Immunoselection of Breast and Ovarian Cancer Cells with Trastuzumab and Natural Killer Cells: Selective Escape of CD44$^{\text{high}}$/CD24$^{\text{low}}$/\text{HER2}^{\text{low}}$ Breast Cancer Stem Cells

Florian Reim, Yvonne Dombrowski, Cathrin Ritter, Mathias Buttmann, Sebastian Häusler, Monika Ossadnik, Mathias Krokenberger, Dagmar Beier, Christoph P. Beier, Johannes Dietl, Jürgen C. Becker, Arnd Höning, and Jörg Wischhusen

Abstract

Although trastuzumab (Herceptin) has substantially improved the overall survival of patients with mammary carcinomas, even initially well-responding tumors often become resistant. Because natural killer (NK) cell–mediated antibody-dependent cell-mediated cytotoxicity (ADCC) is thought to contribute to the therapeutic effects of trastuzumab, we have established a cell culture system to select for ADCC-resistant SK-OV-3 ovarian cancer and MCF7 mammary carcinoma cells. Ovarian cancer cells down-regulated HER2 expression, resulting in a more resistant phenotype. MCF7 breast cancer cells, however, failed to develop resistance in vitro. Instead, treatment with trastuzumab and polyclonal NK cells resulted in the preferential survival of individual sphere-forming cells that displayed a CD44$^{\text{high}}$/CD24$^{\text{low}}$ “cancer stem cell–like” phenotype and expressed significantly less HER2 compared with non–stem cells. Likewise, the CD44$^{\text{high}}$/CD24$^{\text{low}}$ population was also found to be more immunoresistant in SK-BR3, MDA-MB231, and BT474 breast cancer cell lines. When immunoselected MCF7 cells were then re-expanded, they mostly lost the observed phenotype to regenerate a tumor cell culture that displayed the initial HER2 surface expression and ADCC-susceptibility, but was enriched in CD44$^{\text{high}}$/CD24$^{\text{low}}$ cancer stem cells. This translated into increased clonogenicity in vitro and tumorigenicity in vivo. Thus, we provide evidence that the induction of ADCC by trastuzumab and NK cells may spare the actual tumor-initiating cells, which could explain clinical relapse and progress. Moreover, our observation that the “relapsed” in vitro cultures show practically identical HER2 surface expression and susceptibility toward ADCC suggests that the administration of trastuzumab beyond relapse might be considered, especially when combined with an immune-stimulatory treatment that targets the escape variants.

Introduction

The Her-2/neu (c-erbB2, HER2) proto-oncogene belongs to a family of four transmembrane receptor tyrosine kinases that mediate cell growth, differentiation, and survival (1). Overexpression of the HER2 protein, amplification of the her-2/neu gene, or both occurs in 20% to 25% of breast cancers (2, 3), 7 to 13% of newly diagnosed ovarian cancers (3, 4), and in the majority of ovarian cancer cells removed at a second surgery or isolated from ascites of stage III or IV ovarian cancer patients (5). Trastuzumab (Herceptin, Genentech), a humanized monoclonal antibody (rhumAb 4D5) directed against the extracellular domain of HER2, improves disease-free and overall survival in patients with early, metastasized, or recurrent HER2-positive breast cancer significantly (6, 7). Clinically, its most important adverse effect is cardiotoxicity, which is reported in 2.6% to 4.5% of patients (8). The far greater problem is that a significant number of patients with HER2-overexpressing tumors do not respond to trastuzumab (6) or eventually develop resistance after a good initial response (9).

For ovarian cancer treatment, however, trastuzumab does not play a significant role because HER2 overexpression is rare and the objective response rates (7.3%) are low among HER2-overexpressing ovarian cancer patients (10). Data regarding the combination of trastuzumab with chemotherapy are scarce for these patients.

Being an antibody, trastuzumab does not only block HER2 signaling—which could equally be achieved by small-molecule inhibitors (11). Instead, trastuzumab also recruits cytotoxic effector cells via the Fcy-part of this IgG1 antibody (12, 13) and thus induces the so-called “antibody-dependent cell-mediated cytotoxicity” (ADCC), which can be effected by granulocytes, monocytes, macrophages, dendritic cells, and natural killer (NK) cells (14). NK cells that express the activating FcyRIIIA, but no inhibitory Fcy receptors, are widely believed to be the major mediators of ADCC. Accordingly, surgical breast specimens from trastuzumab-treated patients revealed increased numbers of tumor-associated NK cells that expressed higher levels of Granzyme B and Tia1 (15, 16).

Likewise, ADCC and overall NK cell activity were found to correlate with responses to trastuzumab (17). In experimental animal models, trastuzumab reduced the tumor volume by 96% in NK cell competent nude mice, but by <30% in the corresponding FcyRIIIA knockout animals (18). In vitro experiments further showed that trastuzumab-induced ADCC against various tumor cell targets depends on the Fc-part of the antibody (19), the availability of FcyRIIIA or CD16 on NK cells (20), and the presence of interleukin 2 (II-2; ref. 21)—all arguing for a significant contribution from NK cells. Nevertheless, the important quest for mechanisms mediating trastuzumab resistance has largely concentrated on strategies that enable the cancer cells to overcome the growth-inhibitory signals of HER2-blockade.

Cancer stem cells (CSC) have been described in both ovarian and mammary cancers, and may be responsible for resistance against...
therapeutic modalities. Although HER2 is not expressed in normal mouse mammary stem cells (22), its overexpression was found to drive mammary carcinogenesis (23) by converting normal mammary stem or progenitor cells into CSC, i.e., cells that show the ability to initiate and sustain tumor formation, growth, and resistance to chemotherapy. Within breast cancers, these CSC constitute a small subset characterized by a CD44highCD24low phenotype and by stem cell properties such as unlimited self-renewal, differentiation potential, and tumorsphere-like growth (24). In addition, tumor fractionation and subsequent inoculation of nude mice with limiting cell numbers (~100 cells) has shown that only these cells can initiate tumors in vivo (25). Clinically, a correlation between the expression of HER2 and stem cell markers has also been verified (26). For ovarian cancer, in contrast, both the phenotype of putative tumor-initiating cells as well as a potential link to HER2 expression are still unclear (27).

In line with the effect of HER2 on mammary stem cells, HER2-positive breast cancer displays an aggressive phenotype with a high rate of recurrence and short disease-free intervals after adjuvant (postoperative) chemotherapy (2). In ovarian cancer, in contrast, no significant correlation was found between HER2 expression and the generally poor survival (28).

Considering that the “three Es of cancer immunoediting,” i.e., elimination, equilibrium, and escape (29) are likely to occur during treatment with trastuzumab as well as during other immune therapies, we have tested whether treatment with trastuzumab and polyclonal NK cells would select for immune-resistant subclones of HER2-expressing SK-OV-3 ovarian cancer and MCF7 breast cancer cells. This has led to the identification of two different strategies by which cancer cells can acquire resistance against trastuzumab: SK-OV-3 ovarian cancer cells down-regulate HER2 expression, which results in a more resistant phenotype. MCF7 and other breast cancer cell lines, in contrast, contain a highly tumorigenic CSC fraction that displays a reduced HER2 expression and is consequently less susceptible toward ADCC. Moreover, when this fraction survives an immunoselection process, it can regenerate a culture that strikingly resembles the one observed before treatment, but is even more tumorigenic.

Materials and Methods

Cell culture. SK-OV-3 ovarian cancer and MCF7, MDA-MB-231, RT-474, and SK-BR-3 breast cancer cells were obtained from the American Type Culture Collection and cultured as indicated by the supplier. Stable firefly luciferase-expressing transfectants were generated from the parental cell lines by lipofection with FuGene HD (Roche) and subsequent selection with G418 (Carl Roth). The fLuc-neo/zeo plasmid was generously provided by Dr. Michael Jensen (City of Hope National Medical Center, Duarte, CA). To achieve 106 SK-OV-3 fLuc or MCF7 fLuc cancer cells were incubated with 50 ng/ml trastuzumab and 1.5 × 105 polyclonal human NK cells that had been prepared as described above (30). Then, NK cells were removed by washing, and the surviving cells were re-expanded in G418-containing medium. In total, SK-OV-3 ovarian cancer cells were subjected to eight selection cycles and MCF7 mammary carcinoma cells to six selection cycles of about 1 month each.

qRT-PCR. Total RNA was prepared using TriFast (Peqlab) and transcribed with the iScript cDNA Synthesis kit (Bio-Rad). For real-time PCR, cDNA amplification was monitored using the Absolute QPCR SYBR Green Low Rox mix (ABgene) on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The specific primers used were as follows: 18S up, 5′-CCGGCTACCACTACCAAGGAA-3′; 18S down, 5′-GCGTAAGT-TACCCGGGCT-3′; HER2 up, 5′-TGGAAGCCACAAGGTAAACA-3′; HER2 down, 5′-CTACTCTTCCACCAAGGCTAT-3′. Data analysis was done using the ΔΔCt method for relative quantification. Dissociation curves confirmed the presence of a single specific PCR product.

Microscopy. Cells were immunoselected as described above and then analyzed in vivo by phase contrast light microscopy using an Olympus IX70 inverted microscope system at 48 h or 10 d after the NK cell/trastuzumab challenge.

In vivo tumorigenicity assay. Immunoselected (104 or 105; n = 6 for each cell number) or naïve (n = 5) MCF7 breast cancer cells were suspended in 50 µL PBS, mixed with an equal volume of Matrigel (BD Biosciences), and injected into the mammary fat pad of NOD/scid-mice (Charles River). The animals were observed at least thrice per week and tumor formation was recorded. On day 58, all tumors were removed, fixed in paraformaldehyde, and embedded in paraffin. Ten-micrometer sections were stained with H&E and the maximum tumor area was determined using a caliper. HE2 stains were performed in routine diagnostics. All procedures were conducted in accordance with German laws governing animal care.

Statistics. Experiments were performed at least thrice with similar results and representative experiments are shown. In Figs. I A and B, and 24 and B, analysis of significance was performed using one-way ANOVA followed by Tukey’s posttest for multiple comparisons (*, P < 0.05; **, P < 0.01 for rhumAb 4D5 versus control IgG). Tumor-free survival was compared by logrank test, tumor sizes, and clonogenicity by unpaired Student’s t test. All
tests were performed using Statistica (StatSoft). SDs for flow cytometry data were calculated using Summit software (DakoCytomation).

Results

Trastuzumab and polyclonal NK cells show synergistic killing of naïve SK-OV-3 ovarian cancer targets. To assess the effect of trastuzumab on the NK cell–mediated lysis of HER2-expressing ovarian cancer cells, luciferase-transfected SK-OV-3 cells were treated with polyclonal NK cells in the presence of human control IgG and/or trastuzumab at the indicated concentration. Target cell lysis was determined at 4, 8, and 24 hours via the ensuing decrease in ATP-dependent luciferase activity. This showed that trastuzumab enhances the NK cell–mediated killing of HER2-positive tumor targets, thus confirming previous reports of trastuzumab-dependent, NK cell–mediated ADCC (Fig. 1A; ref. 20). The time course of these experiments suggested a rapid effect that cannot be due to growth inhibition. Moreover, the required antibody concentrations were in a range that has been found effective to induce ADCC (33), whereas considerably higher trastuzumab concentrations seem to be required to block proliferation of tumor cell targets (34). Accordingly, trastuzumab showed only minor effects in this assay when applied alone.
Immune-selected SK-OV-3 cells show greatly decreased ADCC and reduced HER2 surface expression. When SK-OV-3 ovarian cancer cells were repeatedly selected for survival in the presence of trastuzumab and polyclonal NK cells, a subline was obtained that was still sensitive to NK cell–mediated killing. However, addition of trastuzumab did not significantly increase target cell lysis any more (except for the highest antibody concentration at the highest effector/target ratio; Fig. 1B). This loss of susceptibility toward trastuzumab and NK cell–mediated ADCC may at least partly be explained by a down-regulation of HER2 mRNA and surface expression (Fig. 1C).

Trastuzumab and polyclonal NK cells show synergistic killing of naïve and immune-selected MCF7 mammary carcinoma cells. Just like SK-OV-3 cells, HER2-expressing MCF7 mammary tumor cells showed increased susceptibility toward NK cell–mediated killing in the presence of trastuzumab (Fig. 2A). However, MCF7 cells that were selected for survival in the presence of trastuzumab and polyclonal NK cells lost luciferase expression. Thus, cytotoxicity was now assessed by modified FATAL assays as described by Krockenberger and colleagues (35). Apart from this unexpected loss of bioluminescence, however, no significant morphologic differences were observed between

![Figure 2. Killing and selection of MCF7 breast cancer cells in the presence of polyclonal human NK cells and monoclonal rhumAb 4D5 (trastuzumab/Herceptin). A, luciferase-transfected MCF7 breast cancer cells were incubated as indicated with polyclonal human NK cells in the absence or presence of rhumAb 4D5 or human control IgG. Target cell lysis was determined via a biophotonic cytotoxicity assay after 4, 8, and 24 h of coculture (n = 3). B, MCF7 breast cancer cells were either just propagated or repeatedly challenged (striped columns) with rhumAb 4D5 (trastuzumab/Herceptin) and polyclonal human NK cells before their susceptibility toward NK cell–mediated killing was assessed in the presence or absence of rhumAb 4D5 or control IgG. Shown is a representative 8-h FATAL assay comparing nonselected MCF7 cancer targets (solid columns) with MCF7 breast cancer cells that had undergone six cycles of immunoselection and re-expansion (n = 3). C, HER2 surface expression was monitored by flow cytometry during the course of the selection process. Shown are representative FACS profiles of unselected and six times selected MCF7 cancer cells. In addition, the HER2 staining intensity was quantified after three, five, and six selection cycles relative to an untreated MCF7 control (right). *, P < 0.05; **, P < 0.01.](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-09-0834)
the immunoselected and the initial cultures: The level of antibody-dependent and antibody-independent target cell killing remained identical over six consecutive selection rounds (data not shown; Fig. 2B). Also HER2 mRNA and surface expression were unaltered in the bulk population (data not shown; Fig. 2C). Thus, we could not select ADCC-resistant subclones of the initial MCF7 culture.

**MCF7 cells surviving an ADCC challenge with NK cells and trastuzumab show a “CSC-like” phenotype.** Although we did not obtain an immune-refractory MCF7 subline, we observed that the cells surviving the challenge initially grew as spheres (Fig. 3A, top right). After a phase of three-dimensional growth (Fig. 3A, bottom right), further expansion and subculturing yielded a tumor cell culture that recapitulated the initial morphology and growth characteristics. Considering that MCF7 cells form rather heterogeneous cultures (36) and that the capability to reconstitute heterogeneous tumor cell cultures has been ascribed to the so-called “CSCs,” we now wondered whether this particular subset (24, 37–39) might have survived the coculture with trastuzumab and NK cells.

In MCF7 and other breast cancer cell lines, CSC were described as CD44\(^\text{high}\)/CD24\(^\text{low}\) cells that can grow as “mammospheres” (40).
Thus, the anchorage-independent growth pattern observed after the immunoselection process (Fig. 3A) prompted us to investigate the expression of CD44 and CD24 on the surface of selected or unselected MCF7 cells.

Briefly (24 hours) after the killing (Fig. 3B, bottom), the proportion of CD44^{high}CD24^{low/-}7-AAD^{negative} putative CSC was found to reach 11% when naïve MCF7 cells (left) and 23.5% when already CSC-enriched immunoselected MCF7 cells (right) were used for the coculture with NK cells and trastuzumab. Although this enrichment for putative tumor-initiating cells was largely transient—the proportion of recognizably stem cell–like cells gradually decreased during re-expansion—MCF7 cells also revealed a significant increase (6.2% versus 0.2%) in CD44^{high}CD24^{low} cells after six cycles of treatment with polyclonal NK cells and trastuzumab. Although this enrichment for putative tumor-initiating cells was largely transient—the proportion of recognizably stem cell–like cells gradually decreased during re-expansion—MCF7 cells also revealed a significant increase (6.2% versus 0.2%) in CD44^{high}CD24^{low} cells after six cycles of treatment with polyclonal NK cells and trastuzumab (Fig. 3B, right), indicating that immunoselection increased the proportion of CSC over time. Likewise, treatment of SK-BR-3, MDA-MB231, and BT474 breast cancer cells with trastuzumab and polyclonal NK induced an enrichment of CD44^{high}CD24^{low} cells (Fig. 3C), suggesting that these cell lines behave similar to MCF7. It should, however, be noted that the association between CSC-like properties and the CD44^{high}CD24^{low} subset has only been validated for the MCF7 cell line.

**CD44^{high}CD24^{low} breast cancer cells show reduced HER2 surface expression and can give rise to CD44^{high}CD24^{high} HER2^{high} cells.** To investigate their differentiation potential, we purified CD44^{high}CD24^{low} MCF7 cells by FACS sorting and found that they can regenerate a culture consisting of mostly CD24^{high} cells within 10 days (Fig. 4, left). Moreover, we observed HER2 surface expression to be significantly lower on the presumed stem cell–like subset. Again, the sorted cell culture reverted quickly to the initial phenotype (Fig. 4, right). Thus, the CD44^{high}CD24^{low} HER2^{low} population can give rise to CD44^{high}CD24^{high} HER2^{high} cells. The hypothesis that the relative resistance of CD44^{high}CD24^{low} mammary carcinoma cells could be due to their low HER2 expression is further supported by the reduced HER2 expression levels that we observed in the CD44^{high}CD24^{low} subsets of our additional cell lines (specific fluorescence intensity values: 7.8 versus 11.6 for SK-BR-3, 2.1 versus 4.0 for MDA-MB-231, 104 versus 168 for BT474).

**Immunoselected MCF7 cells display increased tumorigenicity in vivo.** Finally, we investigated the clonogenicity and tumorigenicity of the MCF7 cells that had undergone six cycles of immunoselection. As shown in Fig. 3B, these cells displayed a stable enrichment of CD44^{high}CD24^{low}HER2^{low} cells. Consequently, they were able to form mammospheres, whereas control cells grew only in small adherent colonies (Fig. 5A). Importantly, the immunoselected cells were also more tumorigenic in vivo. Although only one of five mice injected with 10^5 control MCF7 cells developed a tumor, immunoselected MCF7 cells induced tumor formation in four of six mice (P = 0.18). When the mice were inoculated with 10^6 cells, tumor growth was observed in two of five (control) versus six of six (immunoselected) mice (P = 0.04; Fig. 5B). These differences were confirmed by histologic assessment of the respective tumor sizes (Fig. 5C). Finally, examination of the tumor tissues revealed a far higher cell density and more mitoses in tumors from immunoselected MCF7 cells (Fig. 5D). HER2 expression was low but detectable on all investigated tissues (data not shown).

**Figure 4.** Sorted CD44^{high}CD24^{low} cells express low levels of HER2 and can regenerate a CD44^{high}CD24^{high}HER2^{high} population. Untreated MCF7 breast cancer cells were stained and CD44^{high}CD24^{low} cells were isolated by FACS sorting (top and middle). Expression of HER2 was analyzed simultaneously (right). After 10 d of in vitro culture, the sorted cells were reanalyzed for CD44, CD24, and HER2 expression (n = 3).
Discussion

Experience with traditional chemotherapeutic drugs or modern "targeted" therapies has shown that cancer therapeutics often only delay the progress of the disease. This is frequently observed with the humanized HER2-specific IgG1 antibody trastuzumab that can yield extraordinary initial responses against HER2-overexpressing breast cancer until the disease relapses. Other HER2-positive malignancies altogether fail to respond. Resistance against trastuzumab has largely been explained by redundancy in growth factor receptor signaling that allows a transformed cell to compensate for the blockade of HER2. However, because NK cell–mediated ADCC was shown to be important for the in vivo function of the antibody (18), we wondered whether the failure to respond to trastuzumab might also be related to tumor immune escape, especially because the continuous confrontation with the host immune system is likely to trigger some kind of "immunoediting" (29). Thus, we have confirmed that trastuzumab induces ADCC when SK-OV-3 ovarian cancer and MCF7 mammary carcinoma cells are coincubated with polyclonal NK cells (Figs. 1A and 2A). When we investigated those cells that survived a single or repeated ADCC challenge, two different mechanisms of resistance against ADCC- and ADCC-independent NK cell–mediated killing became apparent:

The ovarian cancer cell line SK-OV-3 down-regulates HER2 surface expression (Fig. 1C) and becomes largely resistant to the ADCC component of the NK cell–mediated killing. Because activated NK cells secrete large amounts of IFN-γ, these effects might be due to a previously described IFN-γ–dependent promoter methylation (41, 42). This effect further suggests that the cells do
not depend on high HER2 expression, which may explain the poor response of ovarian cancer cells to trastuzumab. In the absence of trastuzumab, however, NK cell–mediated lysis did not differ between “naïve” and immunoselected SK-OV-3 cells, indicating that the cells did not become refractory to NK cell killing in general.

Breast cancer cells, however, showed a fundamentally different behavior because neither HER2 surface expression nor sensitivity toward NK cell–mediated killing or ADCC was altered by the immunoselection process. This is in line with studies that showed HER2 expression levels to remain mostly unaltered under therapy with HER2-specific antibodies in humans (43) and in mice (44). However, in vivo experiments based on the transfer of spontaneous tumors from HER2-transgenic mice into congenic wild-type animals showed that IFN-γ–dependent immunoeediting (42) can promote both permanent tumor rejection or the delayed outgrowth of antigen-loss variants with reduced ability to induce “danger signals” (45).

In our experiments, another mechanism of immunoselection became evident: The few surviving cells formed mammosphere-like structures (Fig. 3A) that are characteristic for stem cell–like breast cancer cells (23). Importantly, a small subset of highly tumorigenic CSCs has been described in the MCF7 cell line and found to be characterized by drug efflux (39) and a CD44highCD24low phenotype (24). A flow cytometric analysis in MCF7, SK-BR-3, MDA-MB231, and BT-474 cells indeed revealed a highly significant enrichment of CD44highCD24low cells (Fig. 3B and C) briefly after ADCC. Although this increase largely normalized upon re-expansion after immunoselection or cell sorting (Fig. 4), the repeatedly selected and re-expanded MCF7 cells also showed a lasting enrichment in these putative CSC. Consequently, these cells displayed increased clonogenicity in vitro and tumorigenicity in vivo. Although these data suggest that CSC selectively survived the immunoselection process, we cannot exclude that the ADCC challenge actually induced CSC through dedifferentiation. In fact, it has been shown that both CD4+ (46) and CD8+ T cells can promote epithelial to mesenchymal transition and thereby induce CD44highCD24low breast cancer CSC in HER2-transgenic mice in vivo (47).

Irrespective of the precise mechanism, our findings clearly support that both CD4+ (46) and CD8+ T cells can promote epithelial to mesenchymal transition and thereby induce CD44highCD24low breast cancer CSC in HER2-transgenic mice. A flow cytometric analysis in MCF7, SK-BR-3, MDA-MB231, and BT-474 cells indeed revealed a highly significant enrichment of CD44highCD24low cells (Fig. 3B and C) briefly after ADCC. Although this increase largely normalized upon re-expansion after immunoselection or cell sorting (Fig. 4), the repeatedly selected and re-expanded MCF7 cells also showed a lasting enrichment in these putative CSC. Consequently, these cells displayed increased clonogenicity in vitro and tumorigenicity in vivo. Although these data suggest that CSC selectively survived the immunoselection process, we cannot exclude that the ADCC challenge actually induced CSC through dedifferentiation. In fact, it has been shown that both CD4+ (46) and CD8+ T cells can promote epithelial to mesenchymal transition and thereby induce CD44highCD24low breast cancer CSC in HER2-transgenic mice in vivo (47).

Irrespective of the precise mechanism, our findings clearly support that both CD4+ (46) and CD8+ T cells can promote epithelial to mesenchymal transition and thereby induce CD44highCD24low breast cancer CSC in HER2-transgenic mice in vivo (47).

This observed immune-refractory phenotype of CSC may partly be due to reduced binding of trastuzumab. However, because ADCC-independent NK cell cytotoxicity was also present in our experiment, further investigations regarding the susceptibility of CSCs toward NK cell–mediated immunotherapy are clearly needed (37).

Clinically, our findings have important implications: Although relapse has traditionally been interpreted as the outgrowth of therapy-resistant tumor cell clones, this may only be correct in a subgroup of tumors (represented, e.g., by the ovarian cancer cell line SK-OV-3). In the remainder (represented by our breast cancer cell lines), the resistance against ADCC may be due to the increased resistance of CSC. Accordingly, these CSC would regenerate the tumor after initial therapy-induced regression. Thus, treatment with trastuzumab should be complemented by a therapy that is more effective against CSC (48). However, although such a therapeutic approach is not yet available for breast cancer, our data also suggest that a “rechallenge” with trastuzumab alone or in combination with another HER2-specific antibody like pertuzumab could be beneficial for the treatment of tumors that have initially shown a good response to the antibody and then relapsed. In fact, this is supported by recent clinical observations (49). Moreover, the fact that MCF7 cells maintained their level of HER2 expression despite the selection pressure exerted by trastuzumab and NK cells implicates that HER2 may be much more essential for mammary than for ovarian carcinoma cells—and thus be a much better target in breast cancer (which is again in line with the clinical reality). Finally, the fact that ADCC occurs with HER2-positive tumor cells suggests that tumors that altogether fail to respond to trastuzumab effectively suppress ADCC. Thus, trastuzumab (or an optimized more immunogenic antibody; ref. 33) might still become beneficial for those cases provided that a general immunologic unresponsiveness could be overcome (50). Accordingly, trastuzumab may not only synergize with established chemotherapeutics, but also with experimental immunotherapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 3/4/09; revised 8/12/09; accepted 8/13/09; published OnlineFirst 10/11/09.
Grant support: A grant from the IZKF Würzburg (J. Wischhusen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Susanne Schüler and Christian Adam (KFO124) for excellent assistance with the animal work and Christian Linden (Institute of Immunology, University of Würzburg) for cell sorting and Stefan Rauthe for HER2 tissue staining.

References
1. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat Rev Cancer 2004;4:661–70.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of HER2/neu protein expression with human breast cancer. Cancer Res 1987;47:4270–7.
3. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.
4. Tueffler M, Couturier J, Penault-Llorca F, et al. HER2 status in ovarian carcinomas: a multicenter GINECO study of 320 patients. PLoS ONE 2007;2:e1318.
5. Hellström I, Goodman G, Pullman J, Yang Y, Hellström KE. Overexpression of HER-2 in ovarian carcinomas. Cancer Res 2001;61:420–3.
6. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999;17:2639–48.
7. Jorizzo H, Kellokumpu-Lehtinen PL, Bono P, et al. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. N Engl J Med 2006;354:809–20.
8. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005;353:1673–84.
9. Albanell J, Baselga J. Unraveling resistance to trastuzumab (Herceptin): insulin-like growth factor I receptor, a new suspect. J Natl Cancer Inst 2001;93:1830–2.
10. Bookman MA, Darcy KM, Clarke-Pearson D, Boothby RA, Horwitz IB. Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group. J Clin Oncol 2003;21:283–90.
11. Whenham N, D'Hondt V, Piccart MJ. HER2-positive breast cancer: from trastuzumab to innovatory anti-HER2 strategies. Clin Breast Cancer 2008;8:38–49.
12. Baselga J, Albanell J. Mechanism of action of anti-HER2 monoclonal antibodies. Ann Oncol 2001;12 Suppl 1:S35–41.
13. Hudis CA. Trastuzumab-mechanism of action and use in clinical practice. N Engl J Med 2007;357:39–51.

14. Nimmerjahn F, Ravetch JV. Antibodies, Fc receptors and cancer. Curr Opin Immunol 2007;19:239–45.

15. Gennari R, Menard S, Fagnoni F, et al. Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors over-expressing HER2. Clin Cancer Res 2004;10:5650–5.

16. Arnauld L, Gelly M, Penault-Llorca F, et al. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? Br J Cancer 2006;94:259–67.

17. Beano A, Signorino E, Evangelista A, et al. Correlation between NK function and response to trastuzumab in metastatic breast cancer patients. J Transl Med 2006;4:62.

18. Clynes RA, Towers TL, Presta LG, Ravetch JV. Anti-Fc gamma RIII antibodies deplete cytotoxic T lymphocytes in vivo and augment tumor growth in BALB/c mice. J Immunol 2001;166:559–63.

19. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population in vivo. Cancer Res 2008;68:1129–36.

20. Carson WE, Parhia R, Lindemann MJ, et al. Interleukin-2 enhances the natural killer cell response to Herceptin-coated Her2/new-positive breast cancer cells. J Immunol 2001;166:561–65.

21. Catsimi Y, Kuwahara Y, Tamura S, et al. Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated death of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. Mol Cancer Ther 2007;6:2004–12.

22. Asselin-Labat ML, Shackleton M, Stingl J, et al. Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 2006;98:1011–14.

23. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population during tumorigenesis and invasion. Oncogene 2008;27:6129–38.

24. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 2008;10:R25.

25. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;100:10984–8.

26. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1:555–67.

27. Pan Y, Huang X. Epithelial ovarian cancer stem cell-a review. Int J Clin Exp Pathol 2008;1:260–6.

28. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:7–30.

29. Dunn GP, Old LJ, Schreiber RD. The Three Es of Cancer Immunotherapy. Annu Rev Immunol 2004;22:329–60.

30. Peruzzi B, Ramoni C, Anson I, Cuturi MC, Faust J, Trinchieri G. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat Immunol 1998;1:571–88.

31. Brown CE, Wright CL, Naranjo A, et al. Biophotonic cytotoxicity assay for high-throughput screening of cytolytic killing. J Immunol Methods 2000;237:39–52.

32. Sheehy ME, McDermott AM, Farlan SN, Klemmerer P, Nixon DF. A novel technique for the fluorometric assessment of T lymphocyte antigen specific lysis. J Immunol Methods 2001;249:99–110.

33. Suzuki E, Niwa R, Saji S, et al. A nonfusocyslated anti-HER2 antibody augments antibody-dependent cellular cytotoxicity in breast cancer patients. Clin Cancer Res 2007;13:1875–82.

34. Argiris A, Wang C-X, Whalen SG, DiGiovanna MP. Synergistic Interactions between Tamoxifen and Trastuzumab (Herceptin). Clin Cancer Res 2004;10:1409–20.

35. Kroekenberger M, Dombrowski Y, Weidler C, et al. Macrophage migration inhibitory factor contributes to the immune escape of ovarian cancer by down-regulating NGK2D. J Immunol 2008;180:7338–48.

36. Resnoff C, Medrano EE, Podjasek O, Bravo AL, Boyer L, Mordoh J. Subpopulations of MCF7 cells separated by Percoll gradient centrifugation: a model to analyze the heterogeneity of human breast cancer. Proc Natl Acad Sci U S A 1987;84:2925–9.

37. Mine T, Matuda S, Li X, et al. Breast cancer cells expressing stem cell markers CD44(+)CD24(lo) have a distinct proteomic profile and a reduced ability to induce 'danger signals'. Cancer Immunol Immunother 2009;58:1865–94.

38. Zhou J, Zhang H, Gu P, Margolick JB, Yin D, Zhang Y. Cancer stem/progenitor cell active compound 8-quinolino in combination with paclitaxel achieves an improved cure of breast cancer in the mouse model. Breast Cancer Res Treat 2009;115:269–77.

39. Engelman K, Shen H, Finn OJ. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. Cancer Res 2008;68:3419–26.

40. Fillmore C, Kuperwasser C. Human breast cancer stem cell markers CD44 and CD24: enriching for cells with functional properties in mice or in man? Breast Cancer Res 2007;9:303.

41. Martin C, Müller-Holzner E, Greiter E, et al. Gamma interferon reduces expression of the protooncogene c-erb-B2 in human ovarian carcinoma cells. Cancer Res 1990;50:7037–41.

42. Klieweck M, Knutson KL, Durnn CL, Manjili MH. HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. Eur J Immunol 2007;37:675–85.

43. Hurst BM, Harris NL, Gelman R, et al. Preparative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. J Clin Oncol 2003;21:46–53.

44. Knutson KL, Almand B, Dang Y, Diis ML. Neu antigen-negative variants can be generated after neuspecific antibody therapy in neu transgenic mice. Cancer Res 2004;64:1146–51.

45. Manjili MH, Arnouk H, Knutson KL, et al. Emergence of immune escape variant of mammary tumors that has distinct proteomic profile and a reduced ability to induce 'danger signals'. Breast Cancer Res Treat 2006;96:233–41.

46. Knutson KL, Lu H, Stone B, et al. Immunoeediting of cancers may lead to epithelial to mesenchymal transition. J Immunol 2006;177:1526–33.

47. Santisteban M, Reiman JM, Asiedu MK, et al. Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. Cancer Stem Cells 2009;6:2887–95.

48. Beier D, Röhrsl S, Püllar DR, et al. Temozolomide preferentially depletes cancer stem cells in glioblastoma. Cancer Res 2008;68:5706–15.

49. Portera CC, Walsh JM, Rosing DR, et al. Cardiac toxicity and efficacy of trastuzumab combined with pertuzumab in patients with trastuzumab-insensitive human epidermal growth factor receptor 2-positive metastatic breast cancer. Clin Cancer Res 2008;14:2710–6.

50. Kruschinski A, Moosmann A, Poschke I, et al. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. Proc Natl Acad Sci U S A 2008;105:17481–6.
Immunoselection of Breast and Ovarian Cancer Cells with Trastuzumab and Natural Killer Cells: Selective Escape of CD44high/CD24low/HER2low Breast Cancer Stem Cells

Florian Reim, Yvonne Dombrowski, Cathrin Ritter, et al.

Cancer Res 2009;69:8058-8066. Published OnlineFirst October 13, 2009.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-0834

Cited articles
This article cites 50 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/20/8058.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/20/8058.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/69/20/8058.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.