Antitumor Efficacy of a Bispecific Antibody That Targets HER2 and Activates T Cells

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Abstract
Clinical results from the latest strategies for T-cell activation in cancer have fired interest in combination immunotherapies that can fully engage T-cell immunity. In this study, we describe a trastuzumab-based bispecific antibody, HER2-TDB, which targets HER2 and conditionally activates T cells. HER2-TDB specifically killed HER2-expressing cancer cells at low picomolar concentrations. Because of its unique mechanism of action, which is independent of HER2 signaling or chemotherapeutic sensitivity, HER2-TDB eliminated cells refractory to currently approved HER2 therapies. HER2-TDB exhibited potent antitumor activity in four preclinical model systems, including MMTV-huHER2 and huCD3 transgenic mice. PD-L1 expression in tumors limited HER2-TDB activity, but this resistance could be reversed by anti–PD-L1 treatment. Thus, combining HER2-TDB with anti–PD-L1 yielded a combination immunotherapy that enhanced tumor growth inhibition, increasing the rates and durability of therapeutic response. Cancer Res; 74(19): 1–11. ©2014 AACR.

Introduction
The recent approval of ipilimumab (1) and exciting responses observed during clinical trials of PD-1 and PD-L1 antibodies (2) clearly illustrate the potential of T cell–targeting cancer immunotherapies. The success of strategies that reinvigorate T-cell activity depends on modulation of multiple stimulatory and inhibitory events that enable an antitumor immune response (2). An attractive alternative of leveraging T-cell activity to eliminate cancer is to not rely on existing tumor immune response, but rather to induce T cells to kill tumor cells directly by generating new tumor specificities. Bispecific antibodies can be used to broadly harness the antitumor capacity of T-cell immunity (3). However, successful clinical use of modified and reengineered antibodies and antibody fragments is far from trivial (4). To progress a drug candidate from laboratory to clinic, a vast array of requirements needs to be met in regard to “drug-like” properties. Immunogenicity and short serum half-life are additional problems for bispecific molecules with modified antibody sequences and antibody fragment–based platforms.

Although several tumor targets and bispecific antibody platforms have demonstrated general flexibility and preclinical feasibility for this approach, very few molecules have been implemented in clinical use. In 2009, the European Medicines Agency (EMA) approved the use of a trifunctional EpCam × CD3 bispecific antibody, catumaxomab (Removab), for the intraperitoneal treatment of malignant ascites (5). Promising clinical activity has also been demonstrated with a CD19 × CD3–targeting bispecific scFv antibody fragment, blinatumomab (3). Both catumaxomab and blinatumomab illustrate how technological challenges affect clinical use of bispecific antibodies. Catumaxomab is a mouse IgG2a/rat IgG2b hybrid, and thus it is highly immunogenic in human. Dosing of catumaxomab is limited to maximum of four doses and 20 days. Because of the extremely short serum half-life (1.25 hours) of blinatumomab (3), it is continuously infused via pump throughout the treatment cycle.

Here, we report the properties of a T cell–dependent bispecific antibody targeting HER2 (HER2-TDB), which can induce a polyclonal T-cell response to tumors. The response does not require a preexisting tumor immune response and it has reduced potential for immune escape (e.g., loss of MHC expression). The modality combines extreme potency of the T-cell activity with favorable drug-like properties of IgG1 molecule, including long half-life.

Despite recent advances in treatment of HER2+ breast cancer (6–9), several resistance mechanisms have been identified that engage redundant survival signaling pathways (10–12). De novo or acquired resistance is an expected outcome also for a subset of T-DM1 (ado-trastuzumab emtansine, Kadcyla)–treated patients. Therefore, a significant unmet medical need remains for HER2+ breast cancer. HER2-TDB kills all tested HER2+ tumor cells with low picomolar EC50, and due to its different mechanism of action, effectively kills cells that are refractory to trastuzumab, lapatinib, and T-DM1.
An important outstanding question with T cell–engaging bispecific antibodies is whether, they too may be susceptible to T cell–suppressive resistance mechanisms following the initial T-cell response? Our results demonstrate that PD-1/PD-L1 can inhibit T-cell killing activity induced by bispecific antibodies. Our results using immunocompetent huCD3-transgenic animal model further suggest that combining T cell–recruiting antibodies with anti–PD-L1 antibodies improves outcome of the treatment.

Materials and Methods

**Antibody expression and purification**

The "knob" arm of HER2 huIgG1 TDB is humanized anti-HER2 4D5 (trastuzumab; ref. 13) and "hole" arm is humanized anti-CD3 UCHT1.v9 (14, 15). The huIgG1 bispecific antibodies were produced by two different approaches as described earlier (16); coculture of bacteria expressing each of the two antibody arms, or by expressing each arm separately and then annealing them in vitro.

To avoid immune response toward the TDB, a murine IgG2a (knob-hole, D265A, and N297G) isotype HER2-TDB expressed in Chinese hamster ovary (CHO) cells was used in experiments with immunocompetent mice. In murIgG2a HER2-TDBs, the "knob" arm is murine anti-HER2 4D5 and the "hole" is either chimeric anti-murine CD3 2C11 (4D5/2C11-TDB; ref. 17) or mouse anti-hu CD3 SP34 (4D5/SP34-TDB; ref. 18). Bispecific antibody purification is described elsewhere (16).

**Antibody characterization**

The molecular weight of the bispecific antibody was analyzed by mass spectrometry [liquid chromatography-electrospray ionization/time of flight (LC-ESI/TOF)] as described before (19). The antibodies were also analyzed by analytic size exclusion chromatography in a Zenix SEC-300 column (Sepax Technologies) using an Agilent 1100 HPLC system (Agilent Technologies). The presence of residual antibody fragments was quantified by electrophoresis using a 2100 Bioanalyzer and a Protein 230 Chip (Agilent Technologies).

**HER2-TDB affinity**

The competitive Scatchard assay is described in detail elsewhere (20).

**Breast cancer cell proliferation**

Breast cancer cell proliferation/viability was detected using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). For the assay, 5 × 10⁵ cells per well were plated in 96-well plates and incubated overnight for cell attachment before treatments.

**Blood cell fractionation**

Peripheral blood mononuclear cells (PBMC) were separated from the blood of healthy volunteers using lymphocyte separation medium (MP Biomedicals). CD8⁺ cells were extracted from PBMC using human CD8⁺ Isolation Kit from Miltenyi Biotec (#130-094-156) by negative selection. CD3⁺ depletion was done using CD3⁺ MicroBeads from Miltenyi Biotec (#130-050-101).

**In vitro cytotoxicity assays (in vitro ADCC, T-cell killing)**

*In vitro* cytotoxicity assays [Cytotoxicity Detection Kit; lactate dehydrogenase (LDH); Roche] were performed as previously described (21). Alternatively, *in vitro* cytotoxicity was monitored by flow cytometry. Target cells were labeled with CFSE (Invitrogen; #C34554). The labeled target cells and CD8⁺ cells were mixed with or without TDB for 4 to 26 hours. At the end of the incubation, the cells were lifted by trypsin and collected from the plate. The cells were resuspended in equal volume of PBS + 2% FBS + 1 mmol/L EDTA + propidium iodine (PI). Flow cytometry analysis was done on a FACSCalibur in automation format. The number of live target cells was counted by gating on CFSE⁺/PI⁻ cells. The percentage of cytotoxicity was calculated as follows: %cytotoxicity = (live target cell number without TDB – live target cell number with TDB)/(live target cell number without TDB) × 100. iCell cardiomyocytes (Cellular Dynamics International) were revived from liquid nitrogen and plated at 27,000 cells per 96-well 7 days before the assay and treated as per the manufacturer's instructions. CD8 cells were added in 3:1 ratio on iCells for 24 hours together with the treatment. After 24 hours, cells were gently washed twice with PBS to remove T cells and viability was measured using the CellTiter-Glo Assay.

**Analysis of T-cell activation**

Cells were stained with CD8-FITC (BD Biosciences; 555634), CD69-PE (BD Biosciences; 555531), and CD107a-Alexa Fluor 647 (eBioscience; 51-1079). Alternatively, cells were fixed and permeabilized with Cytotix/CytoPerm solution (BD Biosciences; 54722) and stained with anti-granzyme B-Alexa Fluor 647 (BD Biosciences; 560212).

**Detection of soluble granzymes and perforin**

Soluble perforin (Cell Sciences), granzyme A, and granzyme B (eBioscience) were detected from growth media by ELISA according to the manufacturer's protocols.

**PD-1 induction and effect of PD-L1 expression on TDB activity**

Purified CD8⁺ T cells from human peripheral blood were primed with 100 µg/mL of HER2-TDB and SKBR3 cell at 3:1 ratio for 24 hours. After 24 hours of incubation, the cell pellet was digested with Non-Enzyme Cell Dissociation Solution (Sigma; #C5789) at 37°C for 10 minutes and CD8⁺ T cells were recovered using Human CD8⁺ MicroBeads (Miltenyi Biotec; #130-045-201). The primed-CD8⁺ T cells were used for *in vitro* cytotoxicity assay. In flat-bottomed 96-well plate, CFSE-labeled 293 cells or 293-PD-L1 cells were mixed with primed effector cells in 3:1 ratio in the presence or absence of HER2-TDB and 10 µg/mL anti–PD-L1 antibody (clone 6E11, mlgG2A, D265A, and N297A; Genentech). After 24 hours, cytotoxicity was measured by counting live CFSE⁻ target cells by flow cytometry.

**Pharmacokinetic study in rats**

Sprague-Dawley rats (n = 4/group) were dosed intravenous (i.v.) bolus either HER2-TDB or trastuzumab at 10 mg/kg. Serum samples were assayed for human IgG by ELISA and pharmacokinetic (PK) parameters were determined with a...
two-compartment method (Model 7) using WinNonlin, version 5.2.1 (Pharsight Corp.).

In vivo efficacy

Dosing and monitoring were performed in accordance with guidelines from the Institutional Animal Care and Use Committee at Genentech, Inc. NOD/SCID mice (NOD.CB17-Prkdcscid); The Jackson Laboratory West) were implanted with 0.36 mg, 60-day sustained release estrogen pellets (Innovative Research of America) 1 to 3 days before cell inoculation, subcutaneously over the opposite flank of tumor inoculation. On day 0, 5 million MCF7 neo/HER2 and 10 million nonactivated human PBMCs (huPBMC) in HBSS-Matrigel were inoculated in the right second/third mammary fat pad. The first treatments were administered 2 hours after inoculation. All treatments were administered 1×/week by i.v. tail vein injection for a total of three doses. MMTV-huHER2 transgenic animals maintained on a FVB/N background have been previously described (22). For experiments with syngeneic tumors, 0.1 million CT26-HER2 cells were injected subcutaneously to Balb/c or human CD3e transgenic mice (23). To avoid immune response toward the TDB, a murine IgG2A version of the HER2-TDB was used in experiments with immunocompetent mice. Anti–PD-L1 antibody clone 25A1 (mlgG2A, D265A, and N297A; Genentech) was used for therapeutic blockade of PD-L1.

Results

Generation and purification of full-length HER2-CD3 bispecific antibody (HER2-TDB) using knobs-into-holes technology

HER2-TDB was generated using a knobs-into-holes strategy (Fig. 1A; ref. 14). The anti-CD3 arm (UCHT1 ×9; hole) and the anti-HER2 arm (4D5; trastuzumab; knob) were expressed in separate Escherichia coli cultures and later combined, or alternatively, cocultured from the start (Fig. 1B). The fully assembled antibody was isolated on Protein A and then purified by hydrophobic interaction chromatography. Size exclusion chromatography showed a very low level of aggregation (Fig. 1C, 95.2%; Fig. 1D, 95.9%) and mass spectrometry analysis showed a main mass deconvolution peak corresponding to the heterodimer with an absence of significant amounts of either homodimer (Fig. 1D). These results demonstrate that high-quality HER2-TDB can be efficiently produced using standard expression and purification methods.

T cell–independent properties of HER2-TDB

Target arm binding affinity of HER2-TDB by Scatchard analysis ($K_D = 5.4$ nmol/L; Fig. 1E) was similar to monovalent trastuzumab Fab ($K_D = 3.9$ nmol/L) and lower than the affinity of bivalent trastuzumab to HER2 ($K_D = 0.7$ nmol/L). The $K_D$ for CD3 arm binding affinity to Jurkat cells was 4.7 nmol/L (not shown). The ability of HER2-TDB to directly inhibit SKBR3 proliferation was reduced as compared with bivalent trastuzumab (Fig. 1F). Antibodies produced in E. coli are not glycosylated, which results in impaired FcyRII binding, which is required to mediate antibody-dependent cell-mediated cytotoxicity (ADCC; refs. 24, 25). E. coli produced trastuzumab and HER2-TDB were unable to induce natural killer cell–mediated ADCC (Fig. 1G).

Target-dependent T-cell activation and cytotoxicity

T-cell activation was not detected when CD8+ cells were incubated with HER2-TDB or target cells that do not express human HER2 (BJAB cells; Fig. 2A). A robust T-cell activation was seen when HER2+ SKBR3 cells were used as targets accompanied by release of cytotoxic granules. Soluble perforin, granzyme A, and granzyme B were detected in the growth media by ELISA (Fig. 2B), but only when all the key components (HER2-TDB, T cells, and HER2-expressing cells) were included in the reaction. Granule exocytosis coincided with significant HER2-TDB–induced elevation of caspase-3/7 activity, apoptosis, and cytotoxicity (Fig. 2C).

HER2-TDB does not mediate killing of vector-transfected 3T3-cells (Fig. 2D), in contrast, the HER2-transfected 3T3-cells were very efficiently killed. Addition of HER2-ECD or trastuzumab Fab to the killing assay efficiently inhibited the killing activity (Fig. 2E). To confirm T-cell dependence of killing, we depleted CD8+ cells from the PBMC (Fig. 2F). The depletion resulted in loss of target cell killing activity.

Early signs of T-cell activation (CD69) were detectable 4 hours after HER2-TDB treatment was initiated (Supplementary Fig. S1A). However, late activation markers (extracellular CD107a) were not significantly expressed until the 24-hour time point. Activation of T cells was reflected in killing of HER2+ breast cancer cells (Supplementary Fig. S1A).

These results show that activation of T cells by HER2-TDB is conditional and target dependent. Binding of HER2-TDB to T cells in the absence of target cells is not sufficient to activate T cells.

HER2-TDB induces T-cell proliferation

Cytotoxicity was significantly reduced by effector cell titration (Supplementary Fig. S1B). However, even with an E:T ratio of ≤1:1, a weak LDH signal and robust activation of T cells was detected. We next investigated whether HER2-TDB induces T-cell proliferation by coculturing CD8+ T cells, target cells (SKBR3), and 0.1 μg/mL HER2-TDB followed by T-cell culture in the absence of target cells and HER2-TDB. After 3 days, 75% of the T cells pulsed with TDB and target cells had undergone a cell division (Supplementary Fig. S2); however, the cell number did not increase. Supplementing the growth media with IL2 (20 ng/mL) provided a survival signal to CD8+ cells, and a robust accumulation of T cells was detected, but only if they were exposed to both HER2-TDB and target cells (Supplementary Fig. S2).

HER2-TDB activity correlates with the target cell HER2 expression level

To investigate the relationship between target copy number and TDB activity, we selected a panel of cancer cell lines with predetermined number of HER2 receptors on the cell membrane (Fig. 3A and G; ref. 26). HER2-amplified/overexpressing cell lines were significantly more sensitive to the TDB-mediated killing ($P = 0.007$, t test) and were efficiently lysed at femtomolar to low picomolar concentrations (EC50 = 0.8–3 pmol/L; Fig. 3B). Cell lines expressing low levels of HER2 were significantly less sensitive to HER2-TDB antibody (EC50 = 33–51 pmol/L). Fewer than 1,000 copies of target antigen were
sufficient to support T-cell killing. We have previously reported EC50 4,500 pmol/L as the sensitivity of SKBR-3 to trastuzumab in a 5-day viability assay.

Several normal human tissues express low levels of HER2. Expression level of HER2 in iCell cardiomyocytes (27, 28) is comparable with MCF-7 cells and low level of in vitro activity was detected for HER2-TDB (Fig. 3C and D).

Next, we cotargeted MCF7 (low HER2 expression), or BJAB cells (no HER2 expression) with HER2-amplified SKBR3 cells in the same killing assay. No killing of MCF7 cells was detectable at the EC50 for SKBR3 killing (Fig. 3E). No significant killing of BJAB cells was detectable at any HER2-TDB concentration (Fig. 3F). These results suggest that an overexpression-based therapeutic index may exist for HER2-TDB and demonstrate a lack of bystander killing.

**Figure 1.** Generation and T cell–independent properties of HER2-TDB. A, amino acid substitutions are generated to Cα3 domains of the “knob” (α-HER2 4D5) and “hole” (α-CD3 UCHT1.v9) heavy chains, which selectively promote heterodimerization to generate bispecific full-length IgG1. B, overview of the TDB purification. ProA, Protein A affinity purification; HIC, hydrophobic interaction chromatography; QC, quality control; SEC, size exclusion chromatography. C, size exclusion chromatography demonstrates low levels of aggregate or single arms. D, MS analysis indicates undetectable levels of homodimeric species. E, binding to SKBR-3 was determined by competition binding of 125I-trastuzumab Fab with nonlabeled trastuzumab (black), trastuzumab-Fab (blue), or bispecific HER2-TDB (red). F, direct effect on proliferation of SKBR-3 cells was analyzed after 6 days of treatment with antibodies using the CellTiter-Glo Luminescent Cell Viability Assay. G, the ability of trastuzumab, trastuzumab produced in E. coli, and HER2-TDB to mediate in vitro ADCC by natural killer cells was measured using assay detecting LDH released from lysed cells. Time point, 4 hours. Data points in the figure represent the mean of three samples; error bar, SD.
occupancy was even lower (0.01%–0.05%). The calculated absolute number of TDB bound to HER2 at the EC₅₀ was as low as 10 to 150 molecules in the low expressing cell lines. These results showcase the extreme potency of HER2-TDB and are consistent with studies of T-cell receptor triggering, which suggest that as few as 1 to 25 T-cell receptors need to be engaged to trigger T-cell responses (29–31).

HER2-TDB is efficient in killing of HER2⁺ cancer cells refractory to anti-HER2 therapies

We next selected cell lines previously shown to express high levels of HER2 and be insensitive to the direct cellular effects of trastuzumab and lapatinib in vitro (10, 32). Sensitivity to T-DM1 has been previously reported (32, 33). EC₅₀ for HER2-TDB–mediated killing was in the femtomolar or low picomolar range (Fig. 4A). In addition, HER2-TDB is effective in killing HER2⁺ lung cancer cells, demonstrating that the effect of the molecule is not limited to breast cancer cells. Using two independent cell line models (BT474; Fig. 4B and C; KPL-4, not shown), we demonstrate that acquired resistance to T-DM1 did not affect the sensitivity to HER2-TDB. These results demonstrate that HER2-TDB displays broad subpicomolar killing activity against HER2⁺ cells regardless of the tissue of origin, PI3K pathway activation status or sensitivity to trastuzumab, lapatinib, and T-DM1.

Pharmacokinetics of HER2-TDB in rat

Sprague-Dawley rats were administered a single i.v. dose of 10 mg/kg of either HER2-TDB or trastuzumab. HER2-TDB does not cross-react with rat CD3 or rat HER2 and displayed a biphasic disposition typical of an IgG1 with a short distribution phase and slow elimination phase (Fig. 5). The clearance and half-life of HER2-TDB were within expected ranges for a typical nonbinding human IgG1 antibody.
HER2-TDB inhibits tumor growth in vivo in immunocompromised mice

In vivo efficacy of HER2-TDB was tested in NOD/SCID mice. MCF7-neo/HER2 cells were grafted together with nonactivated huPBMCs from healthy donors to mammary fat pads of mice. Mice were dosed intravenously on a weekly schedule with 0.5 mg/kg of HER2-TDB or control-TDB, starting on the day of tumor cell inoculation. HER2-TDB prevented growth of HER2-expressing tumors (Fig. 6A). As expected, no efficacy was detected in mice when huPBMC were omitted (Supplementary Fig. S3A). A control TDB that shares the same CD3 arm as HER2-TDB, but has an irrelevant target arm had no effect on the tumor growth (Supplementary Fig. S3B).

HER2-TDB causes regression of mammary tumors in huHER2 transgenic mice

We next generated a surrogate TDB using a mouse CD3-reactive antibody clone 2C11 (17) to treat MMTV-huHER2 transgenic mice (22). The in vitro activity of 4D5/2C11-TDB was similar to human CD3-reactive HER2-TDB (Supplementary Fig. S4). With exception of one mouse, 4D5/2C11-TDB resulted in tumor regression in all treated mice (Fig. 6B and C). More than 80% tumor regression was detected in 43% of the mice. Remarkably, responses were noted in mice with tumors that were more than 1,000 mm³ at the start of the treatment (Fig. 6D). Tumor growth was not affected by control TDBs (Fig. 6E) or by treatment with 30 mg/kg bivalent murine 4D5 (precursor of humanized 4D5; trastuzumab; Supplementary Fig. S5).

HER2-TDB has transient antitumor activity in treatment of syngeneic huHER2-expressing tumors

T cells from CD3t transgenic mice (CD3-Tg; ref. 23) express both mouse and human CD3 on approximately 50% level of respective Balb/c mouse or human T cells (Supplementary Fig. S6). CD3-Tg T cells can kill human HER2-expressing target cells in vitro (Supplementary Fig. S4), although killing activity of mouse splenic T cells is consistently lower compared with human peripheral T cells. Human HER2-transfected CT26 tumor cells were grown in the CD3-Tg mice subcutaneously and established tumors were treated with weekly 0.5 mg/kg i.v. doses of HER2-TDB. HER2-TDB clearly inhibited the growth of established tumors, but the effect was transient (Fig. 6F). The activity of HER2-TDB is dependent on T cells, because HER2-TDB had no effect in non-CD3 transgenic mice (Supplementary Fig. S6). The in vivo responses detected in Balb/c mice using 4D5/2C11-TDB were similar to the responses seen in CD3-Tg mice with human-specific CD3 arm–based TDB (Fig. 6F and G). HER2-TDB significantly prolonged the time to tumor progression (log-rank, \( P < 0.0001 \)).
PD-L1 expression in target cells inhibits HER2-TDB activity

We analyzed the cellular composition of the CT26-HER2 tumors to search for an explanation for incomplete tumor response. Of note, 10% to 30% of CD45\(^+\) cells in CT26-HER2 tumors were CD8\(^+\) T cells (Supplementary Figs. S7 and S8). Almost all T cells displayed markers of activation and were positive for PD-1 (80%–95% CD69\(^+\), 95% PD-1\(^+\)). All CD45\(^-\) cells were positive for PD-L1, suggesting that PD-L1 expression by the tumor cells may potentially inhibit the activity of the HER2-TDB. We functionally tested this using human T cells. A clear upregulation of PD-1 in T cells was detected upon overnight coculture with SKBR3 cells and HER2-TDB (Fig. 7A). Induced T cells expressing PD-1 were then transferred on PD-L1 or vector-transfected 293 cells. Because 293 cells express low levels of HER2, the primed T cells efficiently killed the 293 cells, but only when the HER2-TDB was added (Fig. 7B). Expression of PD-L1 in 293 cells significantly inhibited the killing activity and this inhibition was completely reversed by PD-L1-blocking antibody. Together these results demonstrate that PD-L1 expression by the target cells can inhibit HER2-TDB activity and provide a rationale for therapeutic HER2-TDB and anti–PD-L1 combination.
HER2-TDB anti–PD-L1 combination is effective in treatment of established CT26-HER2 tumors

In the next experiment using CD3-TG mice, a similar transient but significant response was seen with the HER2-TDB. In contrast to previous study, we also detected two complete responses (Fig. 7C). Tumor growth was significantly slower in both of the single agent cohorts compared with the control mice, and combination of HER2-TDB with PD-L1 blockade.
further improved the response (Fig. 7C). Combination treatment resulted in durable responses; 60% of the mice lived tumor free until the study was terminated at 80 days after the first dose (not shown). In a repeat study (Fig. 7D), all mice responded to the combination, 82% with complete responses, and tumor growth was controlled by the treatment in all but one mouse in the combination cohort. In summary, combination of HER2-TDB with anti–PD-L1 immune therapy resulted in enhanced inhibition of tumor growth, increased response rates, and durable responses.

Discussion

TDB expression and purification yields were approximately 5 mg/L. These production levels are comparable with previously reported results (16) and similar to the parental antibodies. By optimizing strain and growth conditions, yields sufficient for clinical studies can be obtained. The TDB format retains binding to FcRn receptors (25), which provides a long half-life for the molecule and makes weekly or less frequent dosing schedule attainable. The minor alterations to the antibody that drive the heterodimerization in the knobs-into-holes technology are buried in the CH3–CH3 interaction interface and thus are unlikely to be immunogenic. In support of this view, only minimal and transient antitherapeutic antibody (ATA) responses were detected in onartuzumab-treated patients, without apparent effect on PK profiles (34). This is a striking contrast to the experience with ertuxamomab. Ertu-

maxomab is a HER2-CD3 bispecific mouse IgG2a/rat IgG2b antibody that has been tested in a phase I study demonstrating promising responses. Roughly one third of patients developed antibodies against ertuxamomab (HAMA/HARA) after three doses (35).

The potency of HER2-TDB is consistently in the low picomolar to femtomolar range. Furthermore, as few as 10 to 500 HER2-bound TDBs were sufficient to induce significant in vitro cytotoxicity, which is in agreement with what has been described for the receptor occupancy required to trigger T cell–mediated responses (29–31). With this in mind, it is not surprising that the activity of HER2-TDB was not limited to HER2-overexpressing cells. As a result, on-target T-cell activity on low expressing cells introduces an apparent safety concern for HER2-TDB, because HER2 is expressed in low levels in normal tissues, including mammary gland, kidney, and heart (36). However, our studies demonstrate a clear correlation between target expression levels and in vitro sensitivity to HER2-TDB. This suggests that tumor overexpression of HER2 may provide a therapeutic index for HER2-TDB. Therefore, it may be possible to selectively target the HER2-amplified tumor cells, without affecting normal cells where the expression level is 10- to 100-fold lower. It is noteworthy that the experiments in this article do not specifically address safety of the HER2-TDB. Before dosing human patients, the safety will be addressed using extensive scrutiny and appropriate preclinical models.
Recruitment of T-cell killing activity with HER2-TDB is dependent on HER2 expression, but independent of the HER2 signaling pathway, which predicts that HER2-TDB may be efficient in treatment of tumors that are refractory to current anti-HER2 therapies. Our results suggest that switching to alternative mechanism of action by using HER2-TDB may broadly enable overcoming resistance to antibody–drug conjugates (e.g., T-DM1), targeted small-molecule inhibitors (e.g., lapatinib), and therapeutic monoclonal antibodies that block the pathway signaling (e.g., trastuzumab). We also discovered a potential general resistance mechanism for T cell–recruiting molecules with important diagnostic impact. The finding that PD-L1 expressed by the tumor cells can inhibit the activity of HER2-TDB also provides a mechanistic rationale for combination with PD-L1 blockade.

Taken together, this study presents a new immune therapy for HER2+ breast cancer with an alternative, extremely potent mechanism of action that is broadly effective in cells resistant to current HER2-targeted therapies. Several significant advances are provided to bispecific T cell–recruiting antibodies. We (i) characterize a critical resistance mechanism, (ii) discover a potential diagnostic, (iii) introduce a novel hugCD3 transgenic efficacy model, and (iv) significantly improve the drug-like properties by using technology based on full-length antibodies with a natural architecture. Finally, we demonstrate the benefit of combining two immune therapies: direct polyclonal recruitment of T-cell activity together with inhibiting the T cell–suppressing PD-1/PD-L1 signaling results in enhanced and durable long-term responses.

Disclosure of Potential Conflicts of Interest
All authors have ownership interest in Roche.

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