHO-K1, CHO/dHER2, and SNP) were labeled with 10 μg/ml Calcein AM (31,39-41, 43-53). The target cells (2×10^4 cells) were plated in 96-well plates and mixed with rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories,)

and 100 μg/ml of control dog IgG or H77Bf. Following incubation for 4.5 h at 37˚C, Calcein release into the medium was measured.

Antitumor activity of H77Bf in xenografts of CHO-K1, CHO/dHER2 and SNP cells. BALB/c nude mice (female, 5 weeks old, weighing 14-17 g) were purchased from Charles River Laboratories, Inc. CHO-K1, CHO/dHER2, or SNP cells (5×10^6 cells) were resuspended in DMEM and mixed with BD Matrigel Matrix Growth Factor Reduced (BD Biosciences) were subcutaneously injected into the left flank of mice.

On day 8 post-inoculation, 100 μg of H77Bf (n=8) or control dog IgG (n=8) in 100 μl PBS were intraperitoneally injected. On days 14 and 21, additional antibody inoculations were performed. Furthermore, on days 8, 14 and 21, canine MNCs were injected surrounding the tumors. The tumor volume was measured on days 7, 10, 14, 17, 21, 24 and 28 after the injection of cells. Tumor volumes were determined as previously described (31,37,39-41,50,54).

Statistical analyses. All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted with Welch's t test for ADCC, CDC, and tumor weight. ANOVA with Sidak's post hoc test were conducted for tumor volume and mouse weight. All calculations were performed using GraphPad Prism 8 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Flow cytometric analysis against CHO/dHER2 cells using H77Bf. In our previous study, an anti-HER2 mAb (H_2Mab-77) was established using cancer-specific mAb (CasMab) method (29). H_2Mab-77 was revealed to be very useful for flow cytometry, western blotting and immunohistochemistry (IHC) (29). In the present study, a defucosylated mouse-dog chimeric anti-HER2 mAb (H77Bf) was produced by combining V_H and V_L of H_2Mab-77 with C_H and C_L of dog IgG, respectively (Fig. 1A). H77Bf detected CHO/dHER2 cells dose-dependently, not parental CHO-K1 cells (Fig. 1B), indicating that H77Bf cross-reacted with dHER2.

A kinetic analysis of the interactions of H77Bf with CHO/dHER2 cells was performed via flow cytometry. As revealed in Fig. 1C, the K_D for the interaction of H77Bf with CHO/dHER2 cells was 7.5×10^{-10} M, suggesting that H77Bf exhibits high affinity for CHO/dHER2 cells.

Immunocytochemical analysis against CHO/dHER2 cells using H77Bf. It was examined whether H77Bf is applicable for immunocytochemistry. The H77Bf specificity was evaluated by using CHO/dHER2 and CHO-K1 cells. As revealed in Fig. 1D, H77Bf detected dHER2 on CHO/dHER2 cells, but not CHO-K1 cells. Buffer control showed no signal on both CHO/dHER2 and CHO-K1 cells. These results suggested that H77Bf recognizes exogenous dHER2 in immunocytochemistry.

H77Bf-mediated ADCC and CDC in CHO/dHER2 cells. It was investigated whether H77Bf was capable of mediating
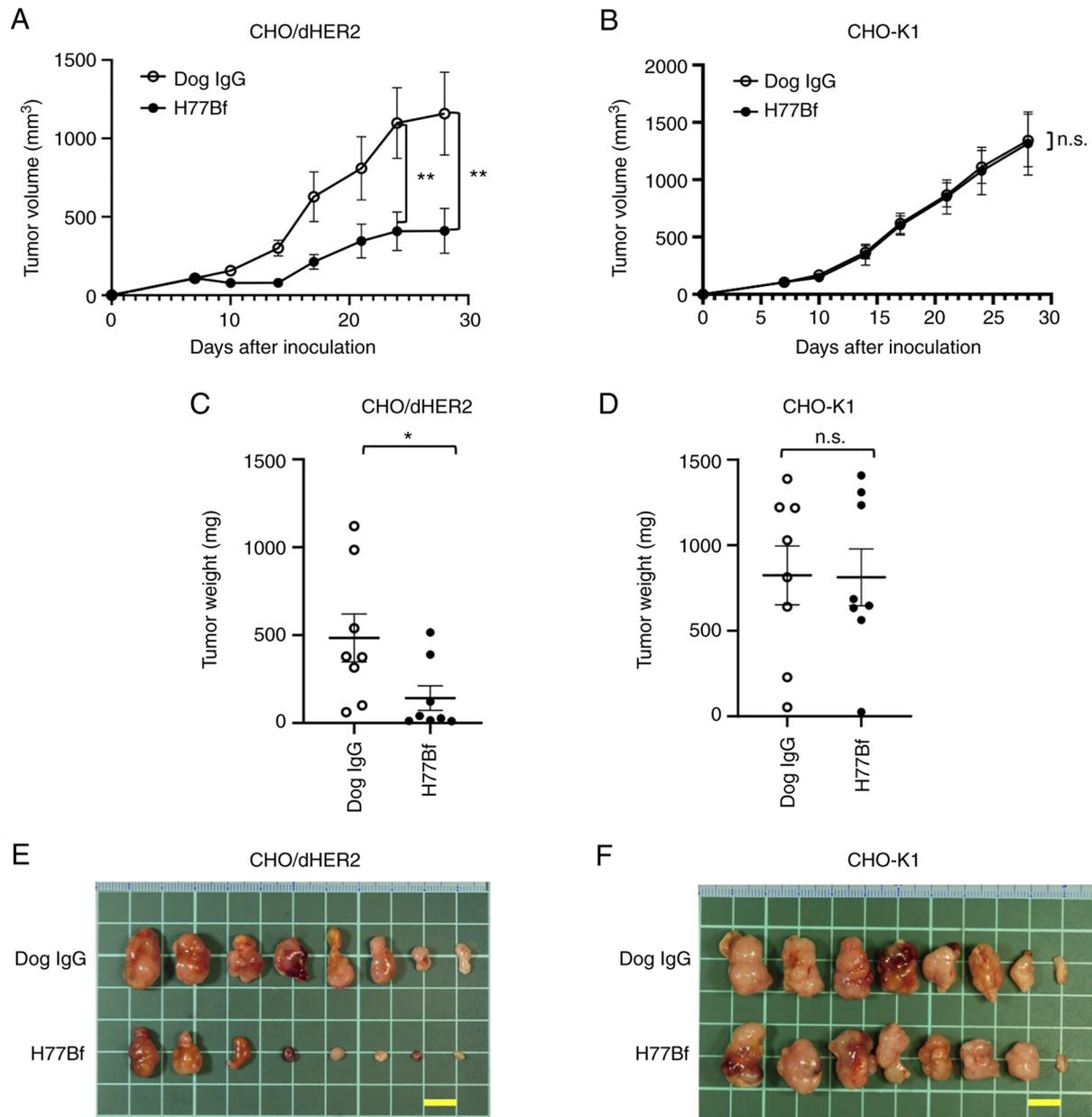
Figure 3. Antitumor activity of H77Bf. (A and B) Evaluation of tumor volume in (A) CHO/dHER2 and (B) CHO-K1 xenograft models. CHO/dHER2 and CHO-K1 cells ($5x10^6$ cells) were subcutaneously injected into mice. On day 8, 100 μg of H77Bf or control dog IgG were injected intraperitoneally into mice. Additional antibodies were injected on days 14 and 21. Mononuclear cells were also injected surrounding the tumors on days 8, 14 and 21. The tumor volume was measured on days 7, 10, 14, 17, 21, 24 and 28 after the injection. Values are presented as the mean ± SEM. **$P<0.01$ (ANOVA and Sidak's multiple comparisons test). (C and D) Tumor weight (day 28) was measured from excised xenografts of (C) CHO/dHER2 and (D) CHO-K1. Values are presented as the mean ± SEM. *$P<0.05$ (Welch's t-test). (E and F) Appearance of resected tumors of (E) CHO/dHER2 and (F) CHO-K1 xenografts from the control dog IgG and H77Bf treated groups on day 28 (scale bar, 1 cm). n.s., not significant.

ADCC against CHO/dHER2 cells. H77Bf showed ADCC (31.8% cytotoxicity) against CHO/dHER2 cells more effectively than the control dog IgG (13.2% cytotoxicity; P<0.05). There was no difference between H77Bf and control dog IgG about ADCC against CHO-K1 (Fig. 2A).

It was then examined whether H77Bf could exert CDC against CHO/dHER2 cells. As revealed in Fig. 2B, H77Bf elicited a higher degree of CDC (50.7% cytotoxicity) in CHO/dHER2 cells compared with that elicited by control dog IgG (33.1% cytotoxicity; P<0.05). There was no difference between H77Bf and control dog IgG about CDC against CHO-K1 (Fig. 2B). These results demonstrated that

H77Bf exhibited higher levels of ADCC and CDC against CHO/dHER2 cells.

Antitumor effects of H77Bf in the mouse xenografts of CHO/dHER2 cells. In the CHO/dHER2 xenograft tumor, H77Bf and control dog IgG were intraperitoneally injected into mice on days 8, 14 and 21, following the CHO/dHER2 cells injection. On days 7, 10, 14, 17, 21, 24 and 28 after the injection, the tumor volume was measured. The H77Bf administration resulted in a significant reduction of tumors on days 24 (P<0.01) and 28 (P<0.01) compared with that of the control dog IgG (Fig. 3A). The H77Bf administration resulted
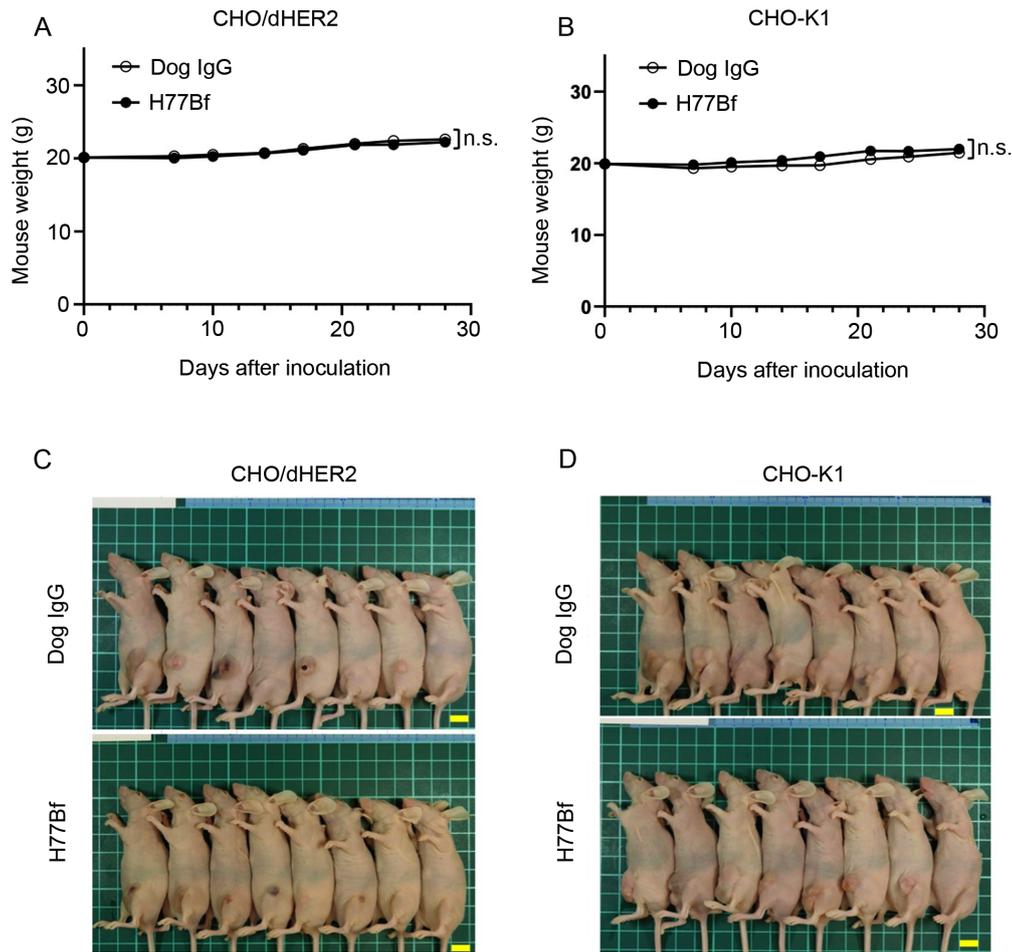
Figure 4. Body weights and appearance of the mice. (A and B) Body weights of mice implanted with (A) CHO/dHER2 and (B) CHO-K1 xenografts on days 7, 10, 14, 17, 21, 24 and 28 (ANOVA and Sidak's multiple comparisons test). (C and D) Body appearance of (C) CHO/dHER2 and (D) CHO-K1-implanted mice on day 28 (scale bar, 1 cm). n.s., not significant.

in a 65% reduction of the volume compared with that of the control dog IgG on day 28 post-injection.

The weight of CHO/dHER2 tumors treated with H77Bf was significantly lower than that treated with control dog IgG (71% reduction; P<0.05; Fig. 3C). CHO/dHER2 tumors that were resected from mice on day 28 are demonstrated in Fig. 3E.

In the CHO-K1 xenograft models, H77Bf and control dog IgG were injected intraperitoneally into mice on days 8, 14 and 21 after the injection of CHO-K1 cells. The tumor volume was measured on days 7, 10, 14, 17, 21, 24 and 28 after the injection of cells. No difference was observed between H77Bf and control dog IgG about CHO-K1 tumor volume (Fig. 3B) and CHO-K1 tumor weight (Fig. 3D). CHO-K1 tumors that were resected from mice on day 28 are demonstrated in Fig. 3F.

The body weights loss and skin disorder were not observed in CHO/dHER2 (Fig. 4A) and CHO-K1 (Fig. 4B) tumor-bearing mice. The mice on day 28 about CHO/dHER2 and CHO-K1 were shown in Fig. 4C and D, respectively.

Flow cytometry and immunocytochemical analysis against SNP cells using H77Bf. As demonstrated in Fig. 5A, H77Bf detected SNP cells dose-dependently. A kinetic analysis of the binding of H77Bf to SNP cells was performed via flow cytometry. The K_D for the interaction of H77Bf with SNP cells was 7.2×10^{-10} M (Fig. 5B), suggesting that H77Bf shows high affinity for SNP cells.

Immunocytochemical analysis was then performed using H77Bf for SNP cells. As a result, H77Bf detected dHER2 on SNP cells (Fig. 5C). Buffer control detected no signal on SNP cells. These results indicated that H77Bf recognizes endogenous dHER2 in immunocytochemistry.

H77Bf-mediated ADCC and CDC in SNP cells. It was investigated whether H77Bf was capable of mediating ADCC against SNP cells. As revealed in Fig. 5D, H77Bf showed ADCC (24.8% cytotoxicity) against SNP cells more potently than did the control dog IgG (6.3% cytotoxicity; P<0.05). It was next investigated whether H77Bf exhibited CDC against SNP cells. H77Bf induced a higher degree of CDC (63.9% cytotoxicity) in SNP cells compared with that induced by control dog IgG (45.7% cytotoxicity; P<0.05) (Fig. 5D). These results demonstrated that H77Bf exhibited higher levels of ADCC and CDC against SNP cells.

Antitumor effects of H77Bf on SNP xenografts. In the SNP xenograft models, H77Bf and control dog IgG were injected
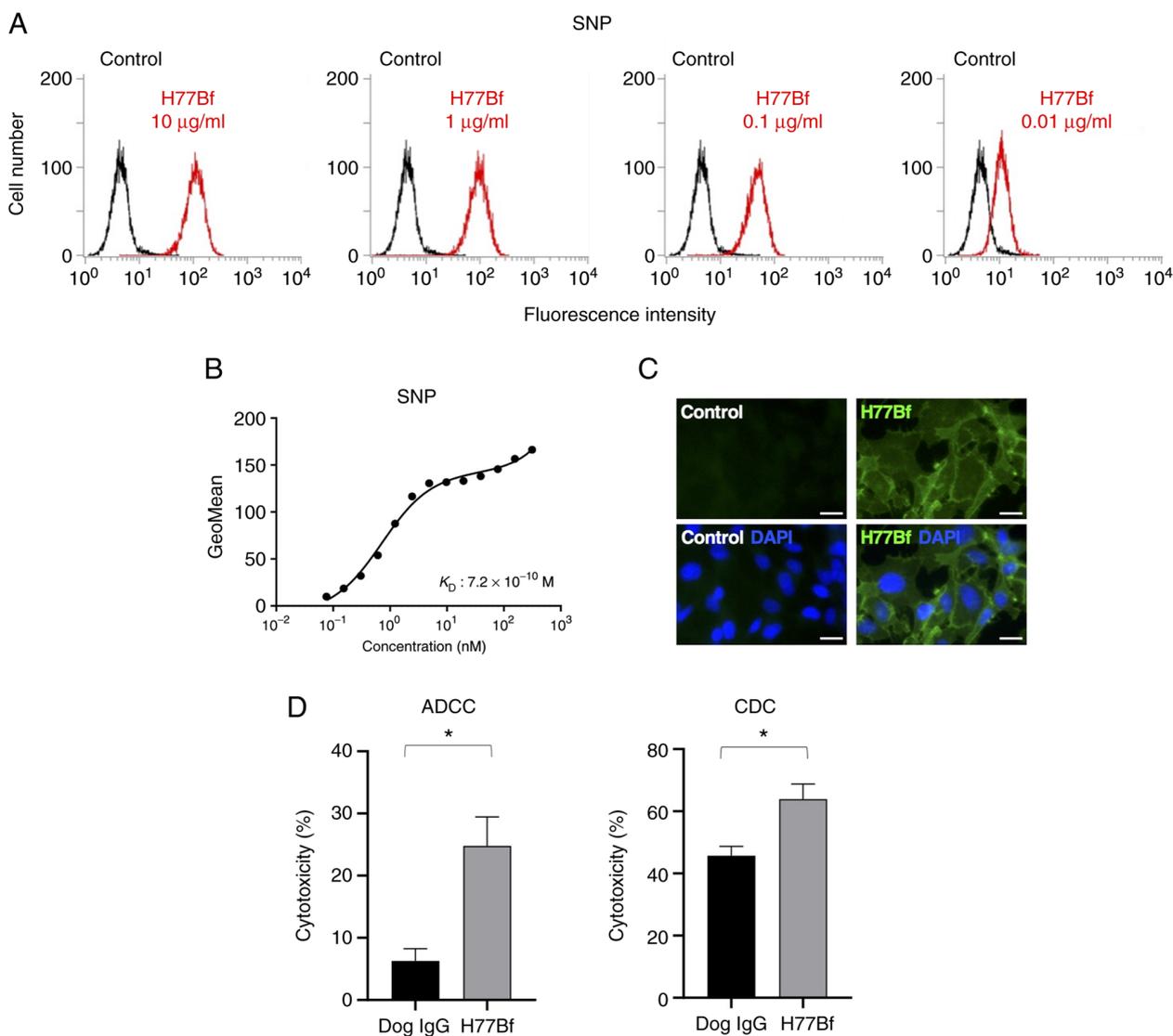
Figure 5. Flow cytometry, ADCC and CDC activity of H77Bf against canine mammary gland tumor cell line, SNP cells. (A) SNP cells were treated with H77Bf or buffer control, followed by FITC-conjugated anti-dog IgG. (B) Determination of the binding affinity of H77Bf for SNP cells using flow cytometry. SNP cells were suspended in 100 μl of serially diluted H77Bf, followed by the addition of FITC-conjugated anti-dog IgG. Fluorescence data were collected using the EC800 Cell Analyzer. (C) Immunocytochemistry using H77Bf. SNP cells were incubated with buffer control or 10 μg/ml H77Bf for 1 h, followed by the incubation with Alexa Fluor 488-conjugated anti-dog IgG and DAPI for 45 min. Fluorescent images were acquired using a fluorescent microscope BZ-X800 (scale bars, 20 μm). (D) ADCC and CDC elicited by H77Bf and control dog IgG targeting SNP cells. Values are presented as the mean ± SEM. [*]P<0.05; Welch's t-test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

intraperitoneally on days 8, 14 and 21, after the injection of SNP cells. The tumor volume was measured on days 7, 10, 14, 17, 21, 24 and 28 after the injection. The H77Bf administration resulted in a significant reduction in tumor growth on days 10 (P<0.01), 14 (P<0.01), 17 (P<0.01), 21 (P<0.01), 24 (P<0.01) and 28 (P<0.01) compared with that of the control dog IgG (Fig. 6A). The H77Bf administration resulted in a 47% reduction of tumor volume compared with that of the control dog IgG on day 28.

Tumors from the H77Bf-treated mice weighed significantly less than those from the control dog IgG-treated mice (35% reduction; P<0.05, Fig. 6B). Tumors that were resected from mice on day 28 are demonstrated in Fig. 6C.

The body weights loss and skin disorder were not observed in SNP tumor-bearing mice (Fig. 7A). The mice on day 28 about SNP xenograft were demonstrated in Fig. 7B.
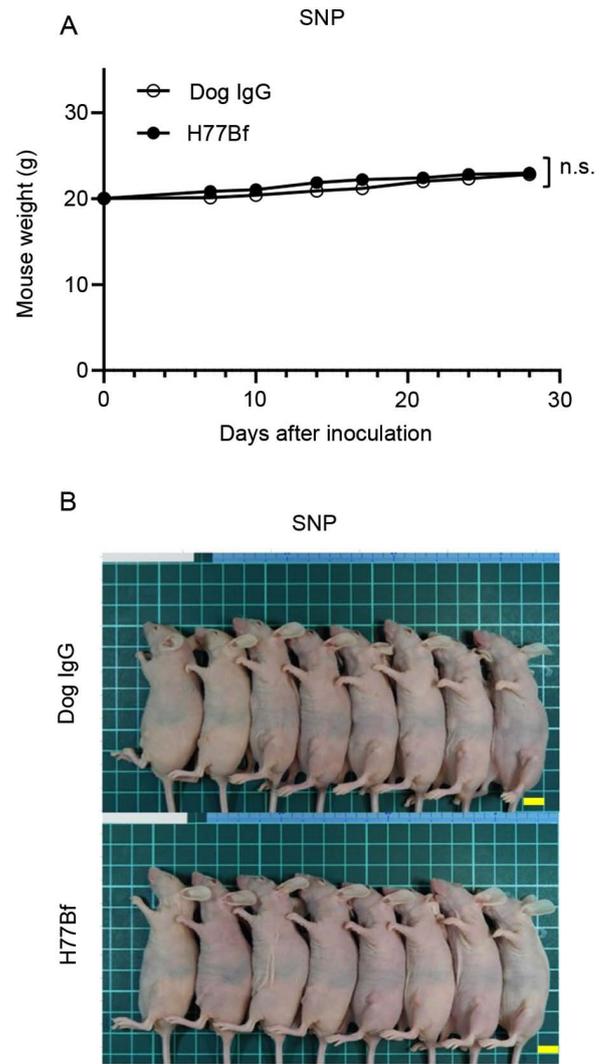
Discussion

Human mAbs that exhibit cross-reactivity to dog have been investigated. It has been suggested that cetuximab (anti-EGFR) and trastuzumab (anti-HER2) can bind to certain canine cancer cell lines (55). The clinical relevance though is limited considering that those antibodies, such as trastuzumab, mostly work through ADCC (56). Furthermore, there is a problem that the humanized mAbs will induce an anti-human immune response in dogs. Therefore, the caninization of mAbs (only the complementarity determining regions are non-canine) is essential to develop antibody therapy for dog. Some caninized mAbs have received conditional approval by the United States Department of Agriculture for lymphoma (for example Blontress, targeting CD20; and Tactress, targeting CD52). However, no peer-reviewed clinical evidence of efficacy for the
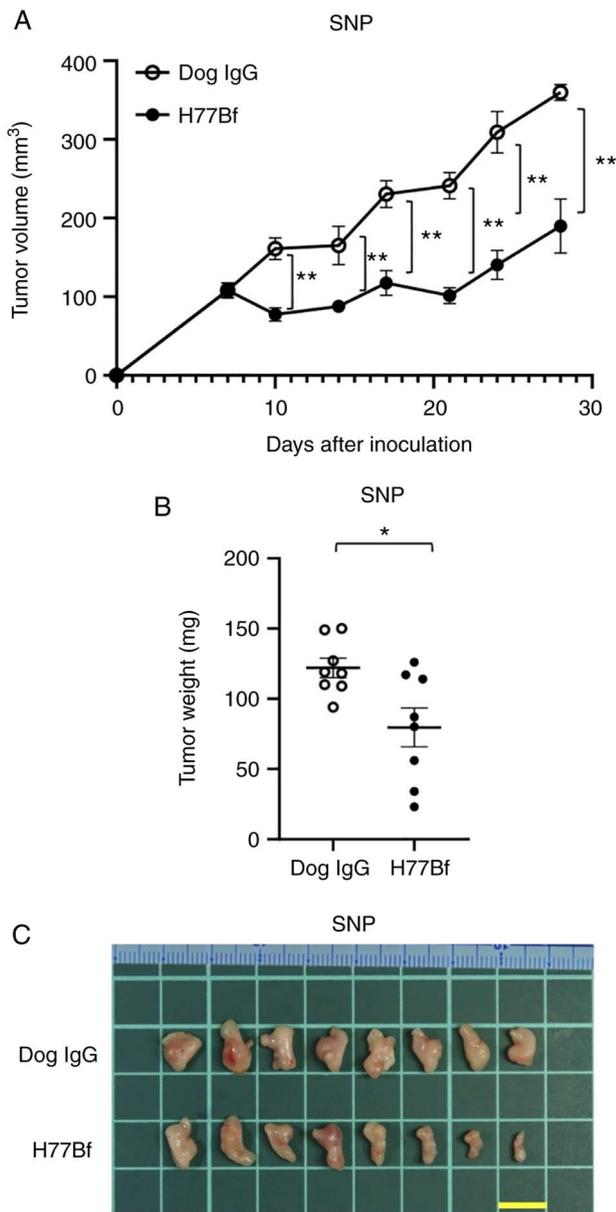
Figure 6. Antitumor activity of H77Bf against SNP xenograft. (A) Evaluation of tumor volume in SNP xenograft models. SNP cells (5×10^6 cells) were injected subcutaneously into mice. On day 8, 100 μg of H77Bf or control dog IgG in 100 μl PBS were injected intraperitoneally into mice. Additional antibodies were injected on days 14 and 21. Mononuclear cells were also injected surrounding the tumors on days 8, 14 and 21. The tumor volume was measured on days 7, 10, 14, 17, 21, 24 and 28 after the inoculation. Values are presented as the mean ± SEM. **$P<0.01$ (ANOVA and Sidak's multiple comparisons test). (B) Tumor weight (day 28) was measured from excised SNP xenografts. Values are presented as the mean ± SEM. *$P<0.05$ (Welch's t-test). (C) Appearance of resected SNP xenografts from the control dog IgG and H77Bf treated groups on day 28 (scale bar, 1 cm).

Figure 7. Body weights and appearance of the mice. (A) Body weights of mice implanted with SNP xenografts on days 7, 10, 14, 17, 21, 24 and 28 (ANOVA and Sidak's multiple comparisons test). (B) Body appearance of SNP-implanted mice on day 28 (scale bar, 1 cm). n.s., not significant.

mAb has been published (57). In the present study, caninized mAb, H77Bf was developed from anti-HER2 mAb H$_2$Mab-77. Among IgG subclasses (A, B, C and D) in dogs, the B and D subclasses were reported to be involved in ADCC (58). Therefore, B type dog IgG was converted and a defucosylated mAb was produced, which has been shown to exhibit more potent ADCC activity through binding to FcγRIIIa on NK cells (59). The cross-reactivity and binding affinity of H77Bf to CHO/dHER2 and SNP cells were first confirmed, and it was found that H77Bf possesses comparable high binding affinity to CHO/dHER2 (7.5×10^{-10} M) and SNP (7.2×10^{-10} M) cells, compared with human cancer A431 (2.1×10^{-9} M by H$_2$Mab-77) and SK-BR-3 (7.3×10^{-9} M by H$_2$Mab-77) cells, as previously reported (29). The quantitative analysis is considered to be essential to apply a human antibody to dog.

In vivo administration of H77Bf and canine MNC resulted in significant growth inhibition for CHO/dHER2 and SNP cells. These results provided evidences to support the suitability of H77Bf as a promising antibody therapy against canine cancers. The ADCC activity was also confirmed *in vitro* using canine MNCs, suggesting that ADCC activity could contribute to the antitumor activity of H77Bf. ADCC in humans is executed predominantly by NK cells through the FcγR that binds to the IgG$_1$ or IgG$_3$ subclass (60). The FcγR-like receptors have not been described on canine NK cells. Recently, a cell line-based assay to measure the ADCC of a canine therapeutic antibody was reported (61). The aforementioned study established a human NK cell line, NK-92 cells expressed with canine FcγR which can be used as effector cells. This system will contribute

to the understanding of NK cell-mediated target cell lysis by canine therapeutic antibodies. Since the knowledge about canine NK cells is incomplete, further studies are needed to reveal the contribution of NK cells to ADCC in dogs. Furthermore, direct cytotoxic mechanisms by the complement system in dogs is also to be determined.

Drug-conjugated mAbs rely on direct cytotoxicity of the payloads through endocytosis of receptor-bound mAbs-drug conjugate (62,63). Trastuzumab deruxtecan (T-DXd, DS-8201) is a HER2-targeting antibody conjugated with a novel DNA topoisomerase I inhibitor (64). T-DXd showed promising clinical outcomes in patients with metastatic breast cancer, who had received multiple anti-HER2-targeting regimens (65). Currently, the clinical efficacy and safety of T-DXd have been evaluated in various clinical trials. T-DXd have been approved in not only HER2-positive breast cancer (65-67), but also HER2-mutant lung cancer (66). A mouse-canine chimeric mAb against dog podoplanin (68-70) (P38B) conjugated with emtansine as the payload (P38B-DM1) was previously generated and challenged for tumor therapy. P38B-DM1 showed cytotoxicity to podoplanin-expressing cells and exhibited higher antitumor activity than P38B in the xenograft model (71). Therefore, H77B-drug conjugate is one more option to treat dHER2-positive CMT. Recently, FDA-approved human immune checkpoint inhibitor against PD-1 and PD-L1 are used in canine tumor treatment (72-74); the combination of immune checkpoint inhibitors with other antibody-drugs is expected to be more effective. H77Bf could contribute to the development of canine cancer treatment, which can be feedback for human cancer treatment.

IHC has played a critical role as a diagnostic tool for the identification of neoplasms with conventional histopathology. In human breast cancer pathology, IHC is routinely used to assist with the prognosis and to determine the specific treatment (e.g. trastuzumab) for patients. Although IHC is not routinely used in CMTs, an increasing number of studies have been looking for reliable diagnostic and/or prognostic IHC biomarkers including dHER2 (21). A positive correlation between dHER2 in serum and tissue expression (by IHC) was reported (26). There is also a positive correlation between dHER2 expression and tumor mitotic index, high histological grade and size (75). However, not all studies have confirmed this, and no difference between dHER2 expression in non-neoplastic and neoplastic lesions was observed (76). Furthermore, in contrast to HER2-positive breast cancer in human, dHER2 amplification and HER2-enrichment subtype are not observed through whole-exome and transcriptome analyses of 191 spontaneous CMTs (77). Therefore, the standardization of dHER2 IHC is essential since those IHC analyses were performed by different Abs. Our established H$_2$Mab-77 mAb is available for IHC (29), and its caninized mAb H77Bf exerts the antitumor activity against dHER2 positive cells, which could contribute to both diagnosis and therapy for dHER2-positive canine tumors.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TO, TT, MS and TA performed the experiments. MKK, MK and YK designed the experiments. TM prepared canine MNCs. TA, HS, TY and YK analyzed the data. HS and YK wrote the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal study protocol was approved (approval no. 2021-056) by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (Numazu, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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