Monoclonal Antibodies in Immunodiagnosis and Immunotherapy > Ahead of Print
Research Article FULL ACCESS Published Online: 2 April 2024

Establishment of a Novel Cancer-Specific Anti-HER2 Monoclonal Antibody H_2Mab-250/H_2CasMab-2 for Breast Cancers

Authors: Mika K. Kaneko, Hiroyuki Suzuki, and Yukinari Kato | AUTHORS INFO & AFFILIATIONS

Publication: Monoclonal Antibodies in Immunodiagnosis and Immunotherapy Preprint

Abstract

Overexpression of human epidermal growth factor receptor 2 (HER2) in breast and gastric cancers is an important target for monoclonal antibody (mAb) therapy. All therapeutic mAbs, including anti-HER2 mAbs, exhibit adverse effects probably due to the recognition of antigens expressed in normal cells. Therefore, tumor-selective or specific mAbs can be beneficial in reducing the adverse effects. In this study, we established a novel cancer-specific anti-HER2 monoclonal antibody, named H_2Mab-250/H_2CasMab-2 (IgG_1, kappa). H_2Mab-250 reacted with HER2-positive breast cancer BT-474 and SK-BR-3 cells. Importantly, H_2Mab-250 did not react with nontransformed normal epithelial cells (HaCaT and MCF 10A) and immortalized normal epithelial cells in flow cytometry. In contrast, most anti-HER2 mAbs, such as H_2Mab-119 and trastuzumab reacted with both cancer and normal epithelial cells. Immunohistochemical analysis demonstrated that H_2Mab-250 possesses much higher reactivity to the HER2-positive breast cancer tissues compared with H_2Mab-119, and did not react with normal tissues, including heart, breast, stomach, lung, colon, kidney, and esophagus. The epitope mapping demonstrated that the Trp614 of HER2 domain IV mainly contributes to the recognition by H_2Mab-250. H_2Mab-250 could contribute to the development of chimeric antigen receptor-T or antibody–drug conjugates without adverse effects for breast cancer therapy.

Introduction

Human epidermal growth factor receptor 2 (HER2) is included in the receptor tyrosine kinase family of human epidermal growth factor receptors. To activate the downstream signaling, HER2 must either form heterodimers with other HER members and their specific ligands or self-assemble into ligand-independent homodimers when overexpressed.[1] The HER2 overexpression is observed in ~20% of breast cancers[2] and 20% of gastric cancers,[3] which are associated with higher rates of recurrence, poor prognosis, and shorter overall survival. A monoclonal antibody (mAb) against HER2, trastuzumab, exhibited an antiproliferating effect *in vitro* and a potent antitumor effect *in vivo*.[4,5] The addition of trastuzumab to chemotherapy improves objective response rates, progression-free survival, and overall survival in HER2-positive breast cancer patients with metastasis.[6] Trastuzumab has become the standard treatment for HER2-positive breast cancers[7] and HER2-positive gastric cancers.[8] Trastuzumab has been the most effective therapy for HER2-positive breast cancer for more than 20 years.[9]

The major adverse effect associated with anti-HER2 therapeutic mAbs is cardiotoxicity, thereby necessitating routine cardiac monitoring in clinics.[10] Furthermore, mice lacking *ErbB2* (ortholog of *HER2*) displayed embryonic lethality due to the dysfunctions associated with a lack of cardiac trabeculae.[11] Ventricular-restricted *ErbB2*-deficient mice showed the features of dilated cardiomyopathy.[12] These results indicate that HER2 is vital for normal heart development and homeostasis. Therefore, more selective anti-HER2 mAbs against tumors, which can reduce heart failures are required.

We previously established several anti-HER2 mAbs, such as H_2Mab-19 (IgG_{2b}, kappa),[13] H_2Mab-41 (IgG_{2b}, kappa),[14] H_2Mab-77 (IgG_1, kappa),[15] H_2Mab-119 (IgG_1, kappa),[16] H_2Mab-139 (IgG_1, kappa),[17] and H_2Mab-181 (IgG_1, kappa)[18] by the immunization of HER2 ectodomain (HER2ec). We further engineered the mAbs into the mouse IgG_{2a} type (H_2Mab-77-mG_{2a}, H_2Mab-119-mG_{2a}, and H_2Mab-139-mG_{2a}, respectively), and produced the core fucose-deficient types (H_2Mab-77-mG_{2a}-f, H_2Mab-119-mG_{2a}-f, and H_2Mab-139-mG_{2a}-f, respectively) to potentiate the antibody-dependent cellular cytotoxicity (ADCC) and antitumor effect *in vivo*.[19–21] In this study, we developed and characterized a novel HER2 mAb, named H_2Mab-250/H_2CasMab-2.

Materials and Methods

Cell culture

Chinese hamster ovaries, (CHO)-K1, BT-474, SK-BR-3, MDA-MB-468, MCF 10A, hTERT TIGKs, HBEC3-KT, hTERT-HME1, RPTEC/TERT1, and P3X63Ag8U.1 (P3U1), were obtained from the American Type Culture

Collection (ATCC, Manassas, VA). Human keratinocyte HaCaT was purchased from Cell Lines Service GmbH (Eppelheim, Germany). The hTCEpi, hTEC/SVTERT24-B, and HCEC-1CT were purchased from EVERCYTE (Vienna, Austria).

The cDNA of HER2 (wild type; WT) and deletion mutants (dN218, dN342, and dN511) were cloned into the pCAG-nPA16 vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). A HER2 point mutant (W614A) and HER2 WT were cloned into the pCAG-nPA-cRAPMAP vector (FUJIFILM Wako Pure Chemical Corporation). CHO-K1 cells were transfected with the abovementioned vectors using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA). A few days after transfection, PA tag-positive cells were sorted by the cell sorter (SH800; Sony Corp., Tokyo, Japan) using NZ-1, which was originally developed as an anti-human podoplanin mAb.[22] Finally, CHO/HER2 and CHO/HER2 (dN218, dN342, dN511, and W614A) cell lines were established.

CHO-K1, CHO/HER2 (WT, deletion, and point mutants), and P3U1 were cultured in Roswell Park Memorial Institute-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), and BT-474, SK-BR-3, MDA-MB-468, HEK293T, and HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Mammary epithelial cell line, MCF 10A was cultured in Mammary Epithelial Cell Basal Medium BulletKit™ (Lonza, Basel, Switzerland) supplemented with 100 ng/mL cholera toxin (Sigma-Aldrich Corp., St. Louis, MO).

Immortalized normal epithelial cell lines were maintained as follows; hTERT TIGKs, Dermal Cell Basal Medium and Keratinocyte Growth Kit (ATCC); HBEC3-KT, Airway Epithelial Cell Basal Medium and Bronchial Epithelial Cell Growth Kit (ATCC); hTERT-HME1, Mammary Epithelial Cell Basal Medium BulletKit without GA-1000 (Lonza); hTCEpi, KGMTM-2 BulletKit (Lonza); hTEC/SVTERT24-B, OptiPRO™ SFM, and GlutaMAX™-I (Thermo Fisher Scientific, Inc.); RPTEC/TERT1, DMEM/F-12, and hTERT Immortalized RPTEC Growth Kit with supplement A and B (ATCC); HCEC-1CT, DMEM/M199 (4:1, Thermo Fisher Scientific, Inc.), 2% Cosmic Calf Serum (Cytiva, Marlborough, MA), 20 ng/mL hEGF (Sigma-Aldrich Corp.), 10 μg/mL insulin (Sigma-Aldrich Corp.), 2 μg/mL apo-transferrin (Sigma-Aldrich Corp.), 5 nM sodium selenite (Sigma-Aldrich Corp.), 1 μg/mL hydrocortisone (Sigma-Aldrich Corp.).

All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

Development of hybridomas

The animal was housed under specific pathogen-free conditions. All animal experiments were approved by

the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Anti-HER2 mAbs, such as H_2Mab-19 (IgG_{2b}, kappa),[13] H_2Mab-41 (IgG_{2b}, kappa),[14] H_2Mab-77 (IgG_1, kappa),[15] H_2Mab-119 (IgG_1, kappa),[16] H_2Mab-139 (IgG_1, kappa),[17] and H_2Mab-181 (IgG_1, kappa),[18] were established previously. In this study, H_2Mab-193 (IgG_1, kappa), H_2Mab-215 (IgG_1, kappa), and H_2Mab-250 (IgG_1, kappa) were established by the same strategy. Briefly, BALB/c mice were immunized with recombinant HER2ec produced by LN229 cells together with Imject Alum (Thermo Fisher Scientific, Inc.). After several additional immunizations, spleen cells were fused with P3U1 cells. The culture supernatants of hybridomas were screened using enzyme-linked immunosorbent assay (ELISA) with recombinant HER2ec and flow cytometry with cell lines.

Production of recombinant mAb

To generate recombinant H_2Mab-250 and H_2Mab-119, their V_H cDNAs and the C_H cDNA of mouse IgG_1 were cloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation). The V_L cDNAs and C_L cDNA of the mouse kappa light chain were also cloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). The vectors were transfected into ExpiCHO-S cells using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc.), and Ab-Capcher (ProteNova, Kagawa, Japan) was used to purify the recombinant H_2Mab-250 and H_2Mab-119.

To generate mouse IgG_{2a}-type H_2Mab-250 (H_2Mab-250-mG_{2a}), we cloned the V_H cDNA of H_2Mab-250 and the C_H of mouse IgG_{2a} into the pCAG-Neo vector. The mouse kappa light chain vector of H_2Mab-250 is described above. To produce the defucosylated form (H_2Mab-250-mG_{2a}-f), the vectors were transfected into BINDS-09 (fucosyltransferase 8-knockout ExpiCHO-S) cells using the ExpiCHO Expression System. H_2Mab-250-mG_{2a}-f was purified using Ab-Capcher.

Flow cytometry

Cells were collected using 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.). The cells (1×10^5 cells/sample) were treated with mouse anti-HER2 mAbs (10 μg/mL), trastuzumab (10 μg/mL), NZ-1 (10 μg/mL), or blocking buffer (control; 0.1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) for 30 minutes at 4°C. Next, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000; Cell Signaling Technology, Danvers, MA) for mouse anti-HER2 mAbs, Alexa Fluor 488-conjugated anti-human IgG (1:1000; Sigma-Aldrich, Corp.) for trastuzumab, or Alexa Fluor 488-conjugated anti-rat IgG (1:1000; Cell Signaling Technology) for NZ-1 for 30 minutes at 4°C. The fluorescence data were collected using EC800 or SA3800 Cell Analyzer (Sony Corp), and the data were analyzed using

FlowJo (BD Biosciences, Franklin Lakes, NJ).

ADCC reporter bioassay

The ADCC reporter bioassay was performed using an ADCC Reporter Bioassay Kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Target cells (BT-474 and HaCaT, 12,500 cells per well) were cultured in a 96-well white solid plate. H_2Mab-250-mG_{2a}-f and trastuzumab (Herceptin; Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) were serially diluted and added to the target cells. Jurkat cells stably expressing the human FcγRIIIa receptor and a nuclear factor of activated T cell (NFAT) response element driving firefly luciferase, were used as effector cells. The engineered Jurkat cells (75,000 cells in 25 μL) were then added and cocultured with antibody-treated target cells at 37°C for 6 hours. Luminescence using the Bio-Glo Luciferase Assay System was measured using a GloMax luminometer (Promega Corporation).

Immunohistochemical analysis

Formalin-fixed paraffin-embedded tissue of HER2-positive breast cancer was obtained from Sendai Medical Center.[15] Informed consent for sample procurement and subsequent data analyses was obtained from the patient or the patient's guardian at Sendai Medical Center. Normal tissues were purchased from BioChain Institute, Inc. (Eureka Drive Newark, CA) or Cybrdi, Inc. (Frederick, MD). The tissue sections were autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. The blocking was performed using SuperBlock T20 (Thermo Fisher Scientific, Inc.). The sections were incubated with H_2Mab-250 (1, 0.5, or 0.1 μg/mL) and H_2Mab-119 (0.5 or 0.1 μg/mL), and then treated with the EnVision+ Kit for mouse (Agilent Technologies, Inc., Santa Clara, CA). The chromogenic reaction was performed using 3,3'-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.). Counterstaining was performed using Hematoxylin (FUJIFILM Wako Pure Chemical Corporation) and Leica DMD108 (Leica Microsystems GmbH, Wetzlar, Germany) was used to obtain images and examine the sections.

ELISA

Synthesized peptides covering the HER2 extracellular domain IV and point mutant peptides were synthesized by Sigma-Aldrich Corp. The peptides (10 μg/mL) and HER2ec (1 μg/mL) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc.). Plate washing was performed with PBS containing 0.05% (v/v) Tween 20 (PBST; Nacalai Tesque, Inc.). After blocking with 1% (w/v) BSA in PBST, H_2Mab-250 (10 μg/mL) was added to each well. Then, the wells were further incubated with peroxidase-conjugated anti-mouse immunoglobulins (1:2000 dilution; Agilent Technologies, Inc.). Enzymatic reactions were conducted using One-Step Ultra TMB. The optical density at 655 nm was measured using an

iMark microplate reader.

Determination of K_D through surface plasmon resonance

Measurement of K_D between H_2Mab-250 and the HER2 peptides was performed using surface plasmon resonance (SPR). H_2Mab-250 was immobilized on the sensor chip CM5 in accordance with the manufacturer's protocol by Cytiva. Immobilization of H_2Mab-250 (10 μg/mL in acetate buffer (pH 4.0); Cytiva) was carried out using an amine coupling reaction. The surface of the flow cell 2 of the sensor chip CM5 was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and N-hydroxysuccinimide, followed by the injection of H_2Mab-250. The K_D between H_2Mab-250 and the peptides was determined using Biacore X100 (Cytiva). The binding signals were measured using a single-cycle kinetics method. Data were analyzed by 1:1 binding kinetics using Biacore X100 evaluation software (Cytiva) to determine the association rate constant (*k*a) and dissociation rate constant (*k*d) and K_D. The affinity constant (K_A) at equilibrium was calculated as $1/K_D$.

Results

Establishment of H_2Mab-250

We immunized mice with HER2ec produced by LN229 cells. The culture supernatants of hybridomas were screened using ELISA with HER2ec. We further screened the reactivity to HER2-positive breast cancers (BT-474 and SK-BR-3) and nontransformed normal epithelial cells, including HaCaT (keratinocyte) and MCF 10A (mammary gland) using flow cytometry. One of the established hybridomas, H_2Mab-250, reacted with CHO/HER2, HER2-positive BT-474, and SK-BR-3 cells, but not with triple-negative MDA-MB-468 cells. H_2Mab-250 did not react with HaCaT and MCF 10A cells (Fig. 1A). In contrast, H_2Mab-119 showed similar reactivity to both cancer and normal epithelial cells (Fig. 1A).

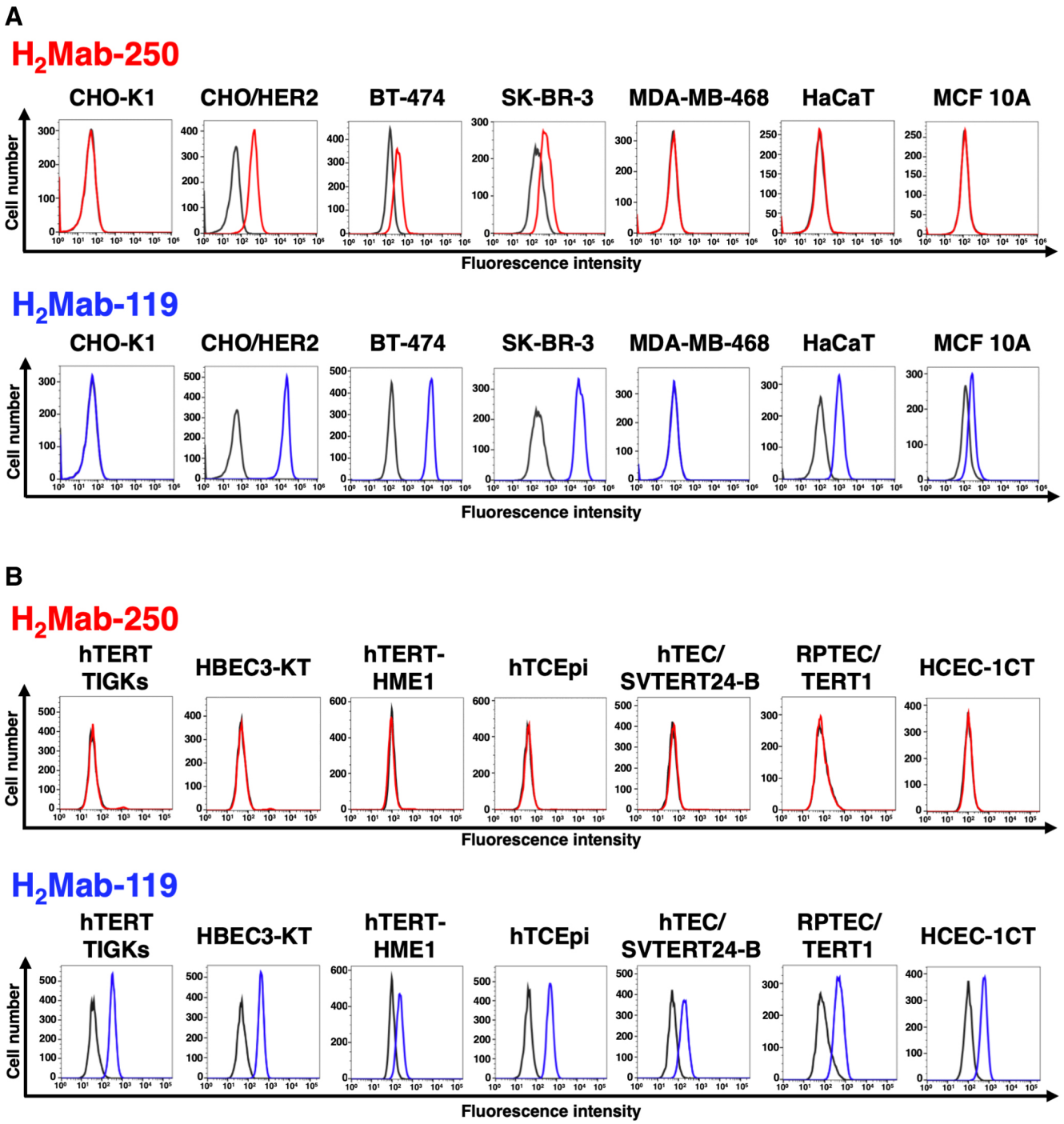


FIG. 1. Flow cytometry using anti-HER2 mAbs. **(A)** Flow cytometry using H_2Mab-250 (10 μg/mL; red line) and H_2Mab-119 (10 μg/mL; blue line) against CHO-K1, CHO/HER2, HER2-positive breast cancers (BT-474 and SK-BR-3), a triple-negative breast cancer (MDA-MB-468), and nontransformed normal epithelial cells (HaCaT and MCF 10A). **(B)** Flow cytometry using H_2Mab-250 (10 μg/mL; red line) and H_2Mab-119 (10 μg/mL; blue line) against immortalized normal epithelial cells, including hTERT TIGKs (gingiva), HBEC3-KT (lung bronchus), hTERT-HME1 (mammary gland), hTCEpi (corneal), hTEC/SVTERT24-B (thymus), RPTEC/TERT1 (kidney proximal tubule), and HCEC-1CT (colon). The black line represents the negative control (blocking buffer). CHO, Chinese hamster ovary; HER2, human epidermal growth factor receptor 2.

We next investigated the difference in the reactivity to immortalized normal epithelial cells, including hTERT TIGKs (gingiva), HBEC3-KT (lung bronchus), hTERT-HME1 (mammary gland), hTCEpi (corneal), hTEC/SVTERT24-B (thymus), RPTEC/TERT1 (kidney proximal tubule), and HCEC-1CT (colon). H_2Mab-250 did not react with those normal cells, while H_2Mab-119 was reactive with all immortalized normal epithelial cells (Fig. 1B), indicating that H_2Mab-250 possesses cancer-specific reactivity against HER2.

We further compared the reactivity of H_2Mab-250 with our established anti-HER2 mAbs and trastuzumab. As shown in Supplementary Figures S1 and S2, anti-HER2 domain I mAbs (H_2Mab-77[15] and H_2Mab-139[17]), anti-HER2 domain II mAbs (H_2Mab-193 and H_2Mab-215), anti-HER2 domain III mAbs (H_2Mab-19[13] and H_2Mab-181[18]), and anti-HER2 domain IV mAbs (H_2Mab-41[14] and trastuzumab) reacted with HER2-positive breast cancers, nontransformed normal epithelial cells, and immortalized normal epithelial cells.[16] These results indicated that H_2Mab-250 exhibits an exceptional reactivity compared with other anti-HER2 mAbs.

The ability of effector cell activation by H_2Mab-250 and trastuzumab

The ADCC reporter bioassay is a bioluminescent reporter gene assay to quantify the biological activity of the antibody through FcγRIIIa-mediated pathway activation in an ADCC mechanism of action.[23] We next produced H_2Mab-250-mG_{2a}-f, the core-fucose deleted IgG_{2a} version of H_2Mab-250 using fucosyltransferase 8-deficient ExpiCHO-S (BINDS-09) cells and examined whether H_2Mab-250-mG_{2a}-f could activate ADCC program in the presence of BT-474 and HaCaT cells. To compare the ADCC pathway activation by H_2Mab-250-mG_{2a}-f and trastuzumab, we treated BT-474 and HaCaT cells with serially diluted mAbs, and then incubated with effector Jurkat cells, which express the human FcγRIIIa receptor and an NFAT response element driving firefly luciferase.

As shown in Figure 2A, H_2Mab-250-mG_{2a}-f could activate the effector (EC_{50}: 9.9 μg/mL), but it was less effective than trastuzumab (EC_{50}: 0.053 μg/mL). Importantly, H_2Mab-250-mG_{2a}-f did not activate the effector in the presence of HaCaT cells. In contrast, trastuzumab activated the effector with similar EC_{50} (0.036 μg/mL) to BT-474 cells (Fig. 2B). These results indicated that H_2Mab-250-mG_{2a}-f selectively activates the effector cells against breast cancer cells.

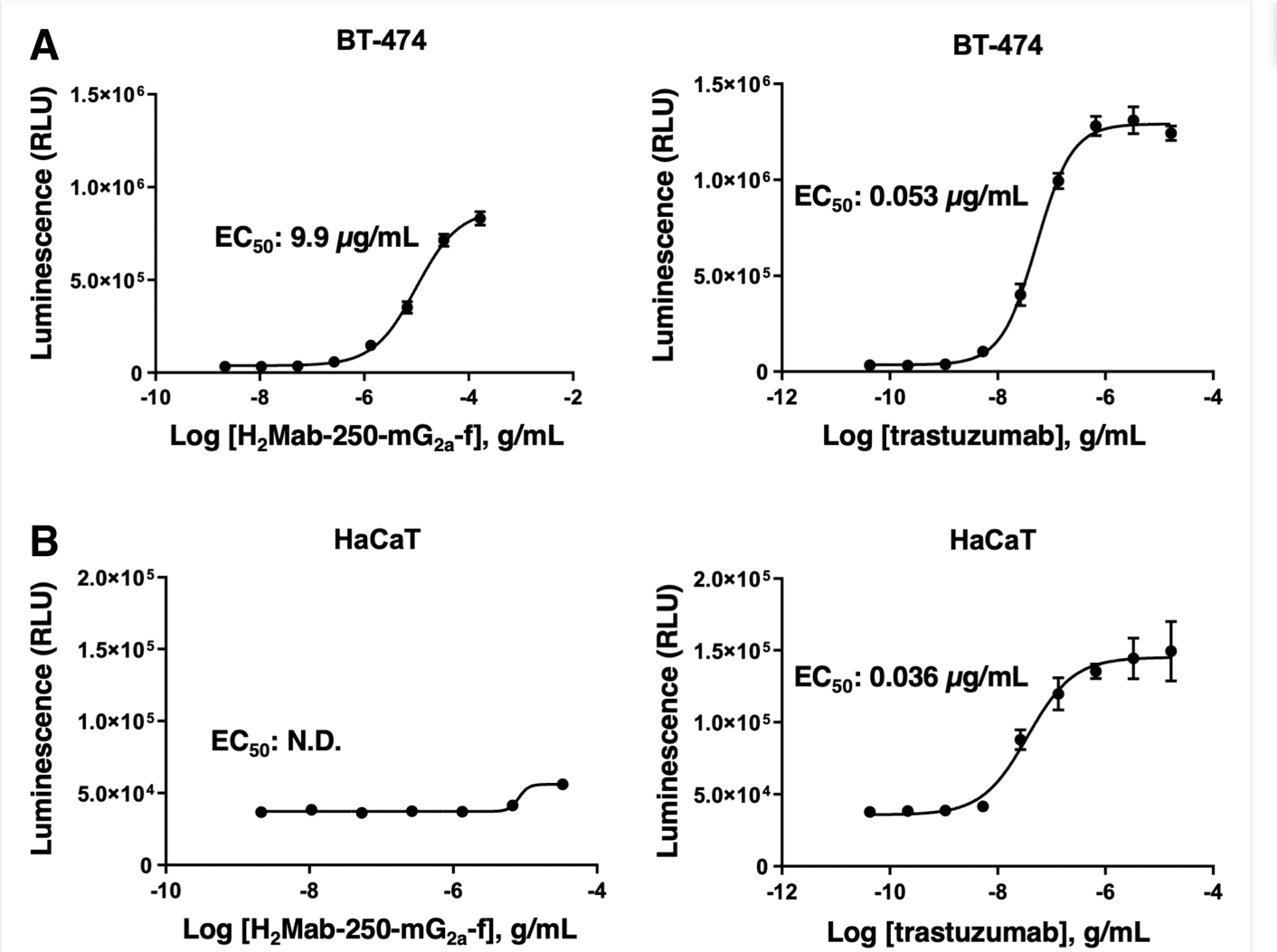
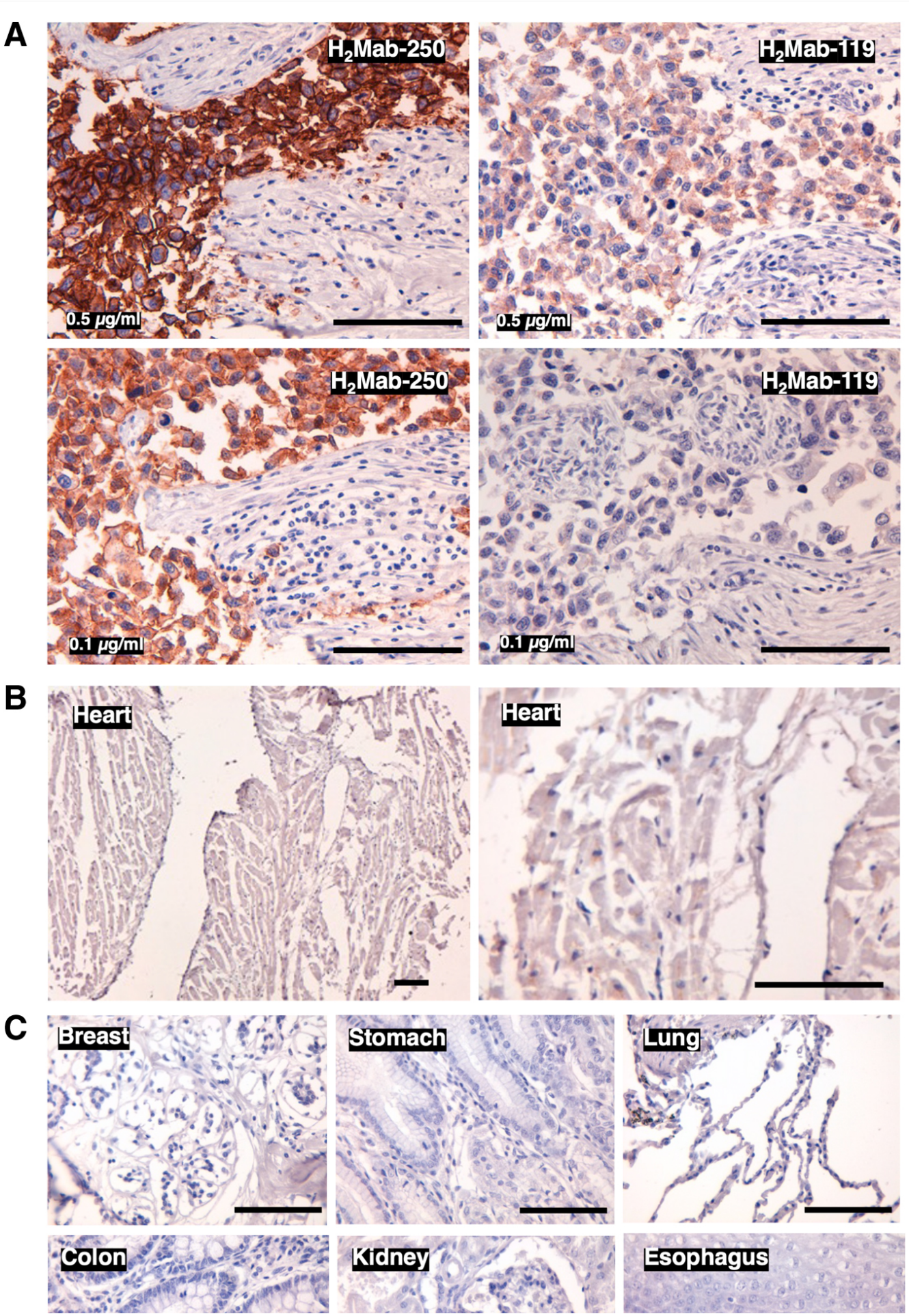


FIG. 2. ADCC reporter assay by H_2Mab-250-mG_{2a}-f and trastuzumab in the presence of BT-474 and HaCaT cells. Target cells such as BT-474 **(A)** or HaCaT **(B)** were cultured in a 96-well white solid plate. H_2Mab-250-mG_{2a}-f and trastuzumab were serially diluted and added to the target cells. The engineered Jurkat cells were then added and cocultured with antibody-treated target cells. Luminescence using the Bio-Glo Luciferase Assay System was measured using a GloMax luminometer. Error bars represent means ± SDs. ADCC, antibody-dependent cellular cytotoxicity; N.D., not determined; SD, standard deviation.

Immunohistochemical analysis of H_2Mab-250 in breast cancer and normal epithelium

Immunohistochemical analysis was performed to examine the reactivity of H_2Mab-250 with normal and tumor tissue sections. In contrast to flow cytometry, H_2Mab-250 exhibited more potent reactivity to the HER2-positive breast cancer section than H_2Mab-119 (Fig. 3A). Since all anti-HER2 therapeutic mAbs are associated with cardiotoxicity, a major adverse effect,[10] the reactivity of H_2Mab-250 to a normal heart was further investigated. Even with higher concentrations of H_2Mab-250 (1 μg/mL), no reactivity with the normal

heart could be detected (Fig. 3B). Finally, the reactivity of H_2Mab-250 to other normal tissues was investigated. As shown in Figure 3C, no reactivity of H_2Mab-250 with any normal tissues, including breast, stomach, lung, colon, kidney, and esophagus could be observed.


A
H2Mab-250
0.5 μg/ml
H2Mab-119
0.5 μg/ml
H2Mab-250
0.1 μg/ml
H2Mab-119
0.1 μg/ml
B
Heart
Heart
C
Breast
Stomach
Lung
Colon
Kidney
Esophagus

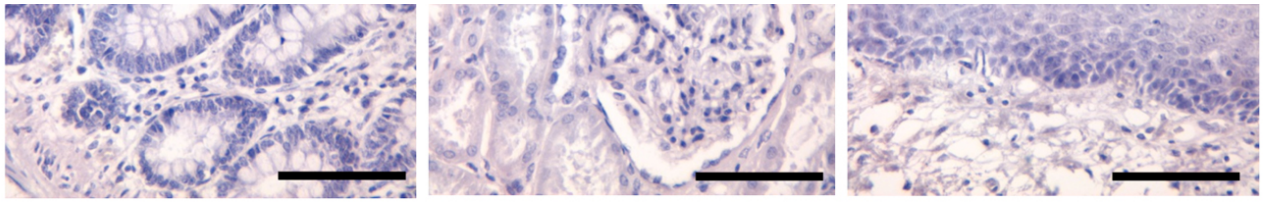


FIG. 3. Immunohistochemical analysis of H_2Mab-250 in breast cancer and normal epithelium. **(A)** The HER2-positive breast cancer tissue sections were treated with H_2Mab-250 or H_2Mab-119 (0.1 or 0.5 μg/mL). **(B)** A normal heart section was treated with H_2Mab-250 (1 μg/mL). **(C)** Sections of normal breast, stomach, lung, colon, kidney, and esophagus were treated with H_2Mab-250 (0.1 μg/mL). The sections were then treated with the Envision+ kit. The chromogenic reaction was performed using DAB, and the sections were counterstained with Hematoxylin. Scale bar = 100 μm. DAB, 3,3'-diaminobenzidine tetrahydrochloride.

Epitope identification for H_2Mab-250

To determine the epitope for H_2Mab-250, we examined the reactivity to CHO/HER2 (WT) and the N-terminal HER2 deletion mutant (dN218, dN342, and dN511)-expressed CHO-K1 cells (Fig. 4A, left). H_2Mab-250 reacted with dN218, dN342, dN511, and HER2 (WT). In contrast, H_2Mab-119 reacted with only WT, but not with dN218, dN342, and dN511. Since HER2 (WT) and the deletion mutants possess PA16 tag at the N-terminus, all expressions on the cell surface could be confirmed by anti-PA16 tag mAb, NZ-1 (Fig. 4A, right). These results suggest that H_2Mab-250 and H_2Mab-119 recognize the domain IV and domain I, respectively.

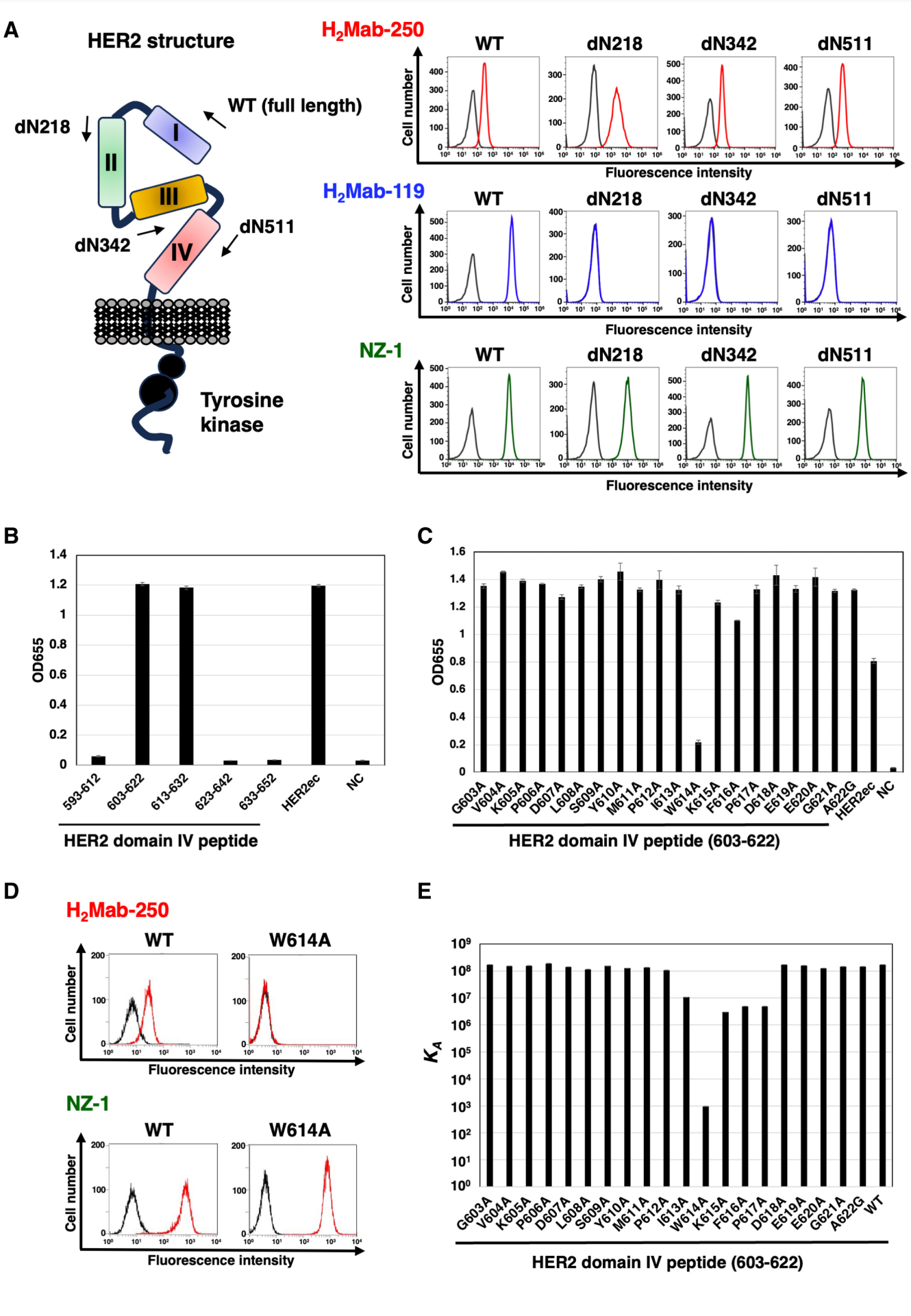

A
HER2 structure
WT (full length)
dN218
dN342
dN511
I
II
III
IV
Tyrosine kinase
H2Mab-250
H2Mab-119
NZ-1
WT
dN218
dN342
dN511
Cell number
Fluorescence intensity
B
OD655
593-612
603-622
613-632
623-642
633-652
HER2ec
NC
HER2 domain IV peptide
C
OD655
HER2 domain IV peptide (603-622)
D
H2Mab-250
NZ-1
WT
W614A
Cell number
Fluorescence intensity
E
KA
HER2 domain IV peptide (603-622)

FIG. 4. Epitope identification for H_2Mab-250. **(A)** Epitope determination of H_2Mab-250 and H_2Mab-119 using flow cytometry. The schematic representation of HER2 and the deletion mutants (left). Flow cytometry using H_2Mab-250 (10 μg/mL; red line) and H_2Mab-119 (10 μg/mL; blue line) against CHO/HER2 (WT and deletion mutants). The cell surface expression was confirmed by an anti-PA tag mAb, NZ-1 (10 μg/mL; green). The cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG for H_2Mab-250 and H_2Mab-119 or Alexa Fluor 488-conjugated anti-rat IgG for NZ-1. The black line represents the negative control (blocking buffer). **(B, C)** Determination of H_2Mab-250 epitope by ELISA. Five synthesized peptides that cover the HER2 domain IV **(B)**, alanine-substituted peptides of HER2 domain IV (603–622) **(C)**, HER2ec, or buffer control (NC) were immobilized on immunoplates. The plates were incubated with H_2Mab-250 (10 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. Optical density was measured at 655 nm. Error bars represent means ± SDs. **(D)** Flow cytometry using H_2Mab-250 (10 μg/mL; red line) against CHO/HER2 (WT and W614A). The cell surface expression was confirmed by an anti-PA tag mAb, NZ-1 (10 μg/mL; red line). The black line represents the negative control (blocking buffer). **(E)** Surface plasmon resonance analysis between H_2Mab-250 and HER2 domain IV (603–622) peptides. The affinity constant (K_A) at equilibrium was calculated as $1/K_D$. ELISA, enzyme-linked immunosorbent assay; HER2ec, HER2 ectodomain; mAb, monoclonal antibody; NC, negative control; SPR, surface plasmon resonance; WT, wild type.

For further assessment of the H_2Mab-250 epitope, ELISA was performed using synthetic peptides that cover the HER2 domain IV. As shown in Figure 4B, H_2Mab-250 reacted with HER2 domain IV peptide, amino acids 603–622 and 613–632, but not with 593–612, 623–642, and 633–652, indicating that H_2Mab-250 recognizes the 613–622 of HER2 domain IV. We further used alanine-substituted peptides of the 603–622 in HER2 domain IV. A potent reduction of the reactivity was observed in the W614A peptide (Fig. 4C). We confirmed that the reactivity of H_2Mab-250 completely disappeared in CHO/HER2 W614A cells in flow cytometry (Fig. 4D).

The K_D of H_2Mab-250 with the alanine-substituted peptides of HER2 domain IV (603–622) was measured using Biacore X100 (Table 1). The affinity constant (K_A) at equilibrium was calculated as $1/K_D$ (Fig. 4E). Compared with the K_A of the 603–622 (WT) peptide, decreased K_A values were observed from the 613–617 region, suggesting that the 613–617 region is involved in the binding to H_2Mab-250. A remarkable reduction was measured in the W614A peptide, indicating that Trp614 is mainly involved in the recognition by H_2Mab-250.

Table 1. Identification of H_2Mab-250 Epitope Using Point Mutants by Biacore

Peptide	Sequence	K_D (M)

Peptide	Sequence	K_D (M)
603–622 (WT)	GVKPDLSYMPIWKFPDEEGA	5.8×10^{-9}
G603A	AVKPDLSYMPIWKFPDEEGA	5.9×10^{-9}
V604A	GAKPDLSYMPIWKFPDEEGA	6.5×10^{-9}
K605A	GVAPDLSYMPIWKFPDEEGA	6.5×10^{-9}
P606A	GVKADLSYMPIWKFPDEEGA	5.3×10^{-9}
D607A	GVKPALSYMPIWKFPDEEGA	7.1×10^{-9}
L608A	GVKPDASYMPIWKFPDEEGA	8.8×10^{-9}
S609A	GVKPDLAYMPIWKFPDEEGA	6.5×10^{-9}
Y610A	GVKPDLSAMPIWKFPDEEGA	7.9×10^{-9}
M611A	GVKPDLSYAPIWKFPDEEGA	7.5×10^{-9}
P612A	GVKPDLSYMAIWKFPDEEGA	9.5×10^{-9}
I613A	GVKPDLSYMPAWKFPDEEGA	9.4×10^{-8}
W614A	GVKPDLSYMPIAKFPDEEGA	1.1×10^{-3}
K615A	GVKPDLSYMPIWAFPDEEGA	3.4×10^{-7}
F616A	GVKPDLSYMPIWKAPDEEGA	2.0×10^{-7}
P617A	GVKPDLSYMPIWKFADEEGA	2.1×10^{-7}
D618A	GVKPDLSYMPIWKFPAEEGA	5.8×10^{-9}
E619A	GVKPDLSYMPIWKFPDAEGA	6.3×10^{-9}
E620A	GVKPDLSYMPIWKFPDEAGA	8.0×10^{-9}
G621A	GVKPDLSYMPIWKFPDEEAA	6.9×10^{-9}
A622G	GVKPDLSYMPIWKFPDEEGG	6.9×10^{-9}

WT, wild type.

Discussion

In this study, we developed a cancer-specific mAb targeting HER2. H_2Mab-250 can recognize breast cancer cells, but not normal cells in flow cytometry (Fig. 1) and immunohistochemistry (Fig. 3). H_2Mab-250-mG_{2a}-f

could activate ADCC against breast cancer cells, but not against normal epithelial cells (Fig. 2). We also identified the H_2Mab-250 epitope sequence ($_{613}$IWKFP$_{617}$) by SPR analysis (Fig. 4). The $_{613}$IWKFP$_{617}$ sequence is partially included with the wider binding epitope of trastuzumab (residues 579–625).[24] Furthermore, no reaction was observed between H_2Mab-250 and CHO/HER2 W614A in flow cytometry (Fig. 4), indicating that Trp614 plays a central role in recognition by H_2Mab-250. Although H_2Mab-250 possesses a high affinity to epitope-containing peptide (603–622) in SPR analysis, the recognition in flow cytometry using cell lines was lower compared with H_2Mab-119 (Fig. 1). In contrast, H_2Mab-250 exhibited a higher reactivity than H_2Mab-119 in the immunohistochemical analysis using breast cancer tissues (Fig. 3).

This discrepancy might be induced by the possibility that the epitope sequence is partially exposed in cancer cells, but not in normal cells in clinical cancer tissues. The mechanism of recognition by H_2Mab-250 should be further investigated in future studies.

For the clinical treatment of metastatic breast cancer, trastuzumab is administered in patients with HER2-overexpressing tumors, which are defined by strong and complete IHC membranous staining of more than 10% of cells (IHC 3+) and/or *in situ* hybridization (ISH) amplified. Furthermore, trastuzumab-based antibody–drug conjugates (ADCs), such as trastuzumab–deruxtecan (T-DXd) have been evaluated in various clinical trials. Based on the studies, T-DXd has been approved in not only HER2-positive breast cancers,[25,26] but also HER2-mutant lung cancer[27] and HER2-low (IHC 1+ or IHC 2+/ISH-nonamplified) advanced breast cancer.[28] A significant number of patients can benefit from T-DXd therapy since approximately half of all breast cancers are classifiable as HER2-low.[29] Meanwhile, cardiotoxicity is the most significant toxicity associated with T-DXd.[30] Further studies are essential to evaluate *in vivo* toxicities of H_2Mab-250.

H_2Mab-250-mG_{2a}-f could trigger the ADCC activity to BT-474 selectively (Fig. 2). Although the effect of H_2Mab-250-mG_{2a}-f is lower compared with trastuzumab, we should consider that effector Jurkat cells express human FcγRIIIa receptor. In contrast, H_2Mab-250 exhibited a superior reactivity to HER2-positive breast cancer tissue sections in immunohistochemistry (Fig. 3). Chimeric antigen receptor (CAR)-T cell therapy against HER2 has been evaluated in clinical studies.[29] It would be worthwhile to investigate the cancer specificity of H_2Mab-250 scFv and the efficacy of CAR-T against HER2-positive tumors in future studies. In the future, H_2Mab-250 could contribute to the development of CAR-T or ADCs without adverse effects for breast cancer therapy.

Supplementary Material

File (suppl_figures1.docx)

DOWNLOAD | 168.96 KB

File (suppl_figures2.docx)

DOWNLOAD | 175.84 KB

References

1. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–137;

Go to Citation | Crossref | PubMed | Google Scholar

2. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–182;

Go to Citation | Crossref | PubMed | Google Scholar

3. Van Cutsem E, Bang YJ, Feng-Yi F, et al. HER2 screening data from ToGA: Targeting HER2 in gastric and gastroesophageal junction cancer. *Gastric Cancer* 2015;18:476–484;

SHOW ALL REFERENCES

VIEW FULL TEXT | DOWNLOAD PDF