Immunology

Immunoselection of Breast and Ovarian Cancer Cells with Trastuzumab and Natural Killer Cells: Selective Escape of CD44high/CD24low/HER2low Breast Cancer Stem Cells

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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 CD44highCD24low "cancer stem cell–like" phenotype and expressed significantly less HER2 compared with non–stem cells. Likewise, the CD44highCD24low 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 CD44highCD24low 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. [Cancer Res 2009;69(20):8058–66]

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 Fcγ-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 FcγRIIIA, but no inhibitory Fcγ 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 FcγRIIIA 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 FcγRIIIA or CD16 on NK cells (20), and the presence of interleukin 2 (IL-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

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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 $CD44^{high}CD24^{low}$ 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, BT-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 investigate clonogenicity, 100 or 500 cells, respectively, were seeded in 24-well plates and the resulting mammospheres were counted after 18 d under an inverted microscope (Leica).

Flow cytometric analysis of surface expression levels and cell sorting. Cells (10^6 per sample) were detached with Accutase (PAA), blocked with 250 μg/mL human control IgG1 (Beriglobin), and stained with 10 μg/mL trastuzumab (Roche) followed by a Cy5-conjugated anti-human IgG (Rockland Immunochemicals) detection antibody. Simultaneously, CD44-RPE (Clone F10-44-2, AbD Serotec), CD24-FITC (clone SN3, Immunotools), and the life stain 7-aminoactinomycin D (Sigma) were applied and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Where appropriate, expression levels are indicated as specific fluorescence intensity values, which are obtained by dividing the fluorescence intensity detected with the specific antibody by the signal measured with the isotype-matched control antibody. For fluorescence-activated cell sorting (FACS) sorting, the stained

cells were separated twice on a Digital FACSVantage (BD Biosciences), first in yield, then in purity mode.

NK cell preparation and cytotoxicity assays. Peripheral blood lymphocytes were obtained from healthy volunteers by density gradient centrifugation (Biocoll, Biochrom) and cultured for 8 to 11 d on irradiated (30 Gy) RPMI 8866 feeder cells to obtain polyclonal NK cell populations (30). CD3, CD56, and CD16 expression were analyzed by flow cytometry, and NK cell purity was found to be >75%. Alternatively, NK cells were purified using the NK cell isolation kit and expanded with the NK cell expansion and activation kit (both from Miltenyi Biotec) or activated by addition of 100 U/mL IL-2 (Peprotech) for 48 h. This yielded NK cell cultures with purities of >90%.

NK cell cytotoxicity against SK-OV-3 and early-passage MCF7 tumor cells was assessed using luciferase-transfected targets seeded into 96-well plates (10^4 per well). NK cells and trastuzumab or irrelevant human control IgG were added in triplicates at the indicated effector/target ratios. After addition of 0.14 mg/mL cell-permeable D-luciferin (PJK), luminescence activity (which is proportional to cell viability) was recorded at different time points using an Orion II luminometer (Berthold; ref. 31).

NK cell lysis of control or immune-selected MCF7 cells (which had lost luciferase expression) was analyzed by modified FATAL assays (32). NK cells were thus labeled with PKH-26 (Sigma), target cells (10^5 per well) were stained with carboxyfluorescein diacetate succinimidyl ester (Invitrogen), cocultures were set up using different effector/target ratios, and lytic activity was assessed after 8 h by flow cytometric detection of carboxyfluorescein diacetate succinimidyl ester^{dim} cells among the PKH-26–negative target cell population. Values were corrected for spontaneous leakage of carboxyfluorescein diacetate succinimidyl ester.

Immunoselection of ADCC-resistant tumor cells using trastuzumab and polyclonal NK cells. To select for ADCC-resistant tumor cell subclones, 3×10^6 SK-OV-3 fLuc or MCF7 fLuc cancer cells were incubated for 12 h with 50 ng/mL trastuzumab and 1.5×10^7 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′-CGGCTACCACATCCAAGGAA-3′; 18S down, 5′-GCTGGAAT-TACCGCGGCT-3′; HER2 up, 5′-TGGAAGCCACAAGGTAAACA-3′; HER2 down, 5′-CCTACTCCATCCCAAGCC TAT-3′. Data analysis was done using the $\Delta\Delta C_T$ 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 IX-70 inverted system microscope at 48 h or 10 d after the NK cell/trastuzumab challenge.

***In vivo* tumorigenicity assay.** Immunoselected (10^3 or 10^4; $n = 6$ 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. HER2 stainings 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. 1*A* and *B*, and 2*A* 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
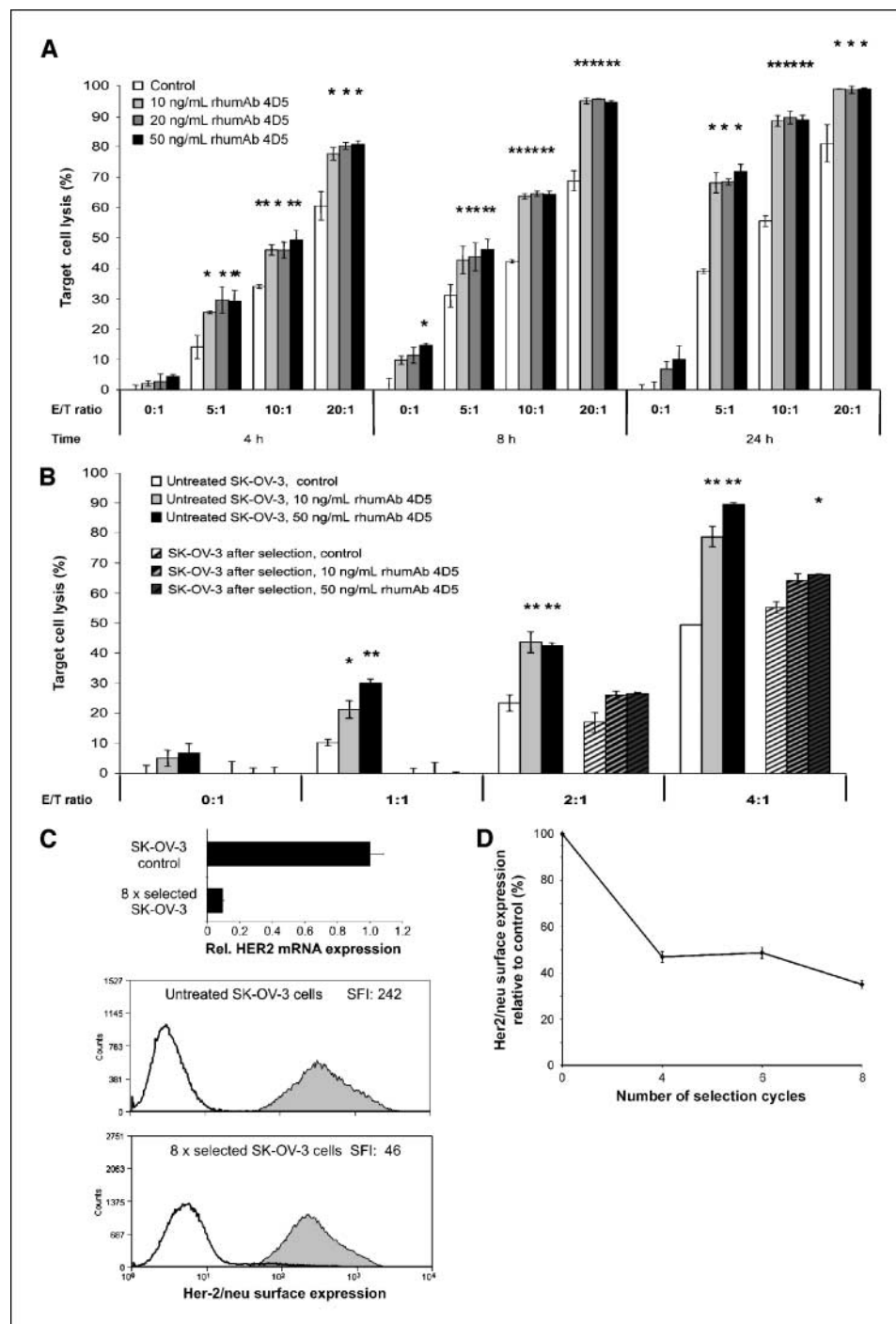
Figure 1. Killing and selection of ADCC-resistant SK-OV-3 ovarian cancer cells in the presence of polyclonal human NK cells and monoclonal rhumAb 4D5 (trastuzumab/Herceptin).
A, luciferase-transfected SK-OV-3 ovarian cancer cells were incubated as indicated with polyclonal human NK cells in the absence or presence of rhumAb 4D5 or human control IgG. Target cell lysis was determined by means of a biophotonic cytotoxicity assay after 4, 8, and 24 h of coculture (*n* = 3). *B,* luciferase-transfected SK-OV-3 ovarian 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 biophotonic lysis assay comparing nonselected SK-OV-3 cancer targets (*solid columns*) with SK-OV-3 ovarian cancer cells that had undergone eight cycles of immunoselection and re-expansion (*n* = 3). *C,* HER2 mRNA and surface expression was monitored by qRT-PCR and flow cytometry during the course of the selection process. Shown are representative results for unselected and eight times selected SK-OV-3 ovarian cancer cells. *D,* in addition, the HER2 staining intensity was quantified after four, six, and eight cycles relative to an untreated SK-OV-3 control. *SFI,* specific fluorescence intensity. *, *P* < 0.05; **, *P* < 0.01.

tests were performed using Statistica (StatSoft). SDs for flow cytometry data were calculated using Summit software (DakoCytomation).
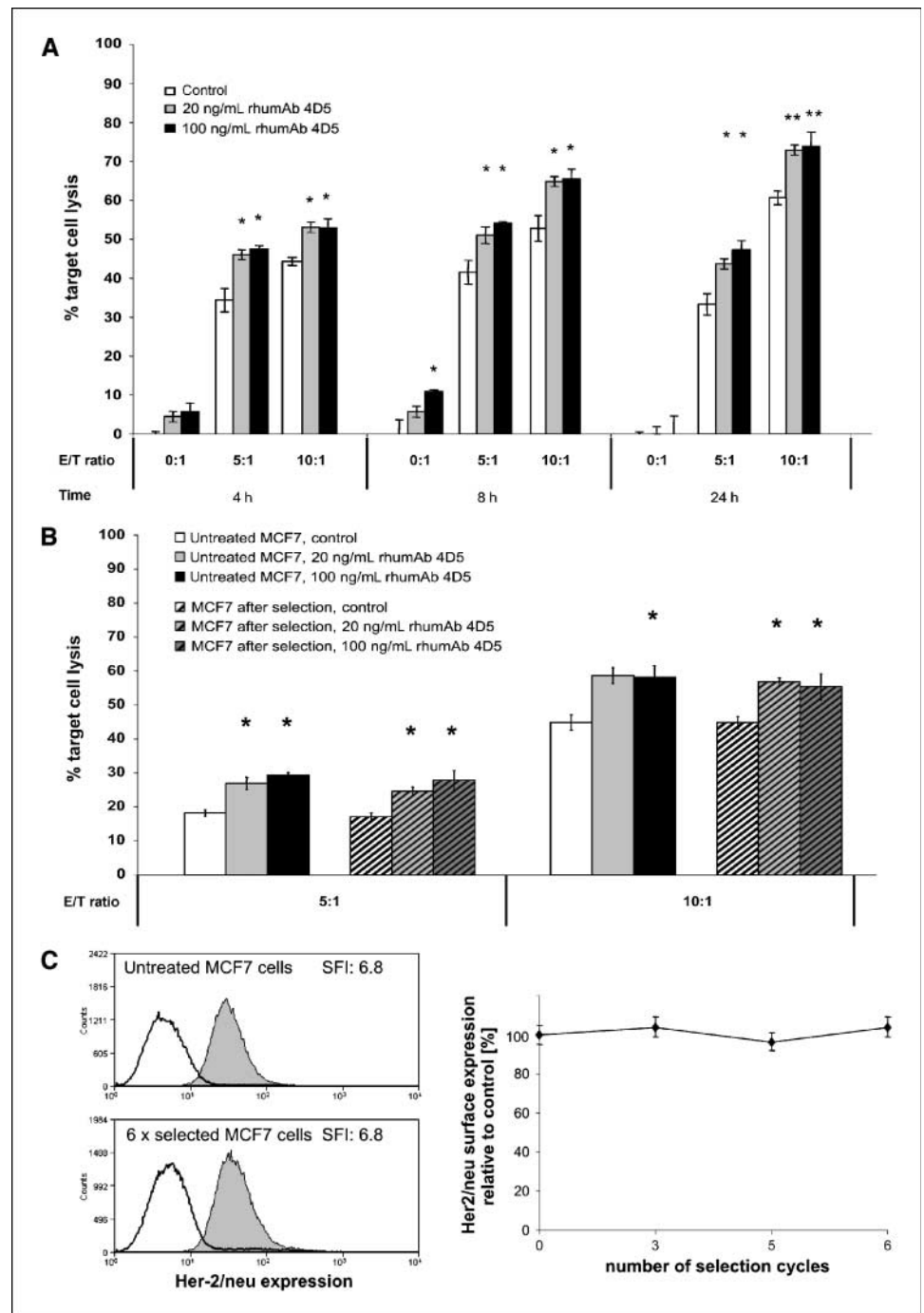
Results

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. 1*A*; 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. 1*B*). 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. 1*C*).

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. 2*A*). 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*). *, $P < 0.05$; **, $P < 0.01$.
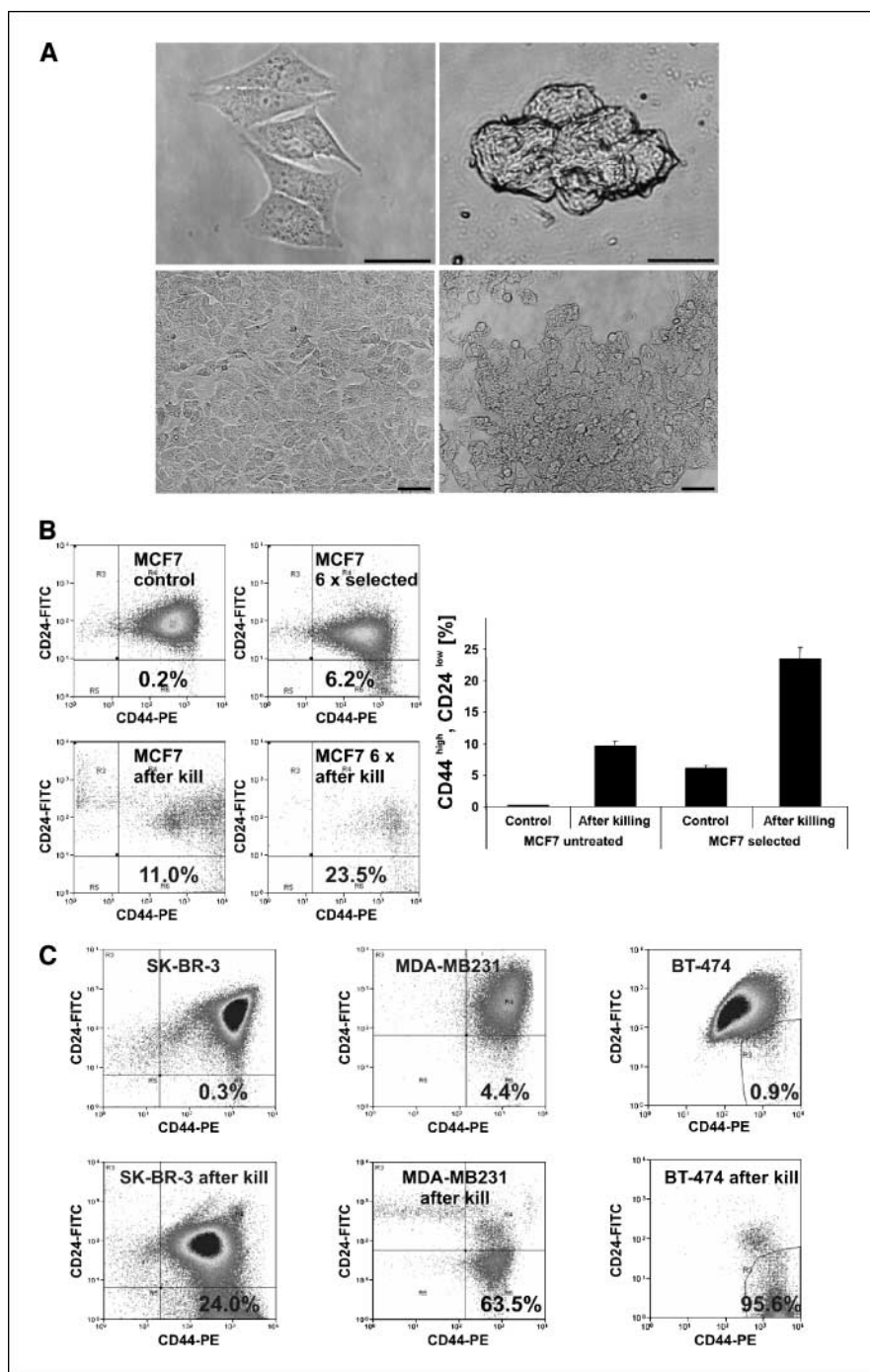
Figure 3. Immunoselection induces an enrichment of CD44^highCD24^low breast cancer cells. *A,* MCF7 breast cancer cells were challenged with rhumAb 4D5 and polyclonal NK cells (*right*). Untreated cells (*left*) were freshly seeded to achieve a similar cell density. The growth pattern of both cultures was then recorded 48 h (*top*) and 10 d after the immunoselection procedure by inverted system phase contrast microscopy ($n = 5$). *Scale bars,* 50 μm (*top*) and 100 μm (*bottom*). *B,* MCF7 cells were analyzed by flow cytometry for cell surface expression of CD44 and CD24, either before (*top left*) or 24 h after (*bottom left*) incubation with rhumAb 4D5 and polyclonal human NK cells. The same experiment was also performed with MCF7 cells that had already undergone six cycles of immunoselection and re-expansion (*right*: preselected cells before (*left*) and 24 h after (*right*) coincubation with NK cells and rhumAb 4D5). FACS settings were kept constant between all groups and dead cells were excluded by staining with 7-aminoactinomycin D. *Right,* the respective percentage of cells displaying a CD44^highCD24^low "breast CSC" phenotype ($n = 3$). *C,* SK-BR-3, MDA-MB231, and BT474 cells were treated and analyzed as in *B* ($n = 3$). The respective percentages of CD44^highCD24^low cells are indicated.

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. 2*B*). Also HER2 mRNA and surface expression were unaltered in the bulk population (data not shown; Fig. 2*C*). 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. 3*A, top*

right). After a phase of three-dimensional growth (Fig. 3*A, 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^highCD24^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.
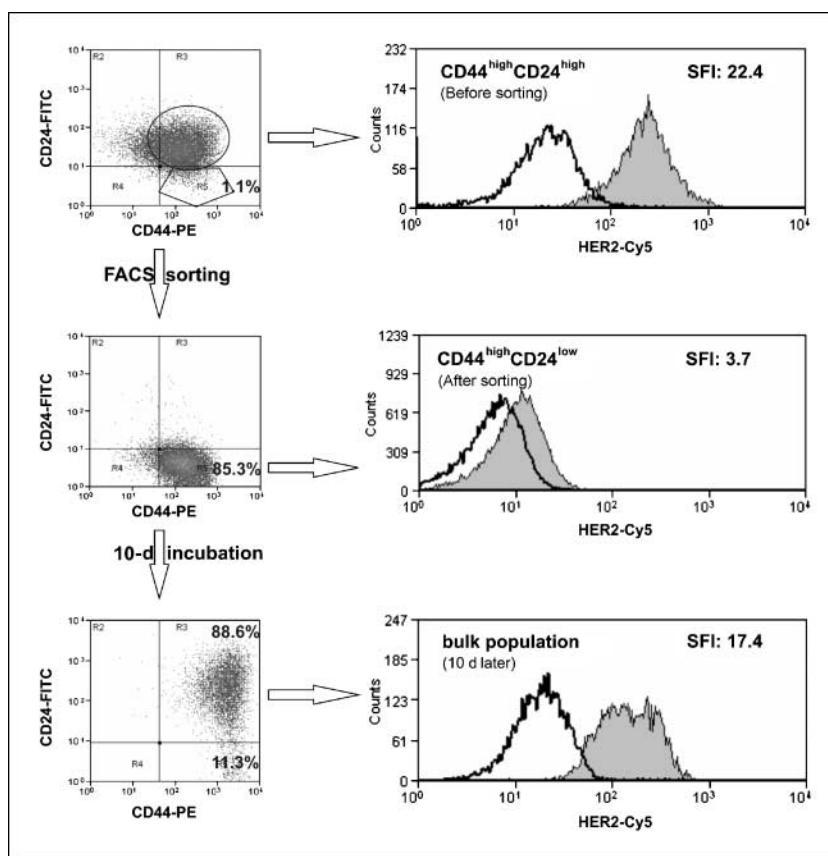
Briefly (24 hours) after the killing (Fig. 3B, *bottom*), the proportion of CD44^{high}CD24^{low}7-AAD^{negative} putative CSC was found to reach 11% when naïve MCF7 cells (*left*) and 23.5% when already CSC-enriched immunoselected MCF7 cells (*right*) were used for the coculture with NK cells and trastuzumab. 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 (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 10^3 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 10^4 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^{low}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 ($n = 3$).
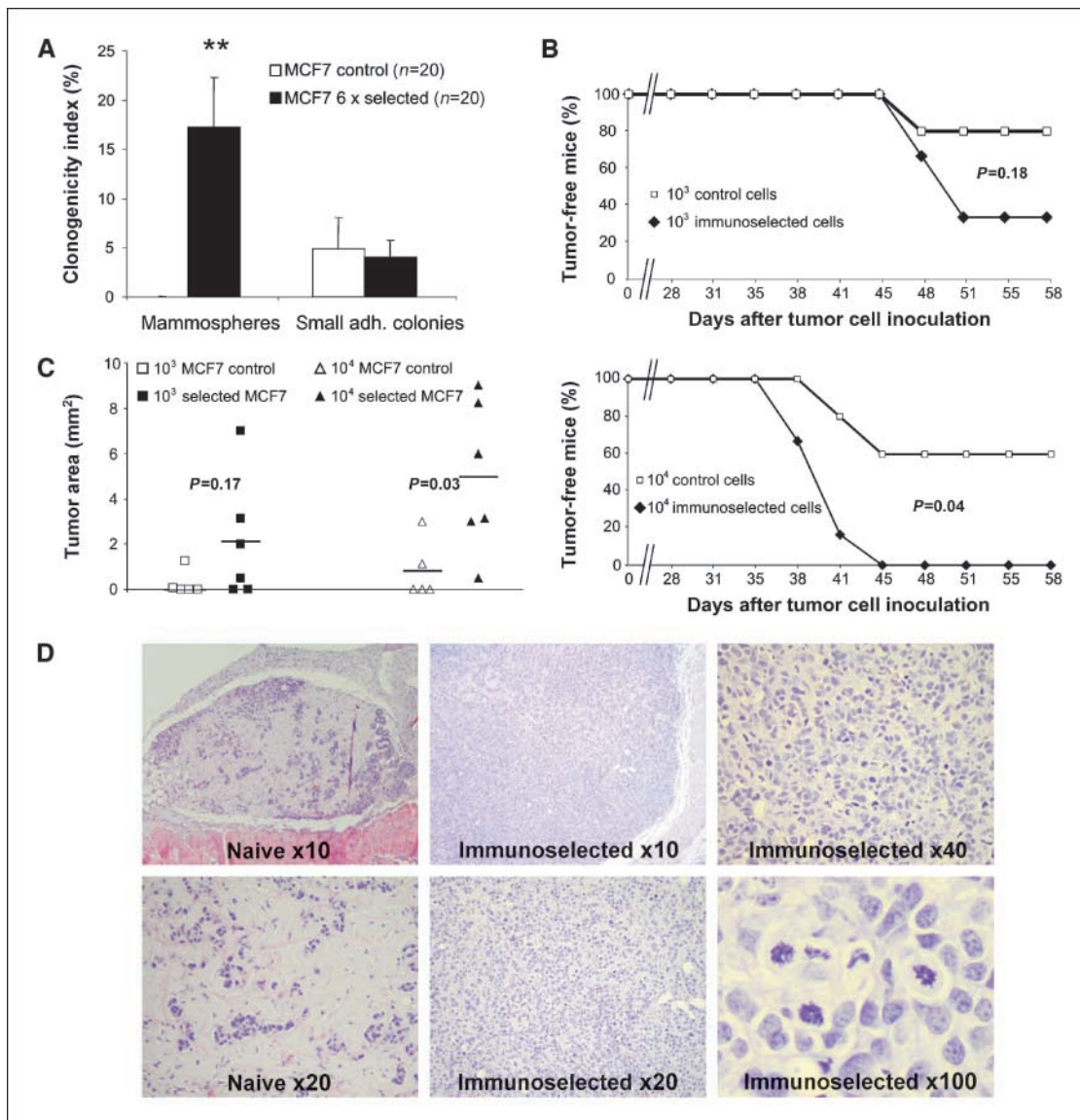
Figure 5. Immunoselected MCF7 cells display increased clonogenicity and tumorigenicity. *A,* 6× immunoselected or naïve MCF7 cells were seeded at low cell densities in 24-well plates (100 and 500 cells per well). After 18 d, the formation of mammospheres and small adherent colonies was determined under an inverted microscope and the percentage of colony-forming cells was calculated. *, $P < 0.01$. *B,* 10^3 (*top*) or 10^4 (*bottom*) naïve ($n = 5$ per cell number) or immunoselected ($n = 6$) MCF7 cells were injected into the mammary fat pads of NOD/Scid-mice. Tumor formation was monitored by palpating. *C,* on day 58, tumors were explanted, embedded in paraffin, cut into 10-μm slices, and stained with H&E. The maximum two-dimensional area of each tumor was measured using a caliper. *D,* representative HE stains of tumors formed from 10^4 naïve or immunoselected MCF7 cells were recorded at ×10, ×20, ×40, and ×100 initial magnification as indicated.

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. 1*A* and 2*A*). 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. 1*C*) 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 immunoediting (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. 3*A*) 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 CD44^{high}CD24^{low} phenotype (24). A flow cytometric analysis in MCF7, SK-BR-3, MDA-MB231, and BT474 cells indeed revealed a highly significant enrichment of CD44^{high}CD24^{low} cells (Fig. 3*B* 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 CD44^{high}CD24^{low} breast cancer CSC in HER2-transgenic mice *in vivo* (47). Irrespective of the precise mechanism, our findings clearly support the CSC model and suggest that breast cancer CSC are resistant to the combination of NK cells and trastuzumab.

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

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

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