A Novel Antibody Engineering Strategy for Making Monovalent Bispecific Heterodimeric IgG Antibodies by Electrostatic Steering Mechanism*

Producing pure and well behaved bispecific antibodies (bsAbs) on a large scale for preclinical and clinical testing is a challenging task. Here, we describe a new strategy for making monovalent bispecific heterodimeric IgG antibodies in mammalian cells. We applied an electrostatic steering mechanism to engineer antibody light chain-heavy chain (LC-HC) interface residues in such a way that each LC strongly favors its cognate HC when two different HCs and two different LCs are co-expressed in the same cell to assemble a functional bispecific antibody. We produced heterodimeric IgGs from transiently and stably transfected mammalian cells. The engineered heterodimeric IgG molecules maintain the overall IgG structure with correct LC-HC pairings, bind to two different antigens with comparable affinity when compared with their parental antibodies, and retain the functionality of parental antibodies in biological assays. In addition, the bispecific heterodimeric IgG derived from anti-HER2 and anti-EGF receptor (EGFR) antibody was shown to induce a higher level of receptor internalization than the combination of two parental antibodies. Mouse xenograft BxPC-3, Panc-1, and Calu-3 human tumor models showed that the heterodimeric IgGs strongly inhibited tumor growth. The described approach can be used to generate tools from two pre-existent antibodies and explore the potential of bispecific antibodies. The asymmetrically engineered Fc variants for antibody-dependent cellular cytotoxicity enhancement could be embedded in monovalent bispecific heterodimeric IgG to make best-in-class therapeutic antibodies.

Pancreatic cancer is the fourth leading cause of cancer death in western countries with a 5-year survival of less than 10% (1); there is a pressing need for developing therapeutic agents to improve the survival rate. Overexpression of EGFR in 40–60% of cases and overexpression of HER2 in some subsets were observed in pancreatic cancer patients (2). Heterodimeric HER2-EGFR is more active than either the HER2 or EGFR homodimer in transducing proliferative signals (3, 4). Blocking the EGFR alone by cetuximab increased HER2 signaling via amplification of HER2 or increased levels of the HER3/HER4 ligand heregulin (5, 6), leading to resistance to the treatment. Dual inhibition of HER2 and EGFR was proposed as a plausible therapeutic strategy to improve treatment outcome (7, 8), because this strategy could take advantage of blocking both HER2- and EGFR-mediated signaling pathways and reduce the chance for tumor cells to develop drug resistance. A promising approach is to make bsAbs against both HER2 and EGFR.

bsAbs that can target two antigens or two epitopes on the same antigen have long been considered as an attractive approach to combine the additive or potentially synergistic effects exhibited by the combination of two monoclonal antibodies (9, 10). Over the past 2 decades, more than 45 different formats of bsAb, including DVD-Ig (11), crossover Ig (12), dual-acting Fab (13), and BiTE (14), have been developed for different biological applications (15). Nevertheless, many of these formats have been limited by some of their liabilities, such as instability, short half-life, poor manufacturability, and immunogenicity.
Heterodimeric IgG, which is based on the heterodimerization of two different IgG molecules in the Fc region, is a promising bsAb format because it maintains the overall size and natural structure of the regular IgG with good bioavailability and pharmacokinetics profile (16). When co-expressing two different HCs and two different LCs in the same cell to generate a functional IgG bsAb, only 1 in 10 combinations has the correct configuration (17). Engineering the C\textsubscript{\textgamma}3 domain of antibody by knob-into-hole (18), or SEED (19), or charge pair residues (20) can promote HC heterodimerization and reduce the combinations to four, but the LC-HC mispairing issue still exists. One solution is to use a common LC (21), but it is time consuming to identify and engineer a promiscuous LC that can accommodate two different HCs while maintaining the desirable functional properties. Catumaxomab, a mouse IgG2a and rat IgG2b hybrid mAb, was produced by quadroma technology by fusing two hybridoma cell lines (22, 23). However, bsAbs derived from quadromas need extensive purification steps and are typically of rodent origin, which limit their applications.

Other approaches have been explored to make monovalent bispecific antibodies. Strop et al. (24) and Labrijn et al. (25) have engineered the hinge region and C\textsubscript{\textgamma}3 domain of Fc, separately expressed the parental antibody, and then assembled to full size bsAb by partial reduction and oxidation. This approach requires generation of two master cell lines and extra post-production purification steps to clean up the final products. Ideally, one would like to produce the bsAb using a single cell line. Spiess et al. (26) have made IgG bsAbs by co-culturing two transformed Escherichia coli cell lines, but the antibodies produced by this approach lack the carbohydrate in the Fc region, which is important for effector functions.

Here, we describe a new method of generating bsAbs from two different pre-existent antibodies by electrostatic steering mechanism. The format of bsAb, which we refer as hetero-IgG, was produced by engineering the HC and LC of the two different antibodies in such a way that they can assemble exclusively into a bsAb without other contaminating species. This was based on the charge pair strategy for C\textsubscript{\textgamma}3 engineering (20) so that two different HCs form a heterodimer exclusively. Similarly by applying an electrostatic steering effect to engineer interface residues between LC and HC, the potential mispairing of LCs to noncognate HCs can be prevented.

The strategy described herein can be used to efficiently produce a full-length bsAb from two pre-existent antibodies in mammalian cells without using any artificial linkers. The resulting bsAbs are stable and amenable to commercial manufacturing without excessive aggregation or loss of yield. As this new version of bsAb can target two different antigens or two different epitopes on the same antigen, it may have significant potential for treating serious diseases with unmet needs such as pancreatic cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell lines BxPC-3, Panc-1, Colo699, JIMT-1, SkBR-3, BT-474, Calu-3, and CHO-K1 were obtained from American Type Culture Collection (ATCC, Manassas, VA). The NCI-N87 (catalog no. ACC 589) was purchased from DSMZ (Braunschweig, Germany). The cells were cultured in appropriate growth medium as recommended by vendors. Anti-EGFR capture antibodies (catalog no. 2646S and NB100-595) was obtained from Cell Signaling Technology and Novus Biologicals, respectively. Anti-Tyr(P) antibody (catalog no. 05-321MG) was purchased from Millipore. HRP-conjugated goat anti-mouse IgG Fc (catalog no. 110-035-164) was purchased from Jackson ImmunoResearch. MSD SULFO-TAG labeled pan-tyrosine (Tyr(P)-20) detection antibody, SULFO-TAG labeled goat anti-rabbit IgG secondary detection antibody (catalog no. R32AB), and phospho-AKT (Ser-473) assay whole cell lysate kit (catalog no. K151CAD-3) were obtained from Meso Scale Discovery (Gaithersburg, MD). 2,2'-Azobis(3-ethylbenzthiazoline-6-sulfonic acid) (catalog no. A9941), TCEP (catalog no. 75259), EGF (catalog no. E5036), polyethyleneimine (catalog no. 408727), and goat anti-human IgG Fc-specific HRP-conjugated polyclonal antibody (catalog no. A0170) were purchased from Sigma. EZ-Link NHS-PEO4 biotinylation kit (catalog no. 21455), SuperSignal West Pico chemiluminescent substrate (catalog no. 34080), CL-X Posure™ x-ray films (catalog no. 34091), streptavidin-HRP (catalog no. 21130), and NeutrAvidin (catalog no. 31005) were obtained from Thermo Scientific (Rockford, IL). QuikChange Lightning multiple site-directed mutagenesis kit (catalog no. 210516) was from Agilent Technologies. Restriction enzymes (Sall, NotI, BamHI, NheI, and BsiWI) and PNGase F were from New England Biolabs. NK cell isolation kit (catalog no. 130-092-657) was purchased from Miltenyi Biotec. Primary human NK cells were isolated from leukopheresis products obtained from Puget Sound Blood Center. Assay plates (catalog no. 3904 and 3368) were purchased from Corning Costar. CellTiter-Glo® (catalog no. G7573) was obtained from Promega. Human immune globulin infusion (huIVIG) (catalog no. NDC 0944-2700-04) was purchased from Baxter (Deerfield, IL). The following reagents were purchased from R & D Systems: recombinant extracellular domain of EGFR (catalog no. 1095-ER); recombinant human EGFR (catalog no. 236-EG); recombinant human neuregulin 1-β1/HRG1-β1 EGF domain (catalog no. 396-HB-050/CF); HER2-Fc (catalog no. 1129-ER); anti-human HER2 capture antibody (catalog no. AF1129); anti-HER3 capture antibody (catalog no. Mab3481); and human phospho-ErbB3 ELISA DuoSet IC kit (catalog no. DYC1769). Biotinylated huHER2 ECD protein (catalog no. HE2 H8225) was purchased from ACRO Biosystems (Newark, DE). Biotinylated huEGFR (ECD)-Fc (rabbit) was made in-house. Pooled normal human serum (catalog no. IPLA-SER) was purchased from Innovative Research, Inc. (Novi, MI). Female CB-17 SCID and female NSG mice were purchased from Charles River Laboratories (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME), respectively. The Rag2−/− MEFcyr4−/−/hCD16a−/−/C57 BL/6 mice (mouse Fcyr4 and Rag2 knockouts and transgenic for human CD16a-158F) were generated at Amgen and bred at Charles River Laboratory (San Diego). All animal experiments were conducted in compliance with Canadian Council on Animal Care specifications.

**Computational Analysis**—Antibody crystal structures were identified from the Protein Data Bank. Two methods were used to identify the residues involved in the light-heavy chain interaction: 1) contact as determined by distance limit criterion and...
2) solvent-accessible surface area analysis. According to the contact based method, interface residues are defined as residues whose side chain heavy atoms are positioned closer than a specified limit (5 Å) from the heavy atoms of any residues in the second chain. The second method involves calculating solvent-accessible surface area of the residues in the presence and absence of the second chain. The residues that show a difference of >1 Å² in accessible surface area between the two calculations are identified as interface residues. Both methods identified a similar set of interface residues. The following criteria were further applied to select VH-VL and C₄₁-CL interface residue pairs for mutagenesis: 1) they should not be in CDRs and do not make contact with the CDR residues; 2) they are highly conserved among IgG antibody subtypes; 3) they are mostly solvent-inaccessible (i.e. buried or partially buried); and 4) they have minimum interference for BiP-C₄₁ binding (27–31). For immunogenicity predictions, the TEPITOPE algorithm was utilized to identify potential nontolerant agpetopes as described by Sturniolo et al. (32). The nontolerant agpetopes could bind to HLA class II molecules to elicit immune responses. Tertiary structural information of the antibody was not considered in this analysis. Rather, an exhaustive search of all linear 9-residue peptides that could bind to HLA class II molecules was performed. Such peptide-HLA complexes could drive T lymphocyte-dependent immune responses. The analysis focused on the eight most common HLA-DRB1 alleles (0101, 0301, 0401, 1101, 1102, 1103, 1104, and 11022), which cover >95% of human populations.

**Gene Synthesis and Mutagenesis to Make Variants**—The amino acid sequences of anti-HER2 (trastuzumab, clone humAb4D5-8) (33), anti-HER2 (pertuzumab, clone humAb2C4) (34), and anti-EGFR (panitumumab, clone E7.6.3) (35) were used to design the DNA sequences after codon optimization for mammalian expression using GeneArt program (Invitrogen). The DNAs encoding Vκ1 O2/O12 signal peptide and variable regions with flanking sequences for restriction enzyme digestion were synthesized by Invitrogen. PCRs using PfuTurbo Hot Start were carried out to amplify the inserts, which were then digested by SalI and NheI for VH and VL, respectively. The double-digested VH fragments were ligated with SalI and NheI-treated pTT5 expression vector (36) in which the human IgG1 C₄₁ + hinge + C₄₂ + C₄₃ domains were already inserted. The double-digested VL fragments were ligated with SalI and BsiWI-treated pTT5 vector in which the human κ constant domain was already inserted. Plasmid DNAs were verified by double strand DNA sequencing. For proof-of-concept studies, an Fn3 tag was inserted in-frame at the N terminus of anti-EGFR HC, and an Fn3-FLAG-Hi6k tag was fused in-frame to the C terminus of anti-EGFR LC. Mutagenesis reactions were carried out to introduce the pairs of charged residues by using QuikChange Lightning multisite-directed mutagenesis kit according to the manufacturer’s recommendations. Double strand DNA sequencing reactions were conducted to confirm the mutant sequences.

**Expression and Purification of Variants**—For 20 ml of medium scale expression testing, a total of 10 μg of plasmid DNAs in pTT5 (1.5 μg of HC1, 3.5 μg of LC1, 1.5 μg of HC2, and 3.5 μg of LC2) were mixed in a 1.5-ml Eppendorf tube; 1 ml of 293 SFM medium containing 10 μl of 3 mg/ml polyethyleneimine, pH 7.0, was added and incubated at room temperature for 20 min. The mixture of DNA/polyethyleneimine was loaded into 19 ml of 293E cells at 1–2 × 10⁶/ml in a 125-ml shaking flask. 0.5 ml of 20% Yeastolate was added to each flask to a final 0.5% the next day. Cells were shaken for 6 more days. The supernatant was harvested by centrifuging cells at 3000 rpm for 15 min. For 5-ml small scale chain drop-out transfections in 24-well plates or for 1-liter large scale production in shaking flasks, the above conditions were scaled down or up proportionally. The harvested supernatant at large scale was purified by protein A column followed by a polishing with Superdex 200 size exclusion column. For chain drop-out experiments, only two plasmid DNAs (either matched or mismatched) were co-transfected in 293E cells using the same condition as above, 6 days post-transfection, and 10 μl/m supernatant was loaded in 8–16% Tris-glycine SDS-PAGE and subjected to Western blotting.

**Dual Antigen-binding Plate ELISA**—huHER2-Fc fusion protein was coated with 100 μl/well at 2 μg/ml in 1× PBS, pH 7.4, in Maxisorp plates at 4 °C overnight. The plates were washed five times with 1× PBS containing 0.05% Tween 20 (1× PBST) and then blocked with 3% nonfat milk/1× PBST at 200 μl/well with shaking at room temperature for 1 h. 100 μl/well of normalized crude supernatants in 1× PBS at 1:3 series dilutions were added. The plates were incubated at room temperature for 1 h with shaking, followed by five washes with 1× PBST. 100 μl/well of 1 μg/ml biotin-huEGFR protein in 1× PBS was added, and plates were shaken at room temperature for 1 h. After five washes with 1× PBST, 100 μl/well of 0.1 μg/ml streptavidin-conjugated HRP in 1× PBS was added. The plates were shaken at room temperature for 1 h followed by five washes with 1× PBST. 100 μl/well 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) substrate in 1× PBS was added for color development. The data were collected in Victor II machine by reading at 405 nm for 0.1 s per well.

**Stable CHO-K1 Cell Line Development**—A DNA fragment encoding a furin recognition site (RRRRRR) and a spacer and a self-cleaving peptide (scp) (37, 38) were linked between the C termini of HC2 and N termini of HC1 by overlapping PCR reactions; the full-length DNA encoding HC2-R6-spacer-scp-HC1 was purified from 1.5% agarose gel and subcloned in pSLX240 puromycin vector treated with SalI and NotI restriction enzymes. Similarly, the same R6-spacer-scp DNA fragment was inserted between the C termini of LC2 and N termini of LC1, and the full-length DNA encoding LC2-R6-spacer-scp-LC1 was subcloned in pSLX240 hygromycin vector treated by SalI and NotI restriction enzymes. Suspension-adapted CHO-K1 cells were transfected with Lipofectamine using a 1:1 ratio (by mass) of expression vectors. Transfected cells were cultured in proprietary media and selected at 10 μg/ml puromycin and 600 μg/ml hygromycin B. Cells were cultivated in suspension format using disposable shake flasks and rotated in a humidified incubator (37 °C 5% CO₂) at 150 rpm. The selection media were replaced every 3–4 days for a duration of ~3 weeks until the pools were fully recovered with >90% viability. Protein production was carried out by inoculating a nutrient-rich proprietary media with 2 × 10⁶ cells/ml and
incubating at 37 °C 5% CO₂ for 7 days. Culture media were harvested from the production by centrifugation (3,000 × g, 5 min) followed by 0.22-μm sterile filter. Concentration of the protein expression in the harvested media was determined by an Octet RED96 (Fortebio) using protein A biosensors.

**SDS-PAGE and Western Blotting—**SDS-PAGE was carried out using NuPAGE 8–16% Tris-glycine gels and corresponding running buffer. Samples were prepared by combining the harvested supernatant with 2× SDS sample buffer and heating for 5 min at 95 °C. Preparation of reduced samples included the addition of NuPAGE reducing agent prior to heating. After electrophoresis, proteins in the gel were stained with either Coomassie Blue or transferred to nitrocellulose membrane using an iBlot (Invitrogen). For supernatant from stable CHO-K1 cells, the nitrocellulose membrane was blocked with fluorescent Western blocking buffer and probed with IR Dye 700 DX-conjugated donkey anti-human IgG antibody. The fluorescent Western blocking buffer and probed with IR Dye using an iBlot (Invitrogen). For supernatant from stable CHO-K1 cells, the nitrocellulose membrane was blocked with fluorescent Western blocking buffer and probed with IR Dye 700 DX-conjugated donkey anti-human IgG antibody. The nitrocellulose membrane was then thoroughly washed in 1× PBST, and images were acquired by using an Odyssey® infrared imaging system from LI-COR Biosciences. For chain drop-out experiments, the nitrocellulose membrane was blocked with 3% milk/1× PBST and probed with HRP-conjugated goat anti-human IgG (Fc-specific) and developed with SuperSignal West Pico chemiluminescent substrate.

**Mass Spectrometry Analysis—**Treatment for deglycosylation and complete reduction and/or partial reduction of bsAb were carried out as follows. 20 μg of bsAb was deglycosylated by incubation with 1 μl of PNGase F in 20 μl of 50 mM Tris buffer, pH 7.2, at 37 °C for 18 h. 5 μg of deglycosylated or nondeglycosylated bsAb was completely reduced by 9 mM DTT in 20 μl of 4 mM guanidine HCl, 50 mM Tris buffer, pH 8.0, at 55 °C for 15 min. 10 μg of deglycosylated bsAb was partially reduced by incubation with 2-fold (at molar ratio) TCEP in 20 μl of 50 mM Tris buffer, pH 7.2, at 37 °C for 50–120 min. Intact mass analysis of deglycosylated and nondeglycosylated whole bsAb and completely reduced or partially reduced bsAb was done in an HPLC-ESI-TOF system (Agilent 6210 TOF mass spectrometer in combination with an Agilent 1200 liquid chromatography system, Santa Clara, CA). A 2.1 × 150-mm Pursuit diphenyl column with 5 μm particle size (Agilent-Varian Inc, Santa Clara, CA) was connected to the liquid chromatography system and operated at 400 ml/min. The column temperature was 75 °C; solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. The gradient started at 25% B and increased linearly to 80% B over 30 min. The TOF mass spectrometer was tuned and calibrated in the range of 100–4500 m/z. The capillary voltage was set at 4500 V, drying gas at 12 liters/min, drying gas temperature at 300 °C, nebulizer gas flow at 40 liters/min, and fragmentor voltage at 375 V for intact antibodies and 300 V for reduced antibodies.

**Thermal Stability Analysis by Differential Scanning Calorimetry (DSC)—**The DSC measurements were obtained using a VP-Capillary DSC system (Microcal Inc., Northampton, MA) equipped with tantalum 61 cells, each with an active volume of 125 μl. Protein samples were diluted to 0.5 mg/ml, and the corresponding buffer was used as a reference. The samples were scanned from 20 to 110 °C at a rate of 20 °C/h with an initial 15 min of equilibration at 20 °C. A filtering period of 16 s was used, and data were analyzed using Origin 7.0 software (OriginLab Corp., Northampton, MA). Thermograms were corrected by subtraction of buffer-only blank scans. The corrected thermograms were normalized for protein concentration. The melting temperatures represent peaks in the experimental thermograms, and the enthalpy of unfolding was obtained using the Origin 7.0 software by integration of the area under the melting curves.

**Surface Plasmon Binding Analysis to Measure the Affinity of Hetero-IgG1 Variants to Antigens—** Biosensor analysis was conducted at 25 °C in an HBS-EP buffer system (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P20) using a ProteOn XPR36 optical biosensor equipped with a GLC sensor chip (Bio-Rad). Goat anti-human IgG capture antibody was immobilized to all channels in the horizontal direction of the sensor chip using standard amine coupling chemistry to a level of 5600–6000 response units. Channels 1–6 in the vertical direction were used to capture antibodies (~100 response units). Five different ruHER2 or ruEGFR concentrations ranging from 25.0 to 0.309 nM (3-fold series dilutions) were prepared in running buffer. Each of the five analyte sample concentrations was injected simultaneously over the chip surface in triplicate in the horizontal direction, as a means of assessing the reproducibility of binding and managing potential systematic bias. Blank buffer injections were run simultaneously with the five analyte concentrations and were used to assess and subtract system artifacts. The association phases were monitored for 420 s each, at a flow rate of 50 μl/min, and the dissociation phases were monitored for 3600 s, at a flow rate of 50 μl/min. The surface was regenerated with 10 mM glycine, pH 1.5, for 30 s, at a flow rate of 50 μl/min. The data were aligned and double-referenced using the ProteOn Manager 3.1.0 version 3.1.0© software (Bio-Rad). The data were then fit using Scrubber version 2.0© software (BioLogic Software Pty. Ltd., Campbell, Australia), which is an SPR nonlinear least squares regression fitting program. First, a dissociation rate coefficient (kd) was determined from the 25 nM ruHER2 or ruEGFR 3600-s dissociation phase data. Second, this value was applied as a fixed parameter in the global fit of the 420-s association phase data to a 1:1 binding model, to determine the association rate coefficient (ka) and the Rmax value.

**ADCC Assay—**Cultured human tumor cells were harvested using pre-warmed trypsin, washed two times with sterile PBS, and seeded at 1 × 10⁷ cells per well in 96-well black/clear bottom plates in 100 μl of their respective growth media. Plates were incubated at 37 °C/5% CO₂ overnight. Hetero-IgG1 antibodies, parental antibodies, and irrelevant antibody were titrated from 6.7 nM to 0.67 fm in Immune Cell Media (ICM) (RPMI 1640, 10% heat-inactivated FBS, 5 mM l-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 55 mM 2-mercaptoethanol). After a wash with PBS, 25 μl of each antibody titration was added to the well containing target cells. Primary human NK cells (FcγRIIIA 158F/F genotype) were washed two times in pre-warmed ICM. 50 μl of NK cells were added to each well at an effector/target ratio of 10:1. Target cells alone and effector cells alone (as controls) were included in each plate. ADCC activity was determined after an overnight incubation at 37 °C/5% CO₂. After washing out the NK cells with 1× PBS,
viable target cells were detected using CellTiter-Glo® as per the manufacturer’s instructions and using a Wallac VICTOR microplate reader. Percent specific lysis was calculated as (RLU values of treated samples – average RLU value of effector alone)/(the average RLU of untreated cells (effector + target) – average RLU of effector alone)×100. Percent specific lysis values were transferred to a graphic program (GraphPad Prism) where the data were transformed in a sigmoidal curve fit graph.

EGF-induced EGFR Phosphorylation Assay and Basal Level HER3 Phosphorylation Assay—MSD assay plates were coated with 30 μl/well of EGFR capture antibody or HER3 capture antibody Mab3481 at 1 μg/ml in 1× TBS at 4 °C overnight. Plates were washed three times with 200 μl/well 1× TBST and then blocked with 200 μl/well of 3% blocker A in 1× TBST. Plates were incubated at room temperature with shaking for 1 h followed by three washes with 200 μl/well 1× TBST. Plates were tapped to dry waiting for the addition of cell lysate. CHO-huEGFR stable cells or BT-474 cells both at 50,000 cells/well were seeded in 160 μl of complete growth medium in 96-well plates and incubated at 37 °C 5% CO2 overnight. The next day, 30 μl of 0.5% BSA was added in triplicate and incubated at 37 °C with 5% CO2 for 30 min. The CHO-huEGFR stable cells were stimulated with 5 nM EGF for 15 min at 37 °C. The liquid was tossed, and 50 μl/well of cell lysate was added to the above treated MSD assay plates that were then shaken for 1 h at room temperature. Plates were washed three times with 200 μl/well 1× TBST, 50 μl/well 3% Blocker A in 1× TBST, 150 μl/well 1% Blocker A/1× PBS at room temperature for 1 h, followed by three washes with Tris wash buffer. MCF-7 cells were treated the same manner as above except MSD SULFO-TAG-labeled pan-tyrosine (Tyr(P))-20 detection antibody diluted to 1.5 μg/ml in 1% Blocker A/1× TBST was added to wells containing the lysate from BT-474 cells. Plates were shaken at room temperature for 1 h followed by three washes with 200 μl/well 1× TBST. 150 μl/well of 1× Read Buffer was added, and the data were collected by reading the plates in MSD Spectra Manager 6000.

Inhibition of EGFR Phosphorylation on BxPC-3 Cells and of HER2, HER3, and AKT Phosphorylation on MCF-7 Cells—96-Well ELISA plates were coated with respective anti-EGFR or HER2 or HER3 capture antibody at 2 μg/ml for EGFR and HER2, or 4 μg/ml for HER3 in 1× PBS at room temperature overnight. The plates were washed three times with 1× PBST and then blocked with 300 μl/well 3% BSA in 1× PBS at room temperature for 1 h followed by three washes with 300 μl/well 1× PBST. BxPC-3 cells at 20,000 cells/well and MCF-7 at 50,000 cells/well were seeded in 96-well tissue culture plates in their completed growth medium and were incubated at 37 °C, 5% CO2 overnight. The next day, BxPC-3 cells were treated with either 15 μg/ml anti-HER2 humAb4D5 IgG1 plus 15 μg/ml hulgG1 isotype, 15 μg/ml anti-EGFR E7.6.3 IgG1 plus 15 μg/ml hulgG1 isotype, 15 μg/ml anti-HER2 humAb4D5–8 IgG1 plus 15 μg/ml anti-EGFR E7.6.3 IgG1, 30 μg/ml anti-HER2 × EGFR hetero-IG2 V23, or 30 μg/ml anti-HER2 × EGFR hetero-IG2 V23_W165 at 1:3 serial dilution titration at room temperature for 1 h. MCF-7 cells were treated with either 15 μg/ml anti-HER2 humAb4D5–8 IgG1 plus 15 μg/ml hulgG1 isotype, 15 μg/ml anti-HER2 humAb2C4 IgG1 plus 15 μg/ml hulgG1 isotype, 15 μg/ml anti-HER2 humAb4D5–8 IgG1 plus 15 μg/ml anti-HER2 humAb2C4 IgG1, 30 μg/ml anti-HER2 (humAb4D5-8) × HER2 (humAb2C4) hetero-IgG1 V23, or 30 μg/ml anti-HER2 (humAb4D5-8) × HER2 (humAb2C4) hetero-IgG1 V23_W165 at 1:3 serial dilution titration at room temperature for 1 h. The end of the antibody treatment, BxPC-3 and MCF-7 cells were incubated with 16.67 nm EGFR and 12.5 nm neuregulin 1-β1 at 37 °C for 7 min, respectively, followed by one wash with ice-cold 1× PBS. The 1× PBS was tossed, and 60 μl/well of ice-cold lysis buffer containing phosphatase and protease inhibitors was added to lyse the cells on ice for 30 min. Aliquot of 50 μl/well cell lysate in triplicate was transferred into ELISA plates that were coated with the capture antibodies. The plates were incubated at 4 °C overnight followed by three washes with 300 μl/well of 1× PBST. Anti-Tyr(P) detection antibody (4G10) at 0.5 μg/ml in 1× PBST at 50 μl/well was added and incubated at room temperature for 2 h. After three washes with PBST, 1 μg/ml HRP-conjugated goat anti-mouse IgG Fc at 50 μl/well was added. Plates were incubated at room temperature for 1 h. After three washes, 50 μl/well of 3,3’,5’,5’-tetramethylbenzidine substrate was added for color development. 25 μl/well of 1 N HCl was added to stop the reactions. Absorbance at 450 nm was recorded in Thermo Multiskan Ascent reader. For the detection of pAkt in MCF-7 cells, MSD plates that have been pre-coated with anti-AKT capture antibody were blocked with 3% BSA in Tris wash buffer at room temperature for 1 h, followed by three washes with Tris wash buffer. MCF-7 cells were treated the same manner as above except MSD SULFO-TAG-labeled pAkt (Ser-473) detection antibody was used. The percent specific inhibition was calculated as (1 – RLU value of treated sample/average RLU of untreated cells)×100.

Internalization Assay—Tumor cell monolayers in a flat bottom 96-well plate were exposed to 100 μl/well of either control human IgG1, anti-HER2 humAb4D5–8 IgG1 alone, anti-EGFR E7.6.3 IgG1 alone, anti-HER2 × EGFR hetero-IgG V23 at final concentration of 5 μg/ml (34 nm), or the combination of anti-HER2 humAb4D5–8 IgG1 and anti-EGFR E7.6.3 IgG1 each at final concentration of 2.5 μg/ml (17 nm). Cells were incubated at 4 °C for 30 min. After washing once with assay buffer (1× PBS containing 5% FBS), a mixture of Alexa 488-conjugated anti-human IgG (H+L) at a dilution of 1:1000 and Hoechst 33342 at a dilution of 1:2000 were added to cells that were then incubated at 4 °C for 30 min. For the cells designated as time point 0 h, they were washed twice with assay buffer, then fixed, and permeabilized with BD cytofix/cytoperm buffer (BD Biosciences). For the cells designated as time point 1, 2, or 4 h, the cells were washed twice with assay buffer and incubated at 37 °C 5% CO2 for 1, 2, or 4 h, respectively, then fixed, and permeabilized with BD cytofix/cytoperm buffer (BD Biosciences). Cells were analyzed by an ArrayScan VTi HCS reader (Cellomics of
**Hetero-IgG Antibody with Cognate LC-HC Pairings**

Thermo Fisher Scientific) with BioApplication “Spot Detector” set at ×40 objective. Images were captured with a Leica fluorescent microscope (DMi6000B) connected to Leica digital camera (CTR6500).

**BxPC-3, Panc-1, and Calu-3 Xenograft Murine Models—** Female CB-17 SCID mice (7–8 weeks old) were implanted subcutaneously with 5 × 10⁶ BxPC-3 cells mixed 2:1 with Matrigel (BD Biosciences) in a total volume of 100 µL. Ten days post-tumor implantation, the tumor volume was measured, and mice were randomly distributed to control and treatment groups with 10 mice in each group, so that the mean tumor size was similar across groups at the beginning of the treatment. Starting on day 10, the mice were treated intraperitoneally in a volume of 200 µL once a week for 5 weeks with either anti-HER2 humAb4D5–8 IgG1 (250 µg), anti-EGFR E7.6.3 IgG1 (250 µg), the combination of these two parental antibodies (250 µg each), anti-HER2 × EGFR hetero-IgG1 (V23, 500 µg), or ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 (V23_W165, 500 µg). Animals receiving saline served as the vehicle control. Rag2−/−/mFcyRII/III−/−/huCD16a−/− C57 BL/6 mice (8–9 weeks old) were implanted subcutaneously with 5 × 10⁶ Panc-1 cells mixed 2:1 with Matrigel in a total volume of 100 µL. Six days post-tumor implantation, the tumor volume was measured, and the mice were randomly distributed to six groups with eight mice in each group. One hour prior to the antibody treatments, mice were injected intraperitoneally with 10 mg of huL1V1G plus 0.2 mg of mouse FcγR II/III blocker 2.4G2, followed by intraperitoneal treatment with either 250 µg of anti-HER2 humAb4D5–8 IgG1 plus 250 µg of huL1V1G isotype, 250 µg of anti-EGFR E7.6.3 IgG1 plus 250 µg of huL1V1G isotype, 250 µg of anti-HER2 humAb4D5–8 IgG1 plus 250 µg of anti-EGFR E7.6.3 IgG1, 500 µg of anti-HER2 × EGFR hetero-IgG1 V23, 500 µg of ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165, or 500 µg of huL1V1G isotype. Treatments were administered once a week for 3 more weeks. Female NSG mice (7–8 weeks old) were implanted subcutaneously with 5 × 10⁶ Calu-3 cells mixed 1:1 with Matrigel in a total volume of 100 µL. Fourteen days post-tumor implantation, the tumor volume was measured, and the mice were randomly distributed to either control or treatment groups with eight mice in each group. The mice were treated similarly as the above Panc-1 xenograft model except that anti-HER2 humAb4D5–8 and anti-HER2 humAb2C4 derived IgG1 antibodies were used.

For all studies, tumor-bearing mice were monitored for weight and for tumor volume twice a week. Tumor volume was calculated using Equation 1.

\[
\text{Volume (mm}^3\text{)} = \text{length} \times \text{width}^2 \times 0.50 \quad \text{(Eq. 1)}
\]

Percent tumor growth inhibition (% TGI) was determined based on Equation 2.

\[
\text{TGI} = 100 - (\text{Tumor size} \times \Delta T / \Delta C) \quad \text{(Eq. 2)}
\]

where \(\Delta C\) or \(\Delta T\) indicates the difference between the average tumor volumes on the last day and the day of initial measurement for the control (\(\Delta C\)) or treatment groups (\(\Delta T\)). Animals receiving saline or isotype huL1V1G served as the control group for these calculations. Antitumor activity is defined as percent TGI ≥50%.

**In Vitro Stability of Hetero-IgG1 Antibodies in Human Serum—** Hetero-IgG1 bsAbs and their parent antibodies at 150 µg/ml in 90% pooled normal human serum were incubated at 37 °C. An equal portion of incubated samples was taken out at different time points (24, 48, 72, 96, and 168 h) from the same tube. At each time point, the samples were briefly spun and stored at −20 °C until the test. 50 µl/well of biotinylated huHER2 (ECD) or huEGFR (ECD)-Fc (rabbit) at 125 ng/ml was added in the NeutrAvidin-immobilized 96-well assay plates. After three washes with 1× PBST, a 1:2 series of diluted hetero-IgG1 or parental IgG1 at 50 µl/well was transferred to the plates, which were then shaken at room temperature for 2 h. After three washes, the HRP-conjugated goat anti-human (Fc-specific) detection antibody was added. The plates were washed again. 3,3′,5,5′-Tetramethylbenzidine substrates were added for color development. The concentration of the antibodies was deduced from the standard curve of each antibody collected and frozen at \(t = 0\). The stability of each antibody was determined by analyzing the percentage retention concentrations (Ab concentration at test time point/Ab initiation concentration-100) over the incubation. The paired Student’s \(t\) test was used to compare retention concentration at the end of incubation \((t = 168 h)\) to the initiation concentration \((t = 0)\).

**Statistical Analysis—** Tumor growth was expressed as the means ± S.E. and plotted as a function of time. Statistical comparison of groups was performed at both overall level and at last measurement time point using the analysis of variance test followed by Dunnett’s or multivariate \(t\) test adjusted for multiple comparisons. Statistical calculations were made through the use of JMP software version 7.0 interfaced with SAS version 9.2 (SAS Institute, Inc., Cary, NC).

**RESULTS**

Selection of Residue Positions for Introducing Charged Amino Acid Pairs— We aimed to make bsAbs in hetero-IgG format from mammalian cells by introducing two different HCs and two different LCs in the same cells. The two different HCs were engineered to strongly favor the formation of heterodimers by using the charge pair mutations in the C4H3 regions (20). Because of the presence of the C4H1 domain, the HCs are retained in the endoplasmic reticulum by BiP proteins before they are engaged by LCs for the assembly of full IgG. To ensure the correct pairing of LC with its cognate HC in the hetero-IgG molecule, it was critical to find an effective way to control the kinetics of LC-HC assembly process so that the LC strongly favors its cognate HC and disfavors the noncognate HC. We attempted to achieve this by engineering the VH-VL and C4H1-CL interfaces as they are both involved in the HC-LC recognition and engagement process (27–31).

Examination of the VH-VL and C4H1-CL interface structures revealed that hydrogen bonds and van der Waals interactions are dominant. Unlike the C4H3-C4H3 interface (20), electrostatic charge-charge residue interaction is rare between the LC and HC. For example, \(\kappa\) C4H1-CL interface has one and \(\lambda\) C4H1-CL interface has two positive-negative charge interactions involving Lys and Glu/Asp residues. All three charged residue pair
interactions involve partially or fully solvent-exposed positions. Because of the solvent molecules interacting with the charged moieties, the electrostatic interaction would be considerably weakened. Hence, to utilize the electrostatic steering effect to drive specific pairing of LC and HC, it is essential to switch the polar or hydrophobic residues with the charged residues at the VH-VL and/or CH1-CL interfaces.

To select appropriate positions for maximal electrostatic steering effect, the following criteria were applied: 1) they should not be in CDRs and not make contact with the CDR residues; 2) they are highly conserved among IgG antibody subtypes; 3) they are mostly solvent-inaccessible (i.e., buried or partially buried); and 4) they have minimum interference with BiP-CH1 binding (27–28). The interface residues that meet the criteria for engineering are listed in Table 1 and were explored to make hetero-IgG antibodies.

As shown in Fig. 1, A and B, Gly-44 and Gln-105 in VH are spatially close to Gln-100 and Ala-43 in VL, respectively, regardless of antibody germ lines. Gly-44 (VH) to Gln-100 (VL) and Gln-105 (VH) to Ala-43 (VL) have been widely mutated to Cys to make a disulfide-stabilized fragment of variable regions (29). The Gln-39 (VH) to Gln-38 (VL) pair, which is located near the center of hydrophobic core of the VH-VL interface, has been mutated to the charged residue pairs to stabilize the diabody (39). In the constant regions shown in Fig. 1, C and D, Ala-141, Pro-171, and Ser-183 in CH1 region are close to residues Phe-116, Ser-162, and Ser-176 in CK, respectively.

**Proof-of-Concept Studies to Validate the Feasibility of Hetero-IgG Approach**—The VH and VL regions of the anti-HER2 trastuzumab and anti-EGFR panitumumab were selected for proof-of-concept studies because trastuzumab and panitumumab are well characterized and are approved drugs, and dual inhibition of HER2 and EGFR may have therapeutic potential for treating pancreatic cancers. To facilitate validation, an Fn3 tag (12 kDa) was inserted at the N termini of anti-EGFR HC2 and an Fn3-FLAG-His6 tag (14 kDa) was fused in-frame to the C termini of anti-EGFR LC2, whereas anti-HER2 HC1 and LC1 were kept as wild type. Four different LC-HC combinations will yield products at three different sizes in the SDS-polyacrylamide gel as follows: 162 kDa for LC1/HC1::LC1/HC1 Fn3_HC2; 176 kDa for the wanted LC1/HC1:: LC2_Fn3-FLAG-His6+Fn3_HC2 or the unwanted LC2_Fn3-FLAG-His6+HC1::LC1+Fn3_HC2; 190 kDa for LC2_Fn3-FLAG-His6+HC1::LC2_Fn3-FLAG-His6+Fn3_HC2. A product with a size of 176 kDa implies the possibility of correct LC-HC pairings. A dual antigen-binding plate ELISA was utilized to quickly screen the favorable variants that may have the wanted LC-HC pairings.

A total of 80 variants in which two pairs of charged residues in VH-VL only; two pairs of charged residues in C_{H1}-CL only; one pair of charged residues in C_{H1}-CL only; two pairs of charged residues in VH-VL and 1 pair of charged residues in C_{H1}-CL (Fig. 1E); two pairs of charged residues in VH-VL; and two pairs of charged residues in C_{H1}-CL (Fig. 1F) were investigated to find variants with high dual antigen binding after nor-

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**TABLE 1**

The amino acid residues closely located at the VH-VL and CH1-Ck interfaces were selected for substitution with charged residues.

| VH | IMGT # | Kabat # | Eu # | AHo # | FW | Residue | contact | IMGT # | Kabat # | Eu # | AHo # | FW | Residue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | 39 | 39 | 46 | 2 | Q/R/T | ←→ | 44 | 38 | 38 | 46 | 2 | Q/G/H/L |
| 49 | 44 | 44 | 51 | 2 | G/R/A | ←→ | * | 100 | 100 | 141 | 4 | Q/G/P |
| * | 105 | 109 | 141 | 4 | Q/K/R/S | ←→ | 49 | 43 | 43 | 51 | 2 | A/G/S/P |

| CH1 | IMGT # | Kabat # | Eu # | AHo # | Ref # | Residue | contact | IMGT # | Kabat # | Eu # | AHo # | Ref # | Residue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | 139 | 141 | * | 176 | A | ←→ | 5 | 116 | 116 | 158 | F | S |
| 82 | 175 | 171 | * | 212 | P | ←→ | 81 | 162 | 162 | 158 | 211 | S |
| 86 | 188 | 183 | * | 230 | S | ←→ | 86 | 176 | 176 | 124 | 216 | Q |
| 26 | 145 | 147 | * | 182 | K | ←→ | 13 | 124 | 124 | 230 | S |
| 26 | 145 | 147 | * | 182 | K | ←→ | 20 | 131 | 131 | 176 | S |
| 26 | 145 | 147 | * | 182 | K | ←→ | 90 | 180 | 180 | 234 | T |

Germline residues in VH and VL are numbered by different numbering systems. The bolded residues are dominant ones. The spatially close residues at VH-VL interface of most antibody germlines are arrayed in the same row as indicated by a double-headed arrow. The spatially close residues in human IgG1 CH1 domain and Ck region are also bolded and arrayed in the same row. FW: Framework. Ref# is the extended AHo # into constant region.
Hetero-IgG Antibody with Cognate LC-HC Pairings

The intact hetero-IgG variant 2B05 was partially reduced by heating at 37 °C for 80 min in the presence of 2-fold molar excess of TCEP. TCEP preferably breaks up the inter-chain disulfide bonds, yielding five different products (Fig. 3, F–I) consisting of HC1 + LC1 (1/2 Ab1); HC1 + Fn3_HC2; HC1 + Fn3_HC2 + LC1 (3/4 Ab1); HC1 + Fn3_HC2 + LC2_Fn3_FLAG-His6 (3/4 Ab2); Fn3_HC2 + LC2_Fn3_FLAG-His6 (1/2 Ab2). The presence of residual full-length antibody indicates that partial reduction happened in the reaction. All components had their theoretical mass with an error of <100 ppm. The carbohydrate attached at Asn-297 was normal as usual (Fig. 3, G and H). Most importantly, no LC1-HC2 or LC2-HC1 product was observed. The 1:1:1 stoichiometry in variant 2B05 showed by mass spectrometry also matched the same band intensity in SDS-polyacrylamide gel under reducing conditions (Fig. 2D). Similar results were obtained for four other purified hetero-IgG variants.

Because the anti-HER2 × EGFR hetero-IgG variants 2B05 and 5D03 (in the presence of Fn3 and Fn3-FLAG-His6 tags) have been identified with ADCC-enhancement mutations W165 by asymmetrical Fc engineering (40), ADCC killing assay was carried out by using human NK cells (FcγRIIIA 158F/F genotype) as effector cells and NCI-N87 cells, a human gastric tumor cell line expressing high levels of HER2 and moderate levels of EGFR, as target cells. As shown in Fig. 2E, at 1 μg/ml the irrelevant human IgG1 control antibody had a background lysis of 30% and did not show a dose-dependent response when it was titrated down, but both 2B05 and 5D03 had much higher specific lysis and showed a dose-dependent manner of response with EC50 at 0.10 and 0.19 μM, respectively. The data suggested that the hetero-IgG variants 2B05 and 5D03 can bind to targets HER2 and/or EGFR with their Fab arms and induce strong killing to NCI-N87 cells by engaging the NK cells.

CHO cells stably expressing human EGFR induce the phosphorylation of EGFR upon EGF stimulation. Although irrelevant human IgG1 did not inhibit the phosphorylation of EGFR (data not shown), the parental anti-EGFR E7.6.3 IgG1 inhibited the phosphorylation of receptor EGFR at IC50 = 2.7 nM (Fig. 2F). The combination of anti-EGFR E7.6.3 IgG1 and anti-HER2

FIGURE 1. Residues at VH-VL and C1-1-CL interfaces for substitutions are buried, conserved, and spatially close. Anti-HER2 trastuzumab crystal structure 1N8Z from Protein Data Bank is illustrated as an example. Structure 1N8Z pdb was loaded into Molecular Operating Environment (Chemical Computing Group, Montreal, Canada). Amber 10:EHT force field was set. Missing atoms were repaired, and charges were applied to the termini as appropriate at pH 7.0 automatically by the structure preparation function. Ribbon rendering was selected with a different color for each of the four domains in the following manner: VH, magenta; VL, orange; C1, blue; C2, green. Selected residues were rendered as ball-and-stick, and the Site View Function isolated a region within 5 Å of all selected residues. Kabat numbering was used for the variable region residues, whereas Eu numbering was used for the constant region residues. Distances were measured between each αC of the indicated (circled) residue pair by the distance tool. A, side view of VH-VL. The selected residues Gly-44, Gln-39, and Gln-105 in VH; Gln-100, Gln-39, and Ala-43 in VL are buried in the hydrophobic core of VH-VL. CDR loops are positioned at the top. 8 VH-VL interface. Circled pairs are substituted with charged residues to drive the electrostatic steaming effect. C, side view of C1–1-CL. The selected residues Pro-171, Ser-183, and Ala-141 in C1 and Ser-162, Ser-176, and Phe-116 in Cx are buried in the hydrophobic core of C1-1-CL. D, C1-1-CL interface. Residues Pro-171, Ser-183, and Ala-141 in C1 and Ser-162, Ser-176, and Phe-116 in Cx are in proximity, respectively. Lys-147 in CH1 and Gln-105 in VH are located in the middle of hydrophobic core (as Ser-183 in C1 and Ser-176 in Cx) but are not shown for view simplicity. Circled pairs are substituted with charged residues to drive the electrostatic steaming mechanism. E, configuration of monovalent bispecific hetero-IgG antibody variants using the electrostatic steaming approach. Two pairs of charged residues, KK-DD, in the variable regions binding to antigen B combined with two pairs of charged residues, KD-KD, in C1-1-CL drive the LC1 to pair with its cognate HC1. Similarly, two pairs of charged residues, DD-KK, in the variable regions binding to antigen A combined with one pair of charged residues, DK-DD, in C1-1-CL drives the LC2 to pair with its cognate HC2. The charged residues for heterodimerization in the C3 domains are also indicated. F, configuration of monovalent bispecific hetero-IgG antibody variants using the electrostatic steaming approach. Two pairs of charged residues, KK-DD, in the variable regions binding to antigen A combined with two pairs of charged residues, DD-KK, in C1-1-CL drive the LC1 to pair with its cognate HC1. Similarly, two pairs of charged residues, KK-DD, in the variable regions binding to antigen B combined with two pairs of charged residues, KD-KD, in C1-1-CL drives the LC2 to pair with its cognate HC2. The charged residues for heterodimerization in the C3 domains are also indicated. More configurations can be found in patent application WO2014081955. The symbol "•" represents positively charged residues and "•" represents negatively charged residues.
Hetero-IgG Antibody with Cognate LC-HC Pairings

A

OD 405

Log [ng/mL]

B

KDa

250

148

96

64

50

C

D

E

M 1 2 3 4 5 6 7

% specific lysis

µg/mL

F

a-EGFR alone
IC50 = 2.7 nM

% inhibition of pEGFR

G

a-Her2 + a-EGFR
IC50 = 3.2 nM

H

a-Her2 x EGFR 2B05
IC50 = 4.2 nM

I

a-Her2 x EGFR 5D03
IC50 = 4.6 nM

J

a-Her2 alone
IC50 = 2.8 nM

% inhibition of pHer2

K

a-Her2 + a-EGFR
IC50 = 5.2 nM

L

a-Her2 x EGFR 2B05
IC50 = 3.0 nM

M

a-Her2 x EGFR 5D03
IC50 = 3.6 nM
Hetero-IgG Antibody with Cognate LC-HC Pairings

TABLE 2
Residue substitutions of anti-HER2 × EGFR hetero-IgG1 variants in the presence of tags showed improved dual antigen binding and single band in Western blot

| Variant | Anti-HER2 HC1 (K392D + K409D) | Anti-HER2 LC1 | Anti-EGFR Fv3 HC2 (E356K + D399K) | Anti-EGFR LC2_Fn3_FLAG_His6 |
|---------|--------------------------------|---------------|---------------------------------|----------------------------|
| 1C02    | Q39K + Q105K + K147D + S183D  | Q38D + A43D   | S131K + S176K                   | G44D + Q105D               |
| 1C04    | Q39K + Q105K + K147D + S183D  | Q38D + A43D + | S176K + T180K                   | G44D + Q105D               |
| 2A05    | Q39K + Q105K + K147D          | Q38D + A43D   | S124K                           | G44D + Q105D               |
| 2B05    | Q39K + Q105K + K147D          | Q38D + A43D   | S180K                           | G44D + Q105D               |
| 5D03    | Q39K + Q105K + K171D          | Q38D + A43D   | S162K                           | G44D + Q105D               |

humAb4D5–8 lgIg1 functioned similarly at IC50 = 3.2 nm (Fig. 2G). Anti-HER2 × EGFR hetero-IgG1 variants 2B05 and 5D03 inhibited the phosphorylation of receptor EGFR at IC50 = 4.2 and IC50 = 4.6 nm, respectively (Fig. 2, H and I), indicating that the anti-EGFR Fab arm in hetero-IgG1 is functioning comparably as that in the wild type anti-EGFR E7.6.3 lgIg1.

BT-474 cells, a human breast tumor cell line, express both HER2 and HER3 on the surface. It was reported that anti-HER2 trastuzumab IgG1 does not decrease HER2 phosphorylation but inhibits the basal HER3 phosphorylation (41). When no ligand was added in the culture medium of BT-474 cells, anti-HER2 humAb4D5–8 lgIg1 alone blocked the phosphorylation of HER3 at IC50 = 2.8 nm (Fig. 2J), whereas irrelevant human IgG1 did not inhibit the pHER3 (data not shown). The combination of anti-EGFR E7.6.3 lgIg1 and anti-HER2 humAb4D5–8 lgIg1 had slightly less potency with IC50 = 5.2 nm (Fig. 2K). Anti-HER2 × EGFR hetero-IgG1 variants 2B05 and 5D03 inhibited the basal phosphorylation of HER3 at IC50 = 3.0 and IC50 = 3.6 nm, respectively (Fig. 2, L and M), indicating that the anti-HER2 Fab arm in hetero-IgG1 is also functioning.

Taken together, the above results suggested that electrostatic steering mechanism allows us to generate monovalent bispecific hetero-lgGs with cognate LC-HC pairings, and both Fab arms in the hetero-lgGs are functioning properly.

Optimization of Hetero-IgG Format in the Absence of Any Tags—The tags of anti-HER2 × EGFR hetero-lgG1 variants 2B05 and 5D03 (Table 2) were removed and then re-tested by chain drop-out transient transfection in mammalian 293E6 cells (Fig. 4A). When all four engineered chains (LC1 and HC1 from anti-HER2; LC2 and HC2 from anti-EGFR) were co-transfected, the main full-size hetero-lgG antibody appeared in the nonreducing SDS-polyacrylamide gel with a smaller amount of half-size antibody. Transfections with two plasmid DNAs encoding the matched LC1 + HC1 or LC2 + HC2 produced the full-size homodimer antibody with a significant amount of half-size antibody. No product was observed when LC1 was co-transfected with the noncognate HC2 for both variants 2B05 and 5D03. However, when the LC2 was co-transfected with the noncognate HC1, there was a faint band at full-size Ab for variant 2B05 but two obvious bands (at full-size Ab and half-size Ab) for variant 5D03, suggesting LC2-HC1 mispairing would occur if two different HCs and two different LCs were present during the production of hetero-lgG. To further improve the design, we explored a series of new variants (Table 3) in most of which symmetrical oppositely charged residue pairs were introduced. Chain drop-out transient transfections and Western blotting were carried out to assess the tolerance of LC-HC mispairings. The mutually reciprocal polarities of charged residues at the same positions of LC-HC interfaces could lead to more stringent LC-HC pairings.

For variants V15 and V20 (Fig. 4B), co-transfection with all four plasmid DNAs or matched two plasmid DNAs produced full-size and half-size antibodies. No product was seen in nonreducing SDS-polyacrylamide gel when LC2 was co-transfected with noncognate HC1, indicating the anti-EGFR LC2 was not tolerated by anti-HER2 HC1. However, high level expression of full-size and half-size antibodies was observed when anti-HER2 LC1 was co-transfected with anti-EGFR HC2. Hetero-lgG variants V21 and V22 had more stringent LC-HC pairings (Fig. 4C), whereas variants V23 and V25 did not tolerate any mis-matched LC-HC pairings (Fig. 4D). Variants (V12, V23, V24, and V25) with strict LC-HC pairings were scaled up by transient transfections; mass spectrometry analysis demonstrated that four different chains were correctly assembled in these hetero-lgG variants (data not shown).

Hetero-lgG Antibody Targeting Two Different Epitopes on the Same Antigen—It was reported that in xenograft HER2-positive human tumor models the combination of anti-HER2 trastu...
zumab and anti-HER2 pertuzumab showed strongly enhanced antitumor activity than trastuzumab alone or pertuzumab alone (42, 43). Anti-HER2 trastuzumab binds to the domain IV of HER2, whereas anti-HER2 pertuzumab binds to the domain II of HER2. We questioned whether anti-HER2 × HER2 hetero-IgG consisting of trastuzumab and pertuzumab could block the signaling pathways synergistically by binding to two different epitopes simultaneously, leading to higher efficacy than the combination of two parental antibodies. The same variant V23 (Table 3) in which two pairs of charged residues in VH-VL and one pair of charged residues in C_j1-CL were reciprocally introduced was tested by either transfecting with four DNAs to make full-length antibody or with only two DNAs to assess the tolerance of mismatched LC-CH pairings. Similar to anti-HER2 × EGFR hetero-IgG variant V23, the anti-HER2 × HER2 hetero-IgG antibody V23 (Fig. 5, V23A) was mainly expressed as the intact antibody after four different chains were translated and assembled. In the presence of two matched chains (anti-HER2 humAb4D5–8 LC1 + HC1 or anti-HER2 humAb2C4 LC2 + HC2), half-antibody and homodimer antibody were produced, indicating that the LCs were compatible with their cognate HCs. In the presence of mismatched chains LC2 + HC1 or LC1 + HC2, no product was formed, indicating that the engineered LCs were not tolerated by their noncognate HCs.

Different Combinations of Charged Residues Affect the Hetero-IgG Expression and LC-CH Pairings—To investigate whether different combinations of charged residues could result in different expression and/or affect the LC-CH pairings, we made and expressed four anti-HER2 (humAb4D5–8) × HER2 (humAb2C4) hetero-IgG1 variants by introducing charged residue pairs with different combinations at the same positions (Table 4 and Fig. 5). V23B also had strict LC-CH pairings as V23A, but the expression level decreased. However, V23C did not produce any antibody when the matched LC2-HC2 was co-transfected, and it produced a low level of antibody when mis-matched LC1-HC2 was co-transfected. V23D produced a low level of antibody when the matched LC2-HC2 was co-transfected, but LC1 was well tolerated by noncognate HC2 as both full-size and half-size antibodies were observed in nonreducing SDS-polyacrylamide gel. This set of data suggested that the electrosstatic steering is not the only mechanism that controls the wanted LC-CH pairings, other mechanisms such as shape complementarity may play a role in this process.

Anti-HER2 × EGFR Hetero-IgG1 Variants Showed Good Thermal Stability—The temperature-induced unfolding of anti-HER2 trastuzumab Ig1G, afucosylated anti-HER2 humAb4D5–8 IgG1, anti-EGFR E7.6.3 IgG1, and four anti-HER2 × EGFR hetero-IgG1 variants V12, V23, V24, and V25 having ADCC enhancement Fc (W165) were assessed under the same solvent conditions by differential scanning calorimetry (Fig. 6). The thermogram of each protein consisted of two or three transitions. Anti-HER2 trastuzumab showed a T_m of Fab/C_j1-3 at 83 °C and a T_m of C_j1-2 at 71 °C; the afucosylated anti-HER2 humAb4D5–8 IgG1 did not change the T_m of separate domains but decreased the enthalpy slightly. The anti-EGFR E7.6.3 IgG1 antibody had a similar profile of temperature-induced unfolding. All four anti-HER2 × EGFR hetero-IgG1 variants had a slightly decreased T_m of merged C_j1-2/C_j1-3 at −69 °C as they all have the ADCC enhancement substitutions in C_j1-2 domains and heterodimerization substitutions in the C_j1-3 domain. In terms of T_m of Fab domains, variants V12 and V24 had the most significant decrease from 83 to 75 °C; variant V25 had two separate peaks at 74 °C and 79 °C, and variant V23 had a single peak at 79 °C. Overall, the four hetero-IgG variants showed good thermal stability. The data suggested the selected positions for substitutions with charged residues in the Fab regions do impact the stability of intact hetero-IgG1 antibodies to some extent, with T_m of separate domains above 68 °C.

**Stable Expression of Hetero-IgG Antibodies in Mammalian CHO-K1 Cells**—As the anti-HER2 × EGFR hetero-IgG variant V23 showed the balanced expression for each half-Ab and strict LC-CH pairings by transient transfection (Fig. 4D) and good DSC profile (Fig. 6), it was chosen to explore the strategy on how to stably express hetero-Igs in mammalian cells and obtain a large amount of material for further characterizations and animal studies. We linked the open reading frame of anti-EGFR E7.6.3 HC2 and anti-HER2 humAb4D5–8 HC1 with a DNA sequence encoding furin cleavage site (R6), a spacer, and scp (37, 38). Similarly, we inserted the same R6-spacer-scp between anti-EGFR E7.6.3 LC2 and anti-HER2 humAb4D5–8 LC1. The two different HCs are designed to integrate into the same chromosome loci to balance the expression of two different HCs as HC heterodimerization is required to form hetero-Igs. The constructs with opposite orientation (anti-HER2 humAb4D5–8 in front of anti-EGFR E7.6.3) were also made. However, transient transfection revealed that, for some unknown reasons, the Fc titer was significant lower (data not shown). Similar constructs were made for anti-HER2 × HER2 hetero-IgG1 with anti-HER2 humAb2C4 in front of anti-HER2 humAb4D5–8. Constructs for hetero-IgGs, either having regular Fc variant V23 or ADCC enhancement Fc variant V23_W165, were transfected in CHO-K1 cells in duplicate and selected under the pressure of puromycin and hygromycin for ~3 weeks. Cell viability decreased to ~10% at day 7 and quickly recovered to >90% at day 22 (data not shown). Compared with transient transfections, CHO-K1 stable pools boosted the Fc titers from 20 to 70 mg/liter to 200–320 mg/liter.

The crude supernatant from two separate stable pools was examined together with the purified hetero-IgG1 from transiently expressed material for further characterizations and animal studies.
sient transfection in SDS-polyacrylamide gel and Western blotting. Under nonreducing condition (Fig. 7A), the dominant band of full-size IgG and bands for half-size IgG, LC dimer, and LC monomer were detected. Under reducing conditions (Fig. 7B), two HCs were separated due to their different size and LCs migrated concurrently due to their identical size. The results indicated that hetero-IgG1 can be stably produced from CHO-K1 cells, although minor half-size IgGs and LCs are present. The conditioned medium was purified by a standard protein A column followed by Superdex 200 size exclusion chromatography. The final products showed ~100% purity by analytical size exclusion chromatography. Mass spectrometry analysis

Figure 4. Chain drop-out transient transfections to assess the LC-HC pairing tolerances for hetero-IgG1 variants in the absence of any tags. 293E cells were transfected with either two or four different plasmid DNAs. Six days post-transfection, crude supernatant was loaded in 8–16% Tris-glycine SDS-polyacrylamide gel and subjected to electrophoresis under nonreducing conditions and Western blotting. The sequence variations of anti-HER2/EGFR hetero-IgG1 variants 2B05 and 5D03 are indicated in Table 2. Variants V15, V20, V21, V22, V23, and V25 are indicated in Table 3. LC1 and HC1 are derived from anti-HER2 trastuzumab, and LC2 and HC2 are derived from ant-EGFR panitumumab. The + symbol indicates the presence of the particular plasmid DNA for transfection, and the − symbol indicates its absence.
was carried out to assess the components in hetero-IgGs. The anti-HER2 × EGFR hetero-IgG1 V23 (Fig. 7C), ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165 (Fig. 7D), anti-HER2 × HER2 hetero-IgG1 V23 (Fig. 7E), and ADCC-enhanced anti-HER2 × HER2 hetero-IgG1 V23_W165 (Fig. 7F) all were revealed to contain additional 1–3 Arg in the presence of traceable half-size Abs. Fig. 7, G–L, shows an example for anti-HER2 × EGFR hetero-IgG1 V23. After deglycosylation

| Variant | Anti-HER2 humAb4D5–8 HC1 (K392D + K409D) | Anti-HER2 humAb4D5–8 LC1 | Anti-EGFR E7.6.3 HC2 (E356K + D399K) | Anti-EGFR 7.6.3 LC2 |
|---------|------------------------------------------|----------------------------|--------------------------------------|-------------------|
| V01     | Q39K + Q105K K147D                      | Q38D + A43D T180K         | Q44D + Q105D K147K                   | G100K + A43K S131D |
| V02*    | Q39K + Q105C K147D                      | Q38D + A43C T180K         | Q44C + Q105D K147K                   | G100C + A43K S131D |
| V03     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V04     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V05     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V06     | Q39K + Q105K K147D                      | Q38D + A43D T180K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V07     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V08     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V09     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V10     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V11     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V12     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V13     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V14     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V15     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V16     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V17     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V18     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V19     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V20     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V21     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V22     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V23     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V24     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |
| V25     | Q39K + Q105K P171D                      | Q38D + A43D S162K         | Q44D + Q105D A141K                   | G100K + A43K F116D |

FIGURE 5. Chain drop-out transient transfections to assess the electrostatic steering effect. 293E cells were transfected with either two or four different plasmid DNAs encoding anti-HER2 trastuzumab and anti-HER2 pertuzumab in which the charged residue pairs in Fab regions were swapped. Six days post-transfection the crude supernatant was loaded in 8–16% Tris-glycine SDS-polyacrylamide gel, subjected to electrophoresis under nonreducing conditions, and Western blotted. The sequence variations V23A, V23B, V23C, and V23D are indicated in Table 4. LC1 and HC1 are derived from anti-HER2 trastuzumab, LC2 and HC2 are derived from anti-HER2 pertuzumab. The * symbol indicates the presence of the particular plasmid DNA for transfection, and the _ symbol indicates its absence.
and complete reduction by DTT, the intact hetero-IgG1 was shown to contain four different chains. The two different HCs were correctly and efficiently processed (Fig. 7, A and J). A ladder of 1–3 extra Arg were found to be retained at the C termini of anti-EGF LC2 (Fig. 7L), whereas anti-HER2 LC1 had been correctly processed (Fig. 7K). Most importantly, no mis-matched HC-LC pairing (LC1 + HC2 or LC2 + HC1) was identified (Fig. 7H). More work is required to improve the homogeneity of hetero-IgG antibodies expressed from stably transfected mammalian cells.

**Hetero-IgG Antibody with Cognate LC-HC Pairings**

**TABLE 4**

Charged residue pairs at the VH-VL and C\textsubscript{\textgamma}1-CL interfaces were swapped to different combinations to explore the electrostatic steering effect

| Variant | Anti-HER2 humAb4D5-8 HC1 (K392D + K409D) | Anti-HER2 humAb4D5-8 LC1 | Anti-HER2 humAb2C4 HC2 (E356K + D399K) | Anti-HER2 humAb2C4 LC2 |
|---------|------------------------------------------|--------------------------|----------------------------------------|------------------------|
| VH1     | Q39K + Q105K                             | Q38D + A43D              | Q39D + Q105D                           | Q38K + A43K           |
| C\textsubscript{\textgamma}1 | S183D                                   | S176K                    | S185K                                  | S176D                 |
| VL1     | Q38D + A43D                             | Q39K + Q105D             | Q39K + Q105D                           | Q38K + A43K           |
| C\textsubscript{\gamma}   | S176D                                   | S176D                    | S185K                                  | S176D                 |
| VH2     | Q39K + Q105K                             | Q39K + Q105K             | Q39K + Q105K                           | Q38K + A43K           |
| C\textsubscript{\gamma}1 | S185K                                   | S185K                    | S185K                                  | S176K                 |
| VL2     | Q38K + A43K                             | Q38K + A43K              | Q38K + A43K                            | S176K                 |
| C\gamma   | S176K                                   | S176K                    | S176K                                  | S176K                 |

and anti-HER2 IgG1 antibodies demonstrated an increase in binding signal only for their respective antigen injection (Fig. 8, C and D). The reciprocal experiments by injecting rhuHER2 first followed by rhuEGFR produced similar results (data not shown). Collectively, these results demonstrated that both Fab arms in the hetero-IgG1 can bind to their specific antigens simultaneously and irrespective of the order of addition in this protein based assay.

**FIGURE 6.** Thermal stability analysis of parental antibodies and anti-HER2 × EGFR hetero-IgG variants by differential scanning calorimetry. All antibodies were produced in 293E cells by transient transfection, purified by protein A, and polished by Superdex 200 size exclusion column. Anti-HER2 trastuzumab IgG1, afucosylated anti-HER2 humAb4D5–8 IgG1, and anti-EGFR E7.6.3 IgG1 were included as internal controls. The sequence variations of anti-HER2 × EGFR hetero-IgG1 variants V12, V23, V24, and V25 are indicated in Table 3. All four anti-HER2 × EGFR hetero-IgG1 have embedded with ADCC enhancement Fc variant W165 (40).

**FIGURE 7.** SPR binding analysis was conducted using the ProteOn XPR36 optical biosensor with an antibody capture format. Interestingly, both anti-HER2 × HER2 hetero-IgGs V23 (Fig. 8A) and V23_W165 (Fig. 8B) demonstrated additive and simultaneous binding as the binding signal increased after the rhuEGFR injection and further increased after the rhuHER2 injection, whereas the parental anti-EGFR and anti-HER2 hetero-IgGs have embedded with ADCC enhancement Fc variant W165 (40).

**FIGURE 8.** SPR binding analysis was conducted using the ProteOn XPR36 optical biosensor with an antibody capture format. Interestingly, both anti-HER2 × HER2 hetero-IgGs V23 (Fig. 8A) and V23_W165 (Fig. 8B) demonstrated additive and simultaneous binding as the binding signal increased after the rhuEGFR injection and further increased after the rhuHER2 injection, whereas the parental anti-EGFR and anti-HER2 hetero-IgGs have embedded with ADCC enhancement Fc variant W165 (40).
**Hetero-IgG Antibody with Cognate LC-HC Pairings**

- **A**: Gel electrophoresis of IgG samples showing bands at different molecular weights (KDa).
- **B**: Another gel electrophoresis showing bands for HCs and LCs.
- **C**: Graph showing intensity vs. desorbed mass for anti-HER2 x EGFR hetero-IgG (V23).
- **D**: Graph for anti-HER2 x EGFR hetero-IgG (V23_W165).
- **E**: Graph for anti-HER2 x HER2 hetero-IgG (V23).
- **F**: Graph for anti-HER2 x HER2 hetero-IgG (V23_W165).
- **G**: Graph for hetero-IgG showing various peaks at different molecular weights.
- **H**: Graph for anti-EGFR E7.6.3 showing peaks for HCs and LCs.
- **I**: Graph for anti-HER2 humanAb409-8 HC1 showing intensity vs. desorbed mass.
- **J**: Graph for anti-EGFR E7.6.3 HC2 showing intensity vs. desorbed mass.
- **K**: Graph for anti-HER2 humanAb409-8 LC1 showing intensity vs. desorbed mass.
- **L**: Graph for anti-EGFR E7.6.3 LC2 showing intensity vs. desorbed mass.

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Asymmetrically Engineered Hetero-IgG Antibodies Elicit Potent ADCC Killing to Tumor Cells—One objective of hetero-IgG antibodies is to kill tumor cells with high potency by the enhanced ADCC effector function, which was achieved by asymmetrical Fc engineering (40). Asymmetrical Fc variant W165 was integrated in anti-HER2 × EGFR and anti-HER2 × HER2 hetero-IgG V23 individually. NCI-N87 cells expressing a high level of HER2 and a moderate level of EGFR were used as target cells for ADCC killing assay (Fig. 10A). The human IgG1 isotype control did not show meaningful killing. The anti-EGFR E7.6.3 IgG1 had 20% specific lysis at the highest concentration of 10 nM, with lower activity when it was titrated down. The anti-HER2 humAb4D5–8 IgG1 showed 80% specific lysis at 10 nM and titrated down in a dose-dependent manner with EC50 = 17.75 pm. The combination of anti-EGFR E7.6.3 IgG1 and anti-HER2 humAb4D5–8 IgG1 showed slightly lower killing (EC50 = 33.64 pm) compared with anti-HER2 humAb4D5–8 IgG1 alone, indicating the ADCC killing was mainly driven by anti-HER2 humAb4D5–8 and is in line with higher HER2 expression on NCI-N87 cells. The anti-HER2 × EGFR hetero-IgG1 V23 having regular Fc showed the killing between the two parental antibodies, whereas a potent killing was observed for the ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165 (EC50 = 2.55 pm). JIMT-1 cells, which express high levels of HER2 but low levels of EGFR and are resistant to anti-HER2 trastuzumab treatment (44), showed low killing by all Ab treatments except that the ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165 had strong killing at EC50 = 4.17 pm (Fig. 10B).

SK-BR-3 cells expressing high levels of HER2 were used to assess the activity of different anti-HER2 antibodies (Fig. 10C). Although the human IgG1 isotype control did not show meaningful killing, anti-HER2 humAb2C4 IgG1 alone had 70% specific lysis at the highest dose with EC50 = 220.4 pm. The anti-HER2 humAb4D5–8 IgG1 alone had stronger killing at EC50 = 19.71 pm. The combination of anti-HER2 humAb2C4 and humAb4D5–8 IgG1s killed SK-BR-3 cells intermediately with EC50 = 46.16 pm. The anti-HER2 × HER2 hetero-IgG1 V23 also killed SK-BR-3 cells at intermediate EC50 = 49.85 pm. However, the anti-HER2 × HER2 hetero-IgG1 V23_W165, which had incorporated ADCC enhancement of Fc variant W165, strongly killed the SK-BR-3 cells with EC50 = 2.272 pm. Similar results were found with BT-474 cells (Fig. 10D). These results confirmed that the asymmetrically engineered Fc variant W165 can enhance the ADCC effector function.

![Figure 7](image_url)

**Hetero-IgG Antibodies Inhibit the Phosphorylation of Receptors and AKT in Downstream Signaling Pathway**—To test how well anti-HER2 × EGFR hetero-IgG1 antibodies inhibit the signaling pathways, BxPC-3 cells were treated with either the titrated parental anti-HER2 humAb4D5–8 IgG1, anti-EGFR E7.6.3 IgG1, or the combination of two parental antibodies, anti-HER2 × EGFR hetero-IgG1 V23, ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165, or the isotype human IgG1 control. For a proper comparison, the total dose of single parental antibody, the combination of the two parental antibodies, and hetero-IgG1s was normalized by their binding valences to each target receptor; hence they have equal binding capacity to the receptors. As shown in Fig. 10E, the phosphorylation of EGFR was neither inhibited by isotype human IgG1 control nor by anti-HER2 humAb4D5–8 IgG1, but it was strongly inhibited by the parental anti-EGFR E7.6.3 IgG1, with 82.11% of inhibition at the plateau dose (IC50 = 1.49 nm). The inhibition from the combination of two parental Abs was slightly weaker, with 77.60% at the plateau dose (IC50 = 5.48 nm). A comparable inhibition of pEGFR was observed for either anti-HER2 × EGFR hetero-IgG1 V23 (IC50 = 4.85 nm) or ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165 (IC50 = 4.45 nm).

The basal expression level of HER2 on BxPC-3 cells was too low to have enough window for the detection of pHER2, regardless of the cells being stimulated with or without 100 ng/ml neuregulin 1, and even the treatment by the combined parental anti-HER2 humAb4D5–8 and anti-EGFR E7.6.3 IgG1s (10 μg/ml each) revealed no effect on pHER3 and pAKT in BxPC-3 cells (data not shown).

To test the inhibitions of pHER2, pHER3, and pAKT by anti-HER2 humAb4D5–8, anti-HER2 humAb2C4 IgG1, the combination of these two parental Abs, anti-HER2 × HER2 hetero-IgG1 V23, ADCC-enhanced anti-HER2 × HER2 hetero-IgG1 V23_W165, or isotype human IgG1 control, MCF-7 cells expressing low level of HER2 (∼35,000 sites per cell) were treated by the titrated Abs at a 1:4 series dilution (starting from 30 μg/ml). As shown in Fig. 10F, no inhibition of pHER2 was detected for the treatment of isotype human IgG1 or anti-HER2 humAb4D5–8 IgG1. The inhibition of pHER2 by anti-HER2 humAb2C4 was observed, with 71.36% inhibition at the plateau dose (IC50 = 4.59 nm), in line with the reports that pertuzumab disrupts the dimerization with other HER family members (7, 8). Both anti-HER2 × HER2 hetero-IgG1s (IC50 = 12.89 and 15.29 nm, respectively) had slightly less inhibition.
when compared with the parental anti-HER2 humAb2C4 IgG1. Only about a 40% inhibition on pHER2 (Fig. 10F), although a strong inhibition on pHER3 (Fig. 10G, IC₅₀ = 5.7 nM) and pAKT (Fig. 10H, IC₅₀ = 6.78 nM), was observed for the combination treatment of the two parental Abs at the plateau dose. Single agent treatment by anti-HER2 humAb2C4 IgG1 had IC₅₀ = 5.44 nM for pHER3 and IC₅₀ = 6.08 nM for pAKT, but single agent treatment by the anti-HER2 humAb4D5–8 IgG1 had a low impact on pHER3 and pAKT (Fig. 10, G and H).

FIGURE 8. Anti-HER2 × EGFR hetero-IgG1 antibodies from stably transfected CHO-K1 cells have comparable binding affinity as parental antibodies. Representative SPR sensorgrams of triplicate injections of 75 nM monomeric rhuEGFR injected at time 0 s followed by 75 nM monomeric rhuHER2 injected at 800 s over the following: A, anti-HER2 × EGFR hetero-IgG1 V23; B, anti-HER2 × EGFR hetero-IgG1 V23_W165; C, anti-EGFR E7.6.3 IgG1; and D, anti-HER2 humAb4D5–8 IgG1. E–G show the SPR sensorgrams (black lines), and the results from nonlinear least squares regression analysis of the data (red lines). Global fits utilize a 1:1 binding model for the triplicate injections of five concentrations of monomeric rhuHER2 ranging between 25.0 and 0.309 nM against captured (E); anti-HER2 × EGFR hetero-IgG1 V23 (F); anti-HER2 × EGFR hetero-IgG1 V23_W165 (G); anti-HER2 humAb4D5–8 IgG1 (H). H–J show the SPR sensorgrams (black lines), and the results from nonlinear least squares regression analysis of the data are shown (red lines). Global fits utilize a 1:1 binding model for the triplicate injections of five concentrations of monomeric rhuEGFR ranging between 25.0 and 0.309 nM against captured anti-HER2 × EGFR hetero-IgG1 V23 (H); anti-HER2 × EGFR hetero-IgG1 V23_W165 (I); and anti-EGFR E7.6.3 IgG1 (J).
Hetero-IgG Antibodies Induce Higher Levels of Receptor Internalization Than Either Parental Antibody Alone or a Combination of Parental Antibodies—Human pancreatic tumor cell lines BxPC-3 and Panc-1 and human lung adenocarcinoma cell line Colo699 were treated with either control human IgG1, anti-HER2 humAb4D5–8 IgG1 alone, anti-EGFR E7.6.3 IgG1 alone, the combination of anti-HER2 humAb4D5–8 IgG1 and anti-EGFR E7.6.3 IgG1, or anti-HER2 × EGFR hetero-IgG1 V23 bound strongly (Fig. 11A) and comparably (Fig. 11F) to BxPC-3 cells. Anti-HER2 humAb4D5–8 IgG1 alone bound with low intensity to BxPC-3 cells (image not shown), correlating with the fact that moderate level of EGFR (about 300,000 sites per cell) and a low level of HER2 (about 20,000 sites per cell) are expressed on BxPC-3 cells (image not shown), correlating with the fact that moderate level of EGFR (about 300,000 sites per cell) and a low level of HER2 (about 20,000 sites per cell) are expressed on BxPC-3 cells (image not shown). At the 4-h time point, no formation of punctate spots was detected for anti-HER2 humAb4D5–8 IgG1 treatment (Fig. 11B), although many punctate spots were observed inside cells for the treatment with anti-EGFR E7.6.3 IgG1.

| Antibody                        | Analyte          | $k_a$          | $k_d$          | $K_d$          |
|---------------------------------|------------------|----------------|----------------|---------------|
| Anti-HER2 × EGFR hetero-IgG1    | rhuHER2          | $5.493 \times 10^4 \pm 4 \times 10^3$ | $3.41 \times 10^{-5} \pm 1 \times 10^{-7}$ | $61.89 \pm 0.05$ |
| Anti-HER2 × EGFR hetero-IgG1    | rhuHER2          | $5.156 \times 10^4 \pm 4 \times 10^3$ | $3.45 \times 10^{-5} \pm 1 \times 10^{-7}$ | $65.95 \pm 0.06$ |
| Anti-HER2 (humAb4D5–8) IgG1     | rhuHER2          | $7.823 \times 10^4 \pm 7 \times 10^3$ | $4.73 \times 10^{-5} \pm 1 \times 10^{-7}$ | $60.08 \pm 0.05$ |
| Anti-HER2 × EGFR hetero-IgG1    | rhuEGFR          | $1.504 \times 10^4 \pm 1 \times 10^3$ | $1.732 \times 10^{-4} \pm 1 \times 10^{-7}$ | $115.06 \pm 0.09$ |
| Anti-HER2 × EGFR hetero-IgG1    | rhuEGFR          | $1.446 \times 10^4 \pm 1 \times 10^3$ | $1.708 \times 10^{-4} \pm 1 \times 10^{-7}$ | $118.28 \pm 0.09$ |
| Anti-EGFR (E7.6.3) IgG1         | rhuEGFR          | $1.408 \times 10^4 \pm 1 \times 10^3$ | $1.643 \times 10^{-4} \pm 1 \times 10^{-7}$ | $116.50 \pm 0.09$ |

**TABLE 5** Kinetic rate binding constants for rhuHER2 and rhuEGFR binding to anti-HER2 × EGFR hetero-IgG1 and parental antibodies

Kinetic rate coefficients were determined from binding analysis experiments performed with a ProteOn XPR36 biosensor. Five concentrations of monomeric rhuHER2 or rhuEGFR ranging between 25.0 and 0.309 nm were run in triplicate against the captured antibodies on a GLC surface. The $k_d$ values were determined by fitting the respective 3600-s dissociation phase data and then using this value as a fixed parameter in the global fits of the association phase data to a 1:1 binding model to calculate the respective $k_a$ and $K_d$ values. The error is the standard error of the global fit for triplicate surfaces. rhuHER2, monomeric recombinant human HER2 extracellular domain; rhuEGFR, monomeric recombinant human EGFR extracellular domain.

**TABLE 6** Kinetic rate binding constants for rhuHER2 binding to anti-HER2 (trastuzumab) × HER2 (pertuzumab) hetero-IgG1 and parental antibodies

Kinetic rate coefficients were determined from binding analysis experiments performed with a ProteOn XPR36 biosensor. Five concentrations of monomeric rhuHER2 ranging between 25.0 and 0.309 nm were run in triplicate against captured antibodies on a GLC surface. The $k_a$ values were determined by fitting the respective 3600-s dissociation phase data and then using this value as a fixed parameter in the global fits of the association phase data to a 1:1 binding model to calculate the respective $k_d$ and $K_d$ values. The error is the standard error of the global fit for triplicate surfaces. rhuHER2, monomeric recombinant human HER2 extracellular domain.
A. Time point 0 hr  B. α-Her2 4 hr  C. α-EGFR 4 hr  D. α-Her2 + α-EGFR 4 hr  E. α-Her2 × EGFR 4 hr

FIGURE 11. Hetero-IgG1 antibodies increase cellular target internalization levels as compared with the levels mediated by either parental antibody alone or a combination of parental antibodies. Monolayer BxPC-3 cells were exposed to either control human IgG1, anti-Her2 humAb4D5–8 IgG1, anti-EGFR E7.6.3 IgG1, anti-Her2 × EGFR hetero-IgG1 V23 at a final concentration of 5 µg/ml (34 nM) or a combination of anti-Her2 humAb4D5–8 IgG1 and anti-EGFR E7.6.3 IgG1 at a final concentration of 2.5 µg/ml (17 nM) for each antibody. A. time point 0 hr. B. anti-Her2 humAb4D5–8 IgG1 at time point 4 hr. C. anti-EGFR 7.6.3 IgG1 at time point 4 hr. D. combination of anti-Her2 humAb4D5–8 IgG1 and anti-EGFR E7.6.3 IgG1 at time point 4 hr. E. anti-Her2 × EGFR hetero-IgG1 V23 at time point 4 hr. F. total detectable cell surface binding of antibodies at time point 0 hr. G. spot intensity per cell over 4 hr of incubation.

Hetero-IgG Antibodies with Cognate LC-HC Pairings

FIGURE 10. Hetero-IgG1 antibodies elicit potent ADCC killing to tumor cells and inhibit phosphorylation of molecules in the signaling pathway. A. NCI-N87 as target cells. B. JIMT-1 as target cells. C. SK-BR-3 as target cells. D. BT-474 as target cells. Percent specific lysis was calculated using (RLU values of treated samples subtracted by average RLU value of effector alone) divided by (the average RLU of untreated cells (effector + target) subtracted by average RLU of effector alone) × 100. E. BxPC-3 cells were used for the peGFR inhibition. F. MCF-7 cells were used for the pHER2 inhibition. G. MCF-7 cells were used for the pHER3 inhibition. H. MCF-7 cells were used for the pAKT inhibition. The level of phosphorylated molecules was detected and analyzed as described under “Experimental Procedures.”

Hetero-IgG Antibodies Strongly Inhibit Tumor Growth in BxPC-3, Panc-1, and Calu-3 Xenograft Tumor Models—BxPC-3 cells were implanted in CB-17 SCID for evaluation of the effect of anti-Her2 × EGFR hetero-IgG1s on tumor growth. As shown in Fig. 12A, a statistical significance of antitumor activity was achieved by anti-Her2 humAb4D5–8 IgG1 (p = 0.0233), anti-EGFR E7.6.3 IgG1 (p = 0.0004), the combination of two parental Abs (p < 0.0001), anti-Her2 × EGFR hetero-IgG1 V23 (p < 0.0001), and ADCC-enhanced anti-Her2 × EGFR hetero-IgG1 V23_W165 (p < 0.0001) when compared with the vehicle saline. Furthermore, the antitumor activity of anti-EGFR E7.6.3 IgG1 showed stronger inhibition on tumor growth than anti-Her2 humAb4D5–8 IgG1 (p = 0.0043), reflecting the fact that BxPC-3 cells express higher EGFR than HER2 on the surface. Importantly, anti-Her2 × EGFR hetero-IgG1 V23 (p = 0.0335) was significantly stronger than anti-Her2 humAb4D5–8 IgG1 alone in inhibiting tumor growth, whereas anti-Her2 × EGFR hetero-IgG1 V23_W165 (p = 0.0726) was marginally different from anti-Her2 humAb4D5–8 IgG1 treatment. At end of the study, treatment by anti-Her2 × EGFR hetero-IgG1 V23 (p = 0.0008), ADCC enhanced anti-Her2 × EGFR hetero-IgG1 V23_W165 (p = 0.0021), and the combination of anti-Her2 humAb4D5–8 IgG1 and anti-EGFR E7.6.3 IgG1 (p = 0.0002) all showed significant difference when compared with the treatment by anti-Her2 humAb4D5–8 IgG1 alone.

To further investigate the mechanisms of tumor growth inhibition by anti-Her2 × EGFR hetero-IgG1 antibodies, Panc-1 cells of pancreatic adenocarcinoma carrying K-RAS mutation
and expressing low HER2/EGFR were implanted in Rag2<sup>−/−</sup>/mFc<sup>−/−</sup> C57BL/6 transgenic mice. Antibody dose for each treatment group was normalized by the binding valence to each target receptor. As shown in Fig 12B, no significant inhibition of tumor growth was observed for the treatments by anti-HER2 humAb4D5–8 IgG1 alone, or anti-EGFR E7.6.3 IgG1 alone, or the combination of the two parental antibodies when compared with the treatment by isotype human IgG1 control. ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23<sub>_W165</sub> (p = 0.0162) significantly reduced the tumor size over the treatment, whereas anti-HER2 × EGFR hetero-IgG1 V23 having regular Fc region did not. At the end of study (day 42), both anti-HER2 × EGFR hetero-IgG1 V23 and ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23<sub>_W165</sub> significantly reduced the tumor size when compared with the isotype human IgG1 control alone (p = 0.0266 and 0.0094, respectively) or anti-HER2 humAb4D5–8 IgG1 alone (p = 0.0441 and 0.0177, respectively). Furthermore, the ADCC-en-
Enhanced anti-HER2 × EGFR hetero-IgG1 V23_W165 significantly reduced the tumor size at the end of study when compared with the combination of two parent IgG1 antibodies \( p = 0.0473 \), whereas the ADCC-norm anti-HER2 × EGFR hetero-IgG1 V23 did not show any significant difference \( p = 0.2701 \). These results showed that anti-HER2 × EGFR hetero-IgG1s strongly inhibited the tumor growth, and ADCC enhancement helps antibody to overcome K-RAS mutation, which is in line with the report by Schlaeth et al. (46). The results also agreed with the finding that anti-HER2 × EGFR hetero-IgG1 induces higher internalization of receptors than single parental Ab or the combination of the two parental Abs in this report.

The effect of anti-HER2 × HER2 hetero-IgG1 on tumor growth was tested in a xenograft tumor model of Calu-3 cells which highly express HER2 at about 300,000 sites per cell. Tumor-bearing NSG mice were treated similarly as those in Panc-1 xenograft tumor model. As shown in Fig. 12C, significant tumor growth inhibition was observed for all antibody treatment groups when compared with the huIgG1 isotype control \( p = 0.0001 \). However, the differences among the treatment by single parental antibody, the combination of two parental antibodies, or anti-HER2 × HER2 hetero-IgG1 could not be demonstrated at the concentrations tested in this study.

Stability of Hetero-IgG1 bsAbs in Human Serum Is Comparable with That of Their Parent Antibodies—To assess the in vitro stability of hetero-IgG1 bsAbs, the bsAbs and their parental antibodies were incubated in 90% human serum at 37 °C up to 168 h. The stability of anti-HER2 × EGFR and anti-HER2 × HER2 hetero-IgG1 was determined by their binding capacity to the captured EGFR and HER2 antigens using ELISA as read-out. As shown in Fig. 13A, anti-HER2 × EGFR hetero-IgG1 V23 retained similar binding capacity to EGFR as the parental anti-EGFR E7.6.3 IgG1 over the incubation in human serum. However, in the HER2 binding assay anti-HER2 × EGFR hetero-IgG1 V23 lost \(~30\)% of HER2 binding after 168 h of incubation in human serum, and a similar trend was observed for the parental anti-HER2 humAb4D5–8 IgG1 \( p = 0.7208 \) (Fig. 13B). Similarly the anti-HER2 × HER2 hetero-IgG1 V23 lost \(~30\)% of HER2 binding at the end of incubation, which tracked the parental anti-HER2 humAb4D5–8 IgG1 because the HER2 binding of a second parental anti-HER2 humAb2C4 IgG1 stayed the same over the incubation (Fig. 13C). There was no statistical difference when comparing anti-HER2 × HER2 hetero-IgG1 V23 with either parental anti-HER2 humAb4D5–8 IgG1 \( p = 0.7027 \) or humAb2C4 IgG1 \( p = 0.0534 \) at the 168-h time point. The data suggested that the hetero-IgG1s had maintained comparable stability in human serum as the parental antibodies.

Prediction of Immunogenicity for Hetero-IgGs—To explore the possibility that the bispecific hetero-IgGs could elicit immunogenicity after the charged residue pairs are introduced in different domains of antibody, we utilized an in silico TEPITOPE algorithm to predict the potential immunogenicity for the parent IgG1 and hetero-IgG1 antibodies (Table 7). Although the parent anti-EGFR E7.6.3 IgG1 did not have any predicted 9-mer peptides binding to DRB1 alleles, one peptide (173–181) in Cκ and one peptide (36–44) in the VH of anti-EGFR E7.6.3 in the context of hetero-IgG1 were predicted to
bind to DRB1_0401 and DRB1_0301, respectively. The parent anti-HER2 humAb4D5–8 IgG1 had three potential DRB1-binding peptides. There were two additional linear peptides (173–181 and 175–183) in C\textsubscript{\textalpha}, one additional peptide (36–44) in VH, and two additional linear peptides (404–412 and 406–414) in C\textsubscript{\textbeta}\textsubscript{3} of the hetero-IgG1 that could bind to DRB1 alleles. Similarly, the parent anti-HER2 humAb2C4 IgG1 had five potential DRB1-binding peptides. There was one additional linear peptide (173–181) in C\textsubscript{\textalpha} that was predicted to bind to DRB1 alleles. It should be noted that the introduced charge residues in all additional DRB1-binding peptides are buried at the VH-VL and CH1-CL interfaces of the cognate HC-LC so that LC strongly favors its cognate HC during the assembly of bsAbs. The BiP protein can retain in the endoplasmic reticulum by the BiP protein through the interaction in the C\textsubscript{\textmu}1 region. The BiP protein can be exchanged by either of the two LCs to form the IgG molecules that are subsequently secreted from cells. It is reasonable to assume that the relative assembly kinetics between HC and the two LCs will determine the final pairing of the HC with each LC. The interactions of both VH-VL and CH1-CL interfaces is generally required to minimize the impact on antibody expression and stability. Among the many charged residue pairs that are introduced in hetero-IgG, the less important to apply a negative design principle in the engineering process so that neither of the two LCs can efficiently assemble with its noncognate HC. For any given pair of antibodies, this can be achieved empirically by using different designs as exemplified in this report. We also developed a very stringent LC-HC mispairing assay in which each design of different LCs is introduced with its noncognate HC by co-transfection of HEK293 cells and tested for its ability to support the secretion of IgG.

**DISCUSSION**

In this report we described an antibody engineering strategy to produce monovalent bispecific heterodimeric IgG from two pre-existent antibodies in mammalian cells. The hetero-IgG bsAbs produced in this format have the same overall size and natural structure as the regular IgG antibody. In particular, we tested multiple designs to engineer LC-HC interface residues so that LC strongly favors its cognate HC during the assembly of bispecific heterodimeric IgGs. This was achieved by introducing charged residues with opposite polarity at the selected positions between VH-VL and C\textsubscript{\textmu}1-C\textsubscript{\textalpha} interfaces. During the assembly process of bsAb inside cells, each HC is retained in the endoplasmic reticulum by the BiP protein through the interaction in the C\textsubscript{\textmu}1 region. The BiP protein can be exchanged by either of the two LCs to form the IgG molecules that are subsequently secreted from cells. It is reasonable to assume that the relative assembly kinetics between HC and the two LCs will determine the final pairing of the HC with each LC. The interactions of both VH-VL and C\textsubscript{\textmu}1-CL domains contribute to final recognition and assembly of HC and LC (27–31).
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human subjects. A fully human anti-TNF antibody adalimumab can elicit immune responses in patients (48). Because the positions for charged pair residue substitutions in this report are buried inside the hydrophobic core of the VH-VL and Cγ1-CL interfaces, it is reasonable to speculate that minimal humoral immune response could be elicited due to the introduction of charged residue pairs. However, more work is needed to determine whether the hetero-IgG1s induce T cell-dependent immune responses in humans.

Most of the LC variants described in this report were based on Cκ chain in the constant region. However, because the Cκ and CA constant domains are structurally conserved, particularly at the LC-HC interfaces, the negative design principle could also be applied in addressing the LC-HC mispairing issues when the ALC(s) is engineered. Alternatively, the constant region can be switched from CA to Cκ to make a Vκ-Cκ chimeric LC. We did observe a slight decrease of expression titer for some antibodies when VΛ-Cκ chimeric LC was used.

The bispecific anti-HER2 × EGFR hetero-IgG1 induces a higher level of receptor internalization than the combination of two parental antibodies. This is likely due to the unique feature of two different Fab arms that could simultaneously bind to two different receptors on the cell surface. Down-regulation of receptors EGFR and HER2 has been proposed as one of the antitumor mechanisms for the combined anti-EGFR and anti-HER2 antibody treatment in mouse xenograft models (7). The bispecific anti-HER2 × EGFR hetero-IgG1 may have the same effect. Each Fab arm in anti-HER2 × HER2 hetero-IgG1 can individually bind antigen HER2. Anti-HER2 × HER2 hetero-IgG1 V23 functions comparably as the combination of two parental antibodies in the ADCC killing assay but less potently than the combination of two parental antibodies in pHER2/ pHER3/pAKT assays. It still remains to be tested whether the two different Fab arms in anti-HER2 × HER2 hetero-IgG1 can simultaneously bind to two different epitopes on HER2.

The bispecific anti-HER2 × EGFR hetero-IgG1s strongly inhibited tumor growth in the mouse xenograft BxPC-3 human tumor model. Higher antitumor activity of anti-HER2 × EGFR hetero-IgG1s was observed than that of the combination of two parental antibodies in the Panc-1 xenograft bearing Rag2-/-/ mFcyR4-/-/hCD16a+ transgenic mice, suggesting that the bispecific anti-HER2 × EGFR hetero-IgG1s may have advantages over the combination of two parental Abs. Furthermore, the ADCC-enhanced anti-HER2 × EGFR hetero-IgG1 V23_ W165 had higher antitumor activity than anti-HER2 × EGFR hetero-IgG1 V23, indicating that ADCC enhancement can elicit potent killing to tumor cells to overcome K-RAS mutation in Panc-1 cells (46). In another aspect, all anti-HER2 antibodies worked well in the Calu-3 xenograft tumor model in female NSG mice, different from the studies by Scheuer et al. (42) in which BALB/c nu/nu female mice were tested. One possible explanation for the increased activity observed in our study is that, unlike BALB/c nu/nu mice, the NSG mice lack serum Ig to block FcγRs on effector cells such as neutrophils, monocytes, and macrophages. Therefore, the effector cells in NSG mice could more effectively bind to Fc fragments of human IgG1, leading to a strong tumor growth inhibition to Calu-3 cells by engaging murine FcγRs.

In summary, we developed an antibody engineering approach to produce bispecific hetero-IgG1 antibodies in mammalian cells. The hetero-IgG1 we generated contains the predicted four different chains, binds to two different antigens with comparable affinity when compared with their parental antibodies, retains their functionality of parental antibodies, and induces higher receptor internalization than the combination of two parental anti-HER2 and anti-EGFR antibodies. Although BxPC-3, Panc-1, and Calu-3 human tumor xenograft models showed that the hetero-IgG1s strongly inhibited the tumor growth, more studies are warranted with different antibodies to demonstrate that our observations are expandable in this hetero-IgG format. The approach described in this report could be applied to the evaluation of bsAb using two pre-existing antibodies as well as for the development of novel therapeutic molecules for treatment of many diseases such as cancers and infectious diseases. The asymmetrically engineered Fc variants for ADCC enhancement could be embedded in monovalent bispecific hetero-IgG1 to make best-in-class therapeutic antibodies.

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