Supporting Information

Labrijn et al. 10.1073/pnas.1220145110

SI Materials and Methods

Cell Lines. FreeStyle 293F (HEK-293F) and FreeStyle CHO-S (CHO-S) cells were cultured in FreeStyle 293 expression medium and FreeStyle CHO expression medium, respectively (Invitrogen). Additional cell lines were obtained from the American Type Culture Collection (ATCC). Daudi (human Burkitt's lymphoma) and Jurkat (human T-cell leukemia) cells were cultured in RPMI 1640 (Lonza) supplemented with 10% (vol/vol) heat inactivated cosmic calf serum (CCS; HyClone), 2 mM L-glutamine (Lonza), 1 mM sodium pyruvate (Lonza), 50 IU/mL penicillin, and 50 μg/mL streptomycin (Lonza). AU565 (human breast carcinoma) and NCI-N87 (human gastric carcinoma) cells were cultured in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated CCS, 1.5 g/L sodium bicarbonate (Lonza), 1 mM sodium pyruvate, 4.5 g/L glucose (Sigma), 50 IU/mL penicillin, and 50 μg/mL streptomycin. All cell lines were maintained at 37 °C in a 5% (vol/vol) CO_2 humidified incubator.

Isolation of Primary Lymphocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation using Leucosep tubes (Greiner Bio-one), according to the manufacturer's instructions. Briefly, buffy coats from standard blood donations (Sanquin Blood Bank) were diluted 1:2 in PBS and layered on top of 15-mL Lymphocyte Separation Medium in 50-mL tubes. Tubes were subsequently centrifuged at $800 \times g$ for 20 min at 20 °C, and PBMCs were recovered from the plasma–medium interface. Collected PBMCs were then washed several times with PBS until supernatant was clear. To obtain purified T cells, cells expressing CD11b, CD16, CD19, CD36, and CD56 were depleted from collected PBMCs using the Dynabead Untouched Human T-cell kit (Invitrogen). Isolated T cells were resuspended in culture medium to a final concentration op 7×10^6 cells/mL.

Cloning and Production of Antibodies. Expression vectors for IgG1-CD20, IgG4-CD20, rhChIgG4-CD20, IgG1-EGFR, IgG4-EGFR, rhChIgG4-EGFR, IgG1-P228S-EGFR, IgG1-P228S-ITL-EGFR, and IgG4-637 have been described previously (1–3). A Quikchange site-directed mutagenesis kit (Stratagene) was used to introduce the T350I/K370T/F405L (ITL), L368X, K370X, D399X, F405L, F405X, Y407X, and K409R mutations (with X representing all natural amino acids with the exception of C and P) in IgG1 using pConG1f2F8 or pConG1f7D8 as templates (EU numbering conventions are used throughout the manuscript). The variable domain heavy (VH) and light (VL) chain coding regions of mAbs 153 and 169 [human epidermal growth factor receptor 2 (HER2) specific] and mAb b12 (HIV-1 gp120-specific) (4) were codon optimized, synthesized de novo by Geneart, and cloned into expression vectors pConG1f (Lonza), for the production of IgG1 heavy chain or pConKappa for the production of light chain. In addition, murine mAb CLB-T3/4 (human CD3 specific) (5) was humanized by CDR grafting on the closest related human germ-line sequences (Table S2) and cloned into pConG1f and pConKappa. Alternatively, VH and VL coding regions were cloned into p33G1f and p33Kappa, respectively, which were obtained by cloning the codon optimized coding regions for the IgG1 heavy chain or kappa light chain constant domains into expression vector pcDNA3.3 (Invitrogen). A Quikchange site-directed mutagenesis kit (Stratagene) was used to introduce the N297Q, T350I/K370T/F405L (ITL), F405L, and K409R mutations.

All antibodies were produced under serum-free conditions (FreeStyle medium) by cotransfecting relevant heavy and light chain expression vectors in HEK-293F cells, using 293fectin (Invitrogen), according to the manufacturer's instructions.

Antibodies were purified by Protein A affinity chromatography (rProtein A FF; GE Healthcare), dialyzed overnight to PBS, and filter-sterilized over 0.2-μM dead-end filters. Concentration of purified IgGs was determined by absorbance at 280 nm (specific extinction coefficients were calculated for each protein). Purified proteins were analyzed by SDS/PAGE and mass spectrometry. Batches of IgG were tested by high-performance size-exclusion chromatography (HP-SEC) and shown to be at least 94% monomeric. Endotoxin levels of batches used in vivo were <0.1 endotoxin units/mg IgG.

Glutathione-Mediated Fab-arm Exchange In Vitro. As described previously (3), combinations of antibodies were mixed and incubated with reduced glutathione (GSH; Sigma) at a final concentration of 4 μg/mL per antibody. The final concentration of GSH was 0.5 mM. The mixtures were incubated at 37 °C for 24 h, and samples were drawn in PBS-TB (PBS, 0.05% Tween-20, 0.2% BSA) at 4 °C, in which bispecific IgG was measured.

2-Mercaptoethylamine–Mediated Controlled Fab-arm Exchange In Vitro. Equimolar amounts of IgG1-F405L (unless indicated otherwise) and IgG1-K409R (unless indicated otherwise) antibodies were mixed and incubated with 2-mercaptoethylamine (2-MEA; Sigma) at a final concentration of 1 mg/mL per antibody (unless indicated otherwise). The final concentration of 2-MEA was 25 mM (unless indicated otherwise). The mixtures were typically incubated for 90 min at 37 °C or 5 h at 25 °C. Depending on the reaction volume, 2-MEA was removed by buffer-exchanging against PBS using Zeba spin desalting plates [100–200 μL; 7-kDa molecular weight cut-off (MWCO); Pierce], PD-10 desalting columns (1–3 mL; 5-kDa MWCO; GE Healthcare), MicroSpin G-25 desalting column (3 mL; 7-kDa MWCO; GE Healthcare), or by diafiltration using a 2.5-m^2 Omega polyethersulfone (PES) ultrafiltration membrane (25 L; 30-kDa MWCO; Pall). Samples were stored overnight at 4 °C to allow reoxidation of the disulfide bonds to occur.

Dual-Binding ELISA for the Detection of EGFR×CD20 Bispecific Antibodies. The presence of EGFR×CD20 bispecific antibodies (bsAbs) was determined using a sandwich ELISA as described previously (3). In short, ELISA plates (Greiner bio-one) were coated overnight with 2 μg/mL of recombinant EGFR (extracellular domain) in PBS at 4 °C. The plates were washed and incubated with serial diluted plasma samples or purified antibody mixtures (in PBS-TB) for 90 min at 20 °C under shaking conditions (300 rpm on MTS 2/4 digital microtiter plate shaker; Fisher Scientific). Next, the plates were washed and incubated with 2 μg/mL of mouse anti-idiotype monoclonal antibody 2F2 SAB1.1 (directed against HuMab-CD20; Genmab) diluted in PBS-TB for 75 min at 20 °C. Bound bsAbs were detected with HRP-labeled goat–anti-mouse IgG (Jackson ImmunoResearch) and ABTS substrate (Roche Diagnostics). The color development reaction was stopped by addition of an equal volume of oxalic acid (Riedel de Haen), and absorbance was measured at 405 nm. bsAbs in plasma samples were quantified by nonlinear regression curve-fitting (GraphPad Software) using an in vitro exchanged antibody mixture as reference.

Detection of Fab-arm Exchange by Electrospray Ionization MS. The presence of bsAbs was determined using electrospray ionization MS (ESI-MS) as described previously (1). Samples containing the

antibody mixtures in 50-μL aliquots (200 μg/mL) were deglycosylated overnight with 1 μL *N*-glycosidase F (Roche Diagnostics). Samples were desalted on an Acquity UPLC (Waters) with a BEH300 C18, 1.7-μm, 2.1 × 50-mm column at 60 °C, and 5 μL was injected and eluted with a gradient from 2% to 95% (vol/vol) acetonitrile (liquid chromatography-MS grade; Biosolve) in deionized water(Millipore) over a 5-min period. The gradient contained 0.1% formic acid as organic modifier (Fluka). ESI-TOF mass spectra were recorded online on a microTOF mass spectrometer (Bruker) operating in the positive ion mode. In each analysis, a 500- to 2,700-*m/z* scale was internally calibrated with ES tuning mix (Agilent). Mass spectra were deconvoluted using the maximum entropy algorithm, provided in DataAnalysis software v3.4 (Bruker). By integrating the peak area corresponding to the bsAb in conjunction to total integrated peak area, the estimated percentage of bsAbs could be deduced.

Cation-Exchange High-Pressure Liquid Chromatography. The efficacy of controlled Fab-arm exchange (cFAE) was assessed by cation exchange high-pressure liquid chromatography (CIEX). For this, samples were diluted to 2 mg/mL in buffer A (10 mM sodium phosphate, pH 7.0), and 25 μL was injected onto an Alliance 2795 HPLC separation module (Waters). The IgG molecules were separated based on charge by using a ProPac WCX-10 column 4 × 250 mm (Dionex Corp.) with a flow rate of 1 mL/min at 30 °C. Elution was performed with a gradient to buffer B (10 mM sodium phosphate with 0.25 M sodium chloride, pH 7.0) using the following gradient conditions: 0% B initial 3 min to 72% B in 55.5 min. Next, 100% eluens C (10 mM sodium phosphate with 0.75 M sodium chloride, pH 7.0) for 5 min was used to clean the column. Protein elution was monitored by measuring the absorbance at 280 nm with a 2,478 dual λ absorbance detector (Waters).

SDS/PAGE. Antibodies were analyzed on SDS/PAGE [4–12% (wt/vol) Bis-Tris; Invitrogen] under nonreducing or reducing conditions at neutral pH according to the manufacturer's instructions. The gels were stained with Coomassie (Invitrogen) and digitally imaged using the Optigo Imaging System (Isogen).

HP-SEC. Antibody batches were analyzed for monomer, multimer and degradation product content by HP-SEC. For this, 50-μL antibody samples (IgG concentration between 1 and 10 mg/mL) were injected into an Alliance 2795 separation unit (Waters) connected to a TSK G3000SW_{xl} column (Toso Biosciences) pre-equilibrated with PBS and kept at 27.5 °C. The samples were run at 1 mL/min for 20 min. Protein detection occurred at 280 nm with a 2,487 dual λ absorbance detector (Waters). Chromatograms were analyzed using Empower software, version 2002 (Waters), and expressed per peak as percentage of total peak area.

Differential Scanning Calorimetry. Changes in thermal stability as a result of the CH3 mutations were determined by differential scanning calorimetry (DSC). Samples were analyzed using an automated VP-capillary DSC system (MicroCal) to determine the melting temperatures (T_m) of the protein (domains). Samples and corresponding buffer (PBS, pH 7.4; Braun) were heated from 10 °C to 100 °C with a scanning rate of 240 °C/h. Samples were run in duplicate, and three buffer scans were acquired in between. Calorimetric enthalpies were calculated using the area under curve (AUC) of the resulting peaks. The T_ms were reported as the average of two experiments with the corresponding SD.

Generation and Purification Fabs. IgG solutions (1 mg/mL in PBS supplemented with 10 mM cysteine and 2 mM EDTA) were prepared, to which 40 μg/mL papaine (Sigma-Aldrich) was added and incubated at 37 °C for 4–8 h depending on the optimal digestion rate. The reaction was stopped by the addition of iodoacetamide (IAA) to a final concentration of 25 mM and incubation for 30 min in the dark at room temperature. The samples were dialyzed overnight at 2–8 °C against a 1,000-fold volume of PBS, pH 7.4 (Braun). Fab fragments were purified by a two-step process, i.e., intact IgG and intact Fc were removed by standard protein A chromatography, followed by a standard gel filtration of protein A flow through to separate the Fab product and degraded Fc.

Complement-Dependent Cytotoxicity Assay. The capacity to induce complement-dependent cytotoxicity (CDC) was assessed essentially as described (6). Briefly, target cells (1×10^5 cells) were preincubated at 21 °C for 15 min with serial diluted antibodies. Pooled human serum [20% (vol/vol)] was added as a source of complement, and cells were incubated at 37 °C for an additional 45 min. Cells were then put on ice, and viability was determined by staining with propidium iodide (PI) and detected using a FACSCanto II flow cytometer (BD Biosciences).

Antibody-Dependent Cellular Cytotoxicity Assay. The capacity to induce effector cell–dependent lysis of tumor cells was evaluated by ^{51}Cr-release assay. Target cells (5×10^5 cells) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Biosciences) under shaking conditions at 37 °C for 1 h. Cells were washed three times with PBS and resuspended in culture medium 1×10^5 cells/mL. Labeled cells were dispensed in 96-well plates (5×10^3, in 50 μL per well) and preincubated (20 °C, 30 min) with 50 μL of 10-fold serial dilutions of mAb in culture medium, ranging from 1 μg/mL to 0.5 ng/mL (final concentrations). Culture medium was added instead of antibody to determine the spontaneous ^{51}Cr release, Triton X-100 [1% (vol/vol) final concentration] was added to determine the maximal ^{51}Cr release. Next, PBMCs were added to the wells (5×10^5 per well), and cells were incubated at 37 °C overnight. The next day, supernatants were collected for measurement of the ^{51}Cr release by determination of the cpm in a gamma counter. Percentage of cellular cytotoxicity was calculated using the following formula: % specific lysis = [experimental release (cpm) − spontaneous release (cpm)]/[maximal release (cpm) − spontaneous release (cpm)] × 100.

Animals. Six- to 11-wk-old female SCID mice (C.B-17/Icr-*Prkdc*^{scid}) or NOD-SCID mice (NOD.CB17-*Prkdc*^{scid}/Ncr) were obtained from Charles River Laboratories and housed in a barrier unit of the Central Laboratory Animal Facility. The mice were kept in filter-top cages with water and food provided ad libitum. Microchips and ear tags were used for mouse identification. Mice participating in experiments were checked daily for signs of toxicity and discomfort. All experiments were approved by the Utrecht University animal ethics committee.

Pharmacokinetic Analysis. bsAbs (100 μg per mouse) were administered i.v. to groups of mice ($n = 3$), and blood samples were drawn from the submandibular vein at 10 min, 3 h, and 1, 2, 7, 14, 21, and 28 d after administration. Blood was collected in heparin-containing vials and centrifuged (5 min at 10,000 × *g*) to separate the plasma from cells. Plasma was transferred to a new vial and stored at −20 °C for determination of (bispecific) antibody levels.

IgG1-CD20 and IgG1-EGFR ELISA. The amount of human IgG1-CD20 antibody in plasma samples from mice was determined by ELISA. In short, ELISA plates were coated overnight with 5 μg/mL of mouse anti-idiotype monoclonal antibody 2F2 SAB1.1 (directed against HuMab-CD20; Genmab) in PBS at 4 °C. The plates were subsequently washed and blocked with PBS-B [PBS/2% (wt/vol) BSA] for 60 min at 20 °C under shaking conditions (300 rpm). Next, the plates were washed and incubated with diluted plasma samples in PBS-TB for 60 min at 20 °C under shaking conditions

(300 rpm). Bound antibodies were detected by HRP-labeled rabbit anti-human kappa (DAKO) and ABTS substrate (Roche Diagnostics). The color development reaction was stopped by addition of an equal volume of oxalic acid, and absorbance was measured at 405 nm. IgG was quantified by nonlinear regression curve fitting (GraphPad Software) using the injected antibodies as reference.

The amount of IgG1-EGFR antibody was detected similarly using ELISA plates coated overnight with 2 μg/mL of recombinant EGFR (extracellular domain) in PBS at 4 °C.

Plasma Clearance. Plasma clearance was calculated for individual mice as dose/ area under the curve (AUC)(0–∞) per kilogram body weight (mL/d/kg). For this, the AUC from time 0 to time t [AUC(0–t)], was determined from the plasma antibody concentration vs. time plot. Additionally, the K_{el} (terminal elimination rate constants) were determined from the log-concentration over time curves using the terminal parts (>2 d) of the curves by nonlinear regression curve fitting (GraphPad Software). AUC(0–∞) was calculated with the formula AUC(0–∞) = AUC(0–t) + [Ct/K_{el}], where Ct is serum concentration at time t.

Flow Cytometric Assay for Simultaneous Binding on Cells. Single cell suspensions of HER2-positive AU565 cells and CD3-positive Jurkat cells were labeled with 1.25 μM CSFE (Carboxyfluorescein diacetate succinimidyl ester; Invitrogen) and 0.2 μM PKH26 (Sigma-Aldrich), respectively, according to the manufacturer's instructions. Cells were washed three times with culture medium and two times with FACS buffer [PBS supplemented with 0.2% (wt/vol) BSA and 0.025% (vol/vol) NaN_3] and resuspended in FACS buffer at 1×10^6 cells/mL. Mixtures of 50,000 CFSE-labeled AU565 cells and 50,000 PKH26-labeled Jurkat cells were incubated for 30 min at 4 °C in a 96-well round bottom plate (Greiner bio-one), with serial diluted antibody (final concentration 0–1,000 ng/mL) in a total volume of 150 μL FACS buffer. Cells were then washed in FACS buffer and resuspended in 100 μL for analysis on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FACSDiva, version 6.1.2 (BD Biosciences), and GraphPad Prism for Windows, version 5.01 (GraphPad) software.

Cytotoxicity Assay. Target (T) cells (5×10^4 AU565 cells) were incubated in 96-well flat-bottomed plates (Greiner) for at least 3 h at 37 °C to allow cells to adhere to the plate. Adhered cells were washed twice and subsequently coincubated with PBMC effector (E) cells at an E/T ratio of 5:1 in culture medium, in the presence or absence of serial diluted bsAbs and control Abs (ranging from 4×10^7 to 20 pg/mL). Wells incubated with Staurosporine [0.5% (vol/vol) final concentration] were included to define maximal cell kill (100%). After incubating for 2 d at 37 °C, supernatants were collected and stored for analysis (Fig. S5*A*). Target cells were washed with PBS, and survival was assessed by incubating (4 h at 37 °C) in 10% (vol/vol) alamarBlue (Invitrogen) in culture medium. Absorbance was measured using an EnVision Multilabel reader (PerkinElmer), and data analysis was performed using Microsoft Office Excel 2003 (Microsoft) and GraphPad Prism for Windows, version 5.01 (GraphPad) software.

T-Cell Activation Assay. Target (T) cells (5×10^4 AU565 cells) were incubated in 96-well flat-bottomed plates for at least 3 h at 37 °C to allow cells to adhere to the plate. Adhered cells were washed twice and subsequently coincubated with purified T lymphocytes (E) at an E/T ratio of 2:1 in culture medium, in the presence or absence of serial diluted bsAbs and control Abs (ranging from 1×10^6 to 1,000 pg/mL). After incubating for 16 h at 37 °C, T lymphocytes were collected, washed in FACS buffer, and resuspended in 150 μL FACS buffer. Expression of CD69, a well-characterized activation marker of cytotoxic T cells, was stained with allophycocyanin (APC)-conjugated mouse anti-human CD69 antibody (clone L78; BD Biosciences) and measured by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FACSDiva, version 6.1.2 (BD Biosciences), and GraphPad Prism for Windows, version 5.01 (GraphPad) software.

Adoptive Transfer Xenograft Tumor Models. Tumor xenografts induction and PBMC reconstitution were performed simultaneously by s.c. inoculation of a mixture of 5×10^6 NCI-N87 cells with 5×10^6 PBMCs (from either of two donors). Within 1 h, mice were randomized (for PBMC donors) over treatment groups, and Ab treatment was started. Abs were injected i.p. or i.v. as indicated in *Results*. During the study, a heparinized blood sample was taken for determination of antibody plasma levels, which were in the expected range. Tumor volumes were measured twice a week and calculated from digital caliper measurements as $0.52 \times \text{length} \times \text{width}^2$ (in mm^3).

Quantitative IgG ELISA. The total amount of human antibodies in the mice was determined by sandwich ELISA. In short, ELISA plates were coated overnight with 2 μg/mL of mouse anti-human IgG (MH16-1; Sanquin) in PBS at 4 °C. The plates were subsequently washed and blocked with PBS-C [PBS/2% (vol/vol) normal chicken serum; Gibco] for 1 h at 37 °C. Next, the plates were washed and incubated with diluted plasma samples in PBS-T (PBS/0.05% Tween-20) for 120 min at 20 °C under shaking conditions (300 rpm). Bound antibodies were detected by HRP-labeled goat anti-human IgG (Jackson ImmunoResearch) and ABTS substrate (Roche Diagnostics). The color development reaction was stopped by addition of an equal volume of oxalic acid, and absorbance was measured at 405 nm. IgG was quantified by nonlinear regression curve fitting (GraphPad Software) using the injection mixtures as reference.

HER2 Down-Modulation Assay. AU565 cells were seeded in 24-well tissue culture plates (100,000 cells per well) in normal cell culture medium and cultured for 3 d at 37 °C in the presence of 10 μg/mL HER2 antibody. Cells were washed in PBS and lysed by incubating 30 min at 20 °C in 110 μL Surefire Lysis buffer (PerkinElmer). Total protein levels were quantified using bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's instructions. HER2 protein levels were analyzed using a HER2-specific sandwich ELISA. In short, ELISA plates were coated overnight with 1,000-fold diluted rabbit anti-human HER2 intracellular domain antibody (Cell Signaling) in PBS at 4 °C. The plates were subsequently washed and blocked with PBS-C [PBS/2% (vol/vol) normal chicken serum] for 1 h at 20 °C. Next, the plates were washed and incubated with undiluted cell lysates for 1 h at 20 °C under shaking conditions (300 rpm). Captured HER2 protein was detected by subsequent incubations (1 h at 20 °C under shaking conditions followed by washing) with 0.15 μg/mL biotinylated goat anti-human HER2 polyclonal antibody (R&D Systems) diluted in PBS-TC (PBS/0.05% Tween-20/0.2% normal chicken serum), followed by 0.1 μg/mL streptavidin-poly-HRP (Sanquin) diluted in PBS-TC. Captured HER2 was visualized by ABTS, and the coloring reaction was stopped with oxalic acid. Fluorescence at 405 nm was measured, and the amount of HER2 was expressed as a percentage relative to untreated cells.

Xenograft Tumor Models. Tumor xenografts were induced by s.c. inoculation of 5×10^6 NCI-N87 cells. When tumor volume reached 200 mm^3 (approximately day 7), mice were randomized over treatment groups, and Ab treatment was started. Ab doses of 40 and 20 mg/kg were injected i.p. at days 7 and 14, respectively. During the study, two heparinized blood samples were taken for determination of antibody plasma levels, which were in the expected range. Tumor volumes were measured twice a week and calculated from digital caliper measurements as $0.52 \times \text{length} \times \text{width}^2$ (in mm^3).

Statistical Analysis. Data sets were compared using one-way ANOVA analysis (Tukey's multiple comparison) at the last day all groups were intact (GraphPad Prism for Windows, version 5.01; GraphPad). Log-rank Mantel-Cox analysis was applied to tumor progression curves (SPSS statistics 20; IBM). Statistical significance was accepted when $P < 0.05$.

1. Labrijn AF, et al. (2009) Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. *Nat Biotechnol* 27(8):767–771.
2. Labrijn AF, et al. (2011) Species-specific determinants in the IgG CH3 domain enable Fab-arm exchange by affecting the noncovalent CH3-CH3 interaction strength. *J Immunol* 187(6):3238–3246.
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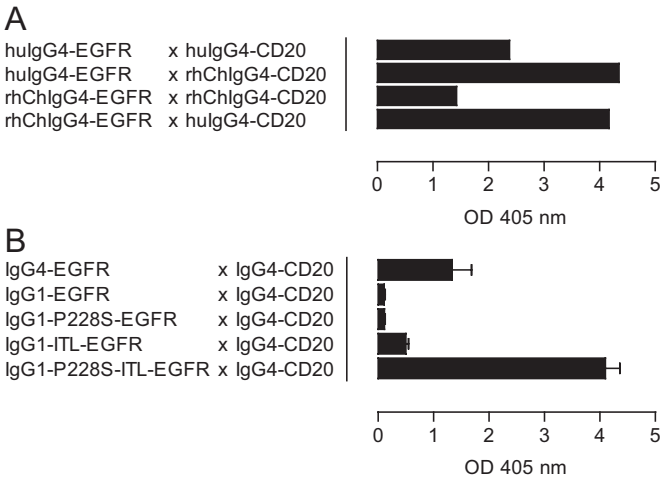


Fig. S1. Interspecies Fab-arm exchange between rhesus and human IgG4 leads to more efficient formation of bispecific antibodies (bsAbs). (*A*) Variable regions of human monoclonal antibodies 2F8 (anti-EGFR) and 7D8 (anti-CD20) were cloned into IgG4 backbones of human (hu) and Chinese rhesus monkey (rhCh) origin (1, 2). (*B*) Rhesus-specific molecular determinants [i.e., I350, T370, and L405 (ITL) (1)] were introduced into human IgG1-EGFR and IgG1-P228S-EGFR. Mixtures of indicated antibodies were incubated in the presence of 0.5 mM GSH at 37 °C. The formation of bsAb after 24 h was measured by dual-binding ELISA. Representative signals at a (total antibody) concentration of 1 μg/mL are shown. The results show that more bsAb is produced in interspecies Fab-arm exchange between human and rhesus monkey IgG4 compared with intraspecies exchange. Furthermore, this increase in efficiency could be transferred to human IgG1-P228S molecules through introduction of the rhesus-specific residues I350, T370, and L405.

1. Labrijn AF, et al. (2011) Species-specific determinants in the IgG CH3 domain enable Fab-arm exchange by affecting the noncovalent CH3-CH3 interaction strength. *J Immunol* 187(6): 3238–3246.
2. van der Neut Kolfschoten M, et al. (2007) Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 317(5844):1554–1557.

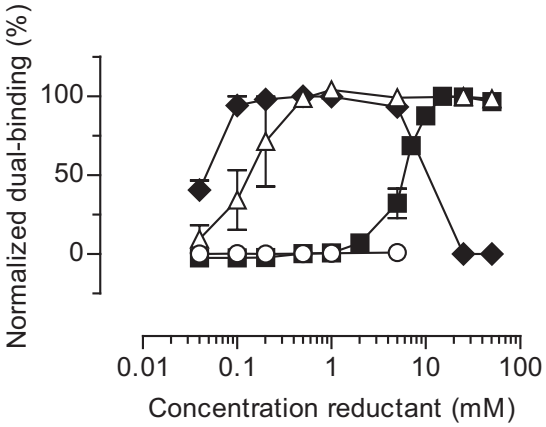
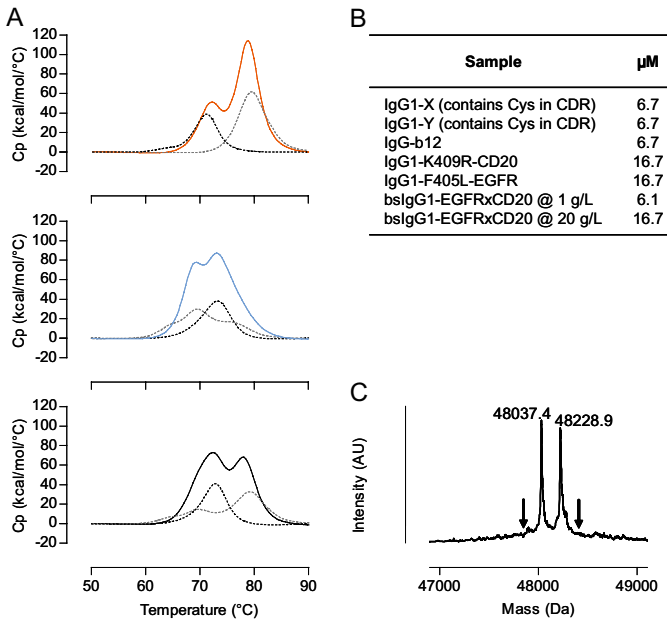


Fig. S2. 2-MEA can efficiently induce FAE in IgG1 molecules (containing WT IgG1 hinges). The rhesus IgG4 F405L mutation in combination with the human IgG4 K409R mutation was found to be sufficient to recapitulate the improvement of FAE. Mixtures of single point mutants (IgG1-F405L-EGFR and IgG4-K409R-CD20) were incubated at 37 °C in the presence of increasing concentrations of different reducing agents. The formation of bsAbs after 90 min was measured by ELISA. The formation of bsAb at a (total antibody) concentration of 1 μg/mL is shown. The results showed that reduced GSH (○) was not a suitable reductant, as concentrations up to 5 mM were not able to induce FAE within the time span tested, and higher concentrations (25–50 mM) resulted in the formation of antibody aggregates (and were therefore excluded from analysis). Tris(2-carboxyethyl)phosphine (TCEP; ◆) was found to be very effective with maximal FAE observed over a concentration range of 0.2–5 mM; however, at higher concentration (25–50 mM), FAE was disturbed. DTT (△) was found to be very effective, with maximal FAE reached at 0.5 mM DDT, which also persisted over higher concentrations of DTT (1–50 mM). Finally, 2-MEA (■) induced effective FAE, with maximal FAE observed at 25 mM 2-MEA, which persisted at higher concentrations. Note that 2-MEA was chosen for further analysis as it is relatively inexpensive and available in clinical grade (Cysteamine bitartrate; Cystagon). Data were normalized to a preformed bsAb (Fig. S1*B*, *Bottom bar*) included on every plate.



Sample	μM	Free SH (μM)	Free SH/ protein
IgG1-X (contains Cys in CDR)	6.7	6.1	0.92
IgG1-Y (contains Cys in CDR)	6.7	13.9	2.09
IgG-b12	6.7	1.4	0.21
IgG1-K409R-CD20	16.7	2.9	0.18
IgG1-F405L-EGFR	16.7	<1	<0.06
bsIgG1-EGFRxCD20 @ 1 g/L	6.1	<1	<0.16
bsIgG1-EGFRxCD20 @ 20 g/L	16.7	<1	<0.06

Fig. S3. Biochemical and physicochemical analysis of bsIgG1-EGFR×CD20. (*A*) Thermostability of bsIgG1 as measured by DSC. Separate Fab (gray dotted lines) and Fc (black dotted lines) fragments were prepared from IgG1-F405L-EGFR (*Top*), IgG1-K409R-CD20 (*Middle*), and bsIgG1-EGFR×CD20 (*Bottom*) by papain digestion and compared by DSC. The intact molecules (solid colored lines) were included in the analysis. Data represent one thermogram from a duplicate measurement. The analyses revealed melting temperatures (T_m) of 71.23 ± 0.00 °C (Fc)/72.01 ± 0.01 °C (intact) for the IgG1-F405L Fc domain and 79.73 ± 0.01 °C (Fab)/78.90 ± 0.00 °C (intact) for the anti-EGFR Fab domain. The IgG1-K409R Fc domain unfolded at a T_m of 72.94 ± 0.01 °C (Fc)/73.35 ± 0.03 °C (intact) and the anti-CD20 Fab domain (main peak) at 69.52 ± 0.01 °C (Fab)/68.40 ± 0.02 °C (intact). In the bsIgG1-EGFR×CD20 intact molecule, the first T_m (71.91 ± 0.01 °C) results from the overlap of bsIgG1 Fc and anti-CD20 Fab domain T_ms; however, analysis of the separate domains revealed a T_m of 72.63 ± 0.01 °C for the bsIgG1-Fc domain and two T_ms in the Fab preparation, representing the anti-CD20 (69.73 ± 0.02 °C) and the anti-EGFR (79.24 ± 0.00 °C) Fab fragments. Separate T_ms for CH2 and CH3 domains could not be determined in this experiment. (*B*) Exchange conditions do not increase the amount of free sulfhydryl (SH) groups in cFAE-derived bsIgG. Free SH groups, potentially induced by the exchange process, were quantitated using the Measure-iT Thiol assay (Invitrogen) under denaturing conditions on an L-cysteine standard. The 25-L scale (1 g/L) batch of bsIgG1-EGFR×CD20 was compared with parental mAbs (IgG1-K409R-CD20 and IgG1-F405L-EGFR) and negative control mAb (IgG1-b12) containing no free cysteine, as well as two positive control mAbs (IgG1-X and IgG1-Y) containing a free cysteine in the CDRs of each heavy chain. Indeed, the latter two batches contained an increased amount of free SH compared with the negative control b12. Free SH levels of bsIgG1 were highly comparable to the levels of free SH detected in the parental mAbs and in IgG1-b12. This comparability confirms that the exchange conditions did not induce the formation of free SH groups. (*C*) No L chain swapping detected in cFAE-derived bsIgG1. Fab fragments were generated from the 25-L scale (1 g/L) batch of bsIgG1-EGFR×CD20 using papain digestion. Nonreduced ESI-MS analysis detected masses of 48,037.4 and 48,228.9 Da, matching the theoretical masses of the parental anti-CD20 and anti-EGFR Fabs, 48,037.7 and 48,230.0 Da, respectively. Masses that would result from L chain swapping, potentially induced by the exchange process, were not detected. Arrows indicate the position of the potential L chain swapped Fab fragments (theoretical mass VH-$CH1_{CD20}$+LC_{EGFR}: 48,415.3 Da, theoretical mass VH-$CH1_{EGFR}$+LC_{CD20}: 47,852.4 Da).

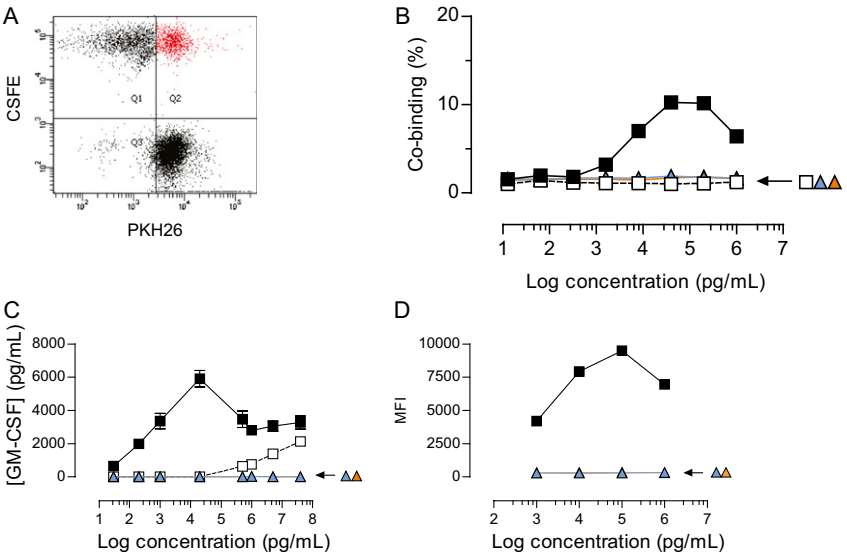


Fig. S4. HER2-specific retargeting leads to T-cell activation. (*A* and *B*) Binding to both HER2 and CD3 was analyzed by incubating mixtures of CSFE-prelabeled HER2-positive AU565 cells and PKH26-prelabeled CD3-positive Jurkat T-cells with bsIgG1-N297Q-CD3×deglycHER2$_{169}$ (obtained by cFAE using IgG1-F405L-N297Q-CD3 and *N*-glycosidase F-treated IgG1-K409R-HER2$_{169}$). A FACSCanto II flow cytometer (BD Biosciences) was used to visualize double (CSFE/PKH26)-positive events representing cell doublets interconnected by simultaneous binding to HER2 and CD3 on either cell type. One representative flow cytometer plot is shown (*A*). The analysis revealed that bsIgG1-N297Q-CD3×deglycHER2$_{169}$ (■), but not control antibody bsIgG1-N297Q-CD3xb12 (□) or parental mAbs IgG1-F405L-N297Q-CD3 (orange triangles), and *N*-glycosidase F-treated IgG1-K409R-HER2$_{169}$ (blue triangles) bound both cell types simultaneously (*B*). A prozone effect is observed that is expected and can be explained by binding competition at high concentration resulting in monovalent binding of the bsIgG1-N297Q-CD3×deglycHER2$_{169}$ antibody. (*C*) Granulocyte macrophage colony-stimulating factor (GM-CSF) is a proinflammatory cytokine that is produced on T-cell activation (1). GM-CSF concentration in culture supernatants derived from cytotoxicity assays (Fig. 5*A*) were measured by human GM-CSF ELISA kit (BD Biosciences). In supernatant derived from cocultures of AU565 cells with PBMCs in the presence of serial diluted bsIgG1-N297Q-CD3×HER2$_{169}$ (■), dose-dependent GM-CSF production could be measured, in line with the cytotoxicity data. No GM-CSF production was measured for control samples, IgG1-K409R-N297Q-HER2$_{169}$ (blue triangle) and IgG1-F405L-N297Q-CD3 (orange triangle); however, higher concentrations of bsIgG1-N297Q-CD3xb12 (□) induced some GM-CSF production. This GM-CSF production could be due to activation by indirect cross-linking via residual FcγR interaction. (*D*) Expression of CD69, a well-characterized activation marker of cytotoxic T cells, was measured by flow cytometry after coculturing isolated human T cells with AU565 tumor cells in the presence of bsIgG1-N297Q-CD3×HER2$_{169}$ (■) for 16 h at 37 °C. A dose-dependent increase in CD69 expression was observed for the antibody concentration in the range of 1,000–100,000 pg/mL, which represents activation of the T cells and correlates with the observed dose-dependent cytotoxicity data. No CD69 expression was measured for control samples: IgG1-K409R-N297Q-HER2$_{169}$ (blue triangle) and IgG1-F405L-N297Q-CD3 (orange triangle).

1. Collins DP, Luebering BJ, Shaut DM (1998) T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry* 33(2):249–255.

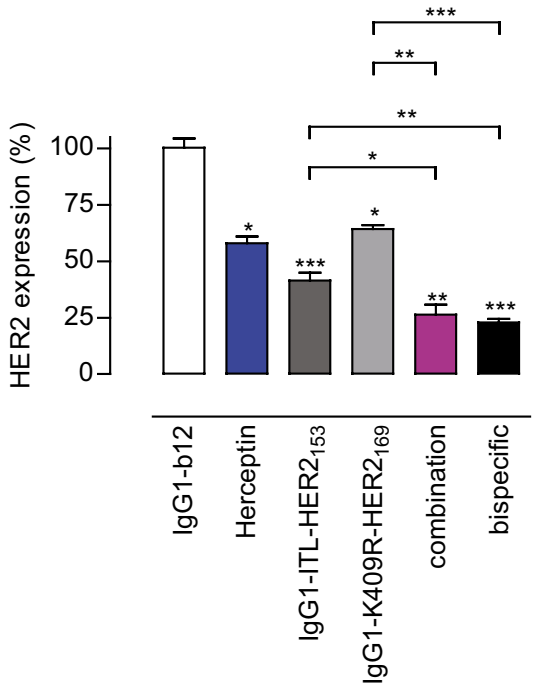


Fig. S5. Dual-targeting HER2 leads to increased down-modulation of HER2. AU565 cells were seeded to confluence (to minimize antibody-induced effects on proliferation) in 24-well tissue culture plates (100,000 cells per well) and incubated for 3 d at 37 °C in the presence of 10 μg/mL of the indicated mAb (total concentration). The amount of HER2 was quantified in AU565 cell lysates using a HER2-specific capture ELISA, normalized to total protein levels, and plotted as a percentage relative to untreated cells. Data represent mean ± SEM of at least two separate experiments. The results showed that the individual HER2-specific mAbs (herceptin, IgG1-ITL-$HER2_{153}$, IgG1-K409R-$HER2_{169}$), the combination of HER2-specific mAbs (IgG1-ITL-$HER2_{153}$ + IgG1-K409R-$HER2_{169}$), and the bsAb (bsIgG1-$HER2_{153}$×$HER2_{169}$) all induced a significant down-modulation of HER2 protein levels compared with untreated cells, whereas irrelevant antibody IgG1-b12 did not. The combination of HER2-specific mAbs and the bsAb, however, showed a significant increase in down-modulation relative to the individual mAbs. Statistical analysis was determined by Student *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

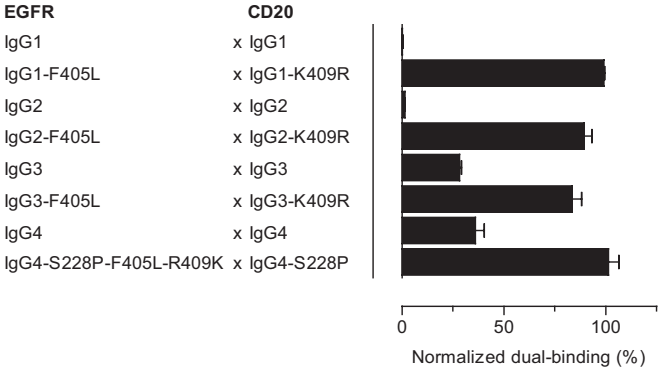


Fig. S6. bsIgG2, bsIgG3, and bsIgG4-S228P can be generated through 2-MEA–induced reduction using the same matched CH3 mutations. The F405L and K409R substitutions were introduced into IgG2 and IgG3 versions of mAbs 2F8 (anti-EGFR) and 7D8 (anti-CD20), respectively. In addition, hinge-stabilized IgG4 versions (i.e., containing the S228P substitution) were generated. As R409 is present in WT IgG4, there was no need to mutate IgG4-S228P-CD20 any further; however, in the matching IgG4-S228P-EGFR molecule, the R409 was removed by introducing a R409K point mutation together with the F405L substitution. WT IgG2, IgG3, and IgG4 versions of both mAbs were included as controls. The formation of bsAb by cFAE (25 mM 2-MEA, 90 min at 37 °C) was measured by dual-binding ELISA. Representative signals at a (total antibody) concentration of 1 μg/mL are shown. The results show that 25 mM 2-MEA is able to reduce the IgG2 and IgG3 hinges containing 4 and 11 disulfide bridges, respectively. Moreover, cFAE in combination with the matched CH3 mutations also enables the efficient generation of bsIgG2, bsIgG3, and bsIgG4-S228P. The lower efficiencies of bsIgG2 and bsIgG3 compared with bsIgG1 and bsIgG4-S228P, both containing two disulfide bridges, however, suggests further optimization of the 2-MEA concentration and incubation time may be required. Furthermore, correct realignment of the disulfide bridges in the complex IgG2 and IgG3 hinges will need to be confirmed.

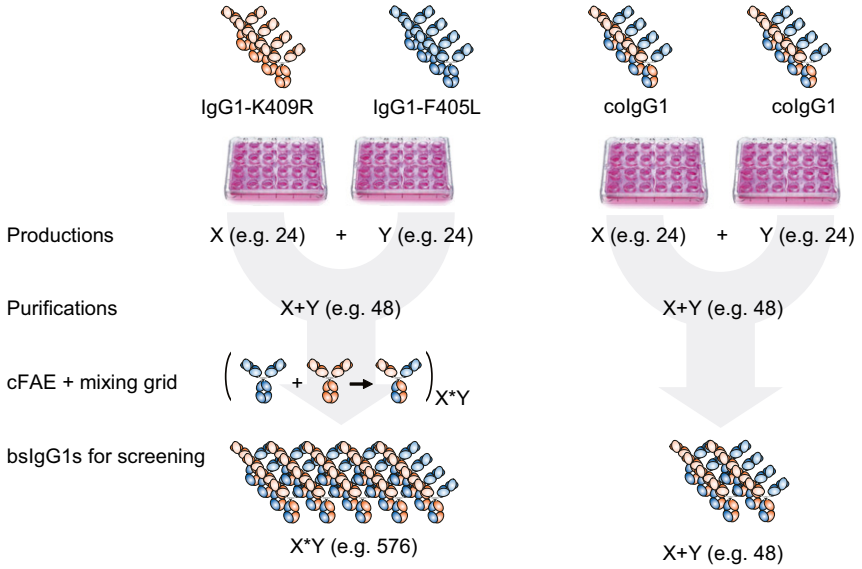


Fig. S7. High-throughput generation and screening of bsAbs. Separate expression of an (*X*) number of IgG1-K409R and (*Y*) number of IgG1-F405L parental antibodies leads to a total of $X + Y$ productions and $X + Y$ purifications (*Left*). By pairwise mixing of the parental antibodies in a matrix-like fashion (at equimolar ratios) and performing cFAE, the number of resulting bsIgG1 products equals $X \times Y$. In a coexpression scenario, the same number ($X + Y$) productions and ($X + Y$) purifications results in smaller number of bsIgG1 products ($X + Y$) (*Right*). Depending on the protein quantity required for the screening strategy, the amount of separately expressed parental antibodies needs to be adjusted, as each of the (*X*) parental antibodies is distributed over mixtures with all (*Y*) parental antibodies.

Table S1. Human mAb panel used for cFAE studies

Clone	Theoretical mass (Da) (ΔDa parental Abs)	Retention time (min) [mean* ± SD (*n*)]
IgG1-F405L-EGFR[†]	146,286.8	18.20 (1)
IgG1-F405L-CD20[‡]	145,902.2	13.51 (1)
IgG1-F405L-HER2$_{153}$[§]	145,293.4	17.52 (1)
IgG1-F405L-N297Q-CD3[¶]	144,547.0	48.17 ± 0.66 (3)
IgG1-F405L-b12[‖]	147,838.2	35.89 (1)
IgG1-K409R-EGFR	146,410.8	18.12 (1)
IgG1-K409R-CD20	146,026.2	13.49 ± 0.08 (4)
IgG1-K409R-HER2$_{169}$[§]	145,778.0	27.03 ± 0.28 (3)
IgG1-K409R-N297Q-HER2$_{169}$	145,804.2	26.73 ± 0.11 (3)
IgG1-K409R-N297Q-b12	147,988.4	35.82 ± 0.31 (4)
bsIgG1-CD20×CD20**	145,964.2 (124.0)	13.57 (1)
bsIgG1-EGFR×CD20	146,456.5 (260.6)	15.83 ± 0.02 (3)
bsIgG1-b12×EGFR	147,124.5 (1427.4)	27.15 (1)
bsIgG1-HER2$_{153}$×HER2$_{169}$	145,535.7 (484.6)	22.59 (1)
bsIgG1-N297Q-CD3×HER2$_{169}$	145,175.6 (1257.2)	37.21 ± 0.13 (2)
bsIgG1-N297Q-CD3×b12	146,267.7 (3441.4)	41.21 ± 0.50 (2)

*Mean RT of the main peak as determined by CIEX.
[†]Human mAb 2F8 (1).
[‡]Human mAb 7D8 (2).
[§]Human mAb 153 (VH:QVQLVESGGGVVQPGRSLRLSCAASGFTFSDYVIHWVRQAPGKGLEWVTVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLSAEDTAMYYCARGGITGTTGVFDYWGQGTLVTVSS;VL:DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYDASSLQSGVPSRFSGSGYGTDFSLTISSLQPEDFAIYYCQQYKSYPITFGQGTRLEIK) and 169 (VH:QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGISWVRQAPGQGLEWMGWLSAYSGNTIYAQKLQGRVTMTTDTSTTTAYMELRSLRSDDTAVYYCARDRIVVRPDYFDYWGQGTLVTVSS;VL:EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK) are directed against nonoverlapping epitopes.
[¶]Humanized mAb CLB-T3/4 (3) (see Table S2).
[‖]Human mAb b12 (gp120 specific) (4).
**Because mass and charge differences were insufficient to distinguish between parental mAbs and the bsAb product, bsIgG1-EGFR×CD20 was prepared simultaneously to act as surrogate for exchange efficiency.

1. Bleeker WK, et al. (2004) Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. *J Immunol* 173(7):4699–4707.
2. Teeling JL, et al. (2004) Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* 104(6):1793–1800.
3. Parren PW, Geerts ME, Boeije LC, Aarden LA (1991) Induction of T-cell proliferation by recombinant mouse and chimeric mouse/human anti-CD3 monoclonal antibodies. *Res Immunol* 142(9):749–763.
4. Roben P, et al. (1994) Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. *J Virol* 68(8):4821–4828.

Table S2. Humanization of human CD3-specific mouse antibody CLB-T3/4

	Anti-human-CD3ε VH sequences
KABAT CDRs	111 11 2 22222222 22222222 3333333333
IMGT CDRs	11111111 22222222 333333333333
CLB-T3/4 (murine)	EVQLVESGGDLVKPGGSLKLSCAAS GFTFSSYG MFWVRQTPDKRLEWVAT ISRYSRYI YYPDSVKGRFTISRDNVKNTLYLQMSSLKSEDTAIYYC ARRPLYGSSPDY WGQGTTLTVSS
IGHV3-21*01	---------G--------R------ -------S -N----A-G-G----SS --SS-S-- --A-------------A--S-----N--RA----V--- --
huCLB-T3/4	EVQLVESGGGLVKPGGSLRLSCAAS GFTFSSYG MFWVRQAPGKGLEWVAT ISRYSRYI YYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYC ARRPLYGSSPDY WGQGTLVTVSS
	Anti-human CD3ε VL sequences
KABAT CDRs	111 11111 11 222 2222 333333333
IMGT CDRs	11111 222 333333333
CLB-T3/4 (murine)	ENVLTQSPAIMSASPGEKVTMTCSAS SSVTY VHWYQQKSNTSPKLWIY DTS KLASGVPGRVSGSGSGNSYSLTISSMEAEDVATYYC FQGSGYPLT FGSGTKLEMR
IGKV3-11*01+IGKJ4*02	-I-------TL-L----RA-LS-R-- Q--SSY LA-----PGQA-R-L-- -A- NR-T-I-A-F------TDFT-----L-P--F-V--- Q-R-NW--- ----------
huCLB-T3/4 LC	EIVLTQSPATLSLSPGERATLSCSAS SSVTY VHWYQQKPGQAPRLLIY DTS KLASGIPARFSGSGSGTDFTLTISSLEPEDFAVYYC FQGSGYPLT FGSGTKLEMR

The mouse VH and VL sequences of CLB-T3/4 (1) were aligned to human germ-line sequences (indicated in red) using the IMGT/V-QUEST database (2), and the closest related human germ-line sequences were identified, that is IGHV3-21*01 for the VH gene and IGKV3-11*01(+IGKJ4*02) for the VL gene (– indicate identical amino acid residues). Humanized sequences of both VH and VL were obtained by replacing amino acid sequence differences in the mouse framework regions (FR) by human germ-line equivalent (indicated in red) without compromising the complementarity determining regions (CDRs; for this purpose, CDRs by both Kabat (3) and IMGT (4) definitions were combined). As no related J-region was found for the VH sequence, the common WGQGTLVTVSS sequence was used for the FR4 region of the heavy chain.

1. Parren PW, Geerts ME, Boeije LC, Aarden LA (1991) Induction of T-cell proliferation by recombinant mouse and chimeric mouse/human anti-CD3 monoclonal antibodies. *Res Immunol* 142(9):749–763.
2. Brochet X, Lefranc MP, Giudicelli V (2008) IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res* 36(Web Server issue):W503–W508.
3. Kabat EA (1991) *Sequences of Proteins of Immunological Interest.* 5th Ed (National Institutes of Health, Bethesda, MD).
4. Lefranc MP, et al. (2003) IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. *Dev Comp Immunol* 27(1):55–77.