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Original Article

Targeting breast cancer stem cells with HER2-specific antibodies and natural killer cells

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Abstract: Breast cancer is the most common cancer among women worldwide. Every year, nearly 1.4 million new cases of breast cancer are diagnosed, and about 450.000 women die of the disease. Approximately 15-25% of breast cancer cases exhibit increased quantities of the trans-membrane receptor tyrosine kinase human epidermal growth factor receptor 2 (HER2) on the tumor cell surface. Previous studies showed that blockade of this HER2 proto-oncogene with the antibody trastuzumab substantially improved the overall survival of patients with this aggressive type of breast cancer. Recruitment of natural killer (NK) cells and subsequent induction of antibody-dependent cell-mediated cytotoxicity (ADCC) contributed to this beneficial effect. We hypothesized that antibody binding to HER2-positive breast cancer cells and thus ADCC might be further improved by synergistically applying two different HER2-specific antibodies, trastuzumab and pertuzumab. We found that tumor cell killing via ADCC was increased when the combination of trastuzumab, pertuzumab, and NK cells was applied to HER2-positive breast cancer cells, as compared to the extent of ADCC induced by a single antibody. Furthermore, a subset of $CD44^{high}CD24^{low}HER2^{low}$ cells, which possessed characteristics of cancer stem cells, could be targeted more efficiently by the combination of two HER2-specific antibodies compared to the efficiency of one antibody. These *in vitro* results demonstrated the immunotherapeutic benefit achieved by the combined application of trastuzumab and pertuzumab. These findings are consistent with the positive results of the clinical studies, CLEOPATRA and NEOSPHERE, conducted with patients that had HER2-positive breast cancer. Compared to a single antibody treatment, the combined application of trastuzumab and pertuzumab showed a stronger ADCC effect and improved the targeting of breast cancer stem cells.

Keywords: Trastuzumab, pertuzumab, ADCC, tumor stem cells, breast cancer

Introduction

Human epidermal growth factor receptor-2 (HER2/neu, or c-erbB2) is a proto-oncogene, which belongs to a family of four trans-membrane receptor tyrosine kinases that mediate critical functions, like cell growth, differentiation, and survival, in malignant and normal breast epithelial cells [1]. HER2 is often involved in facilitating the interaction between epidermal growth factor receptor (EGFR) or HER3 and their corresponding ligands. In addition to the homodimerization of HER2, the heterodimerization of HER2 and HER3 or EGF can initiate oncogenic signaling [2-4].

Approximately 15%-25% of primary breast cancers overexpress the HER2 protein due to amplification in the HER2 gene. The overexpression of HER2 is associated with an aggressive clinical phenotype, increased disease recurrence, and an unfavorable prognosis [5]. Many of these negative effects of HER2 overexpression can be reversed by the application of monoclonal antibodies directed against the HER2 antigen. The humanized monoclonal antibody trastuzumab (Herceptin®, Genentech) is directed against the extracellular domain of HER2. It is administered for the treatment of patients with HER2-positive breast and gastric cancers. The antibody improves disease-free and overall survival in the adjuvant, metastasized setting [6, 7]. When trastuzumab is used as a monotherapy, it causes tumor cell growth inhibition. In combination with chemotherapeu-

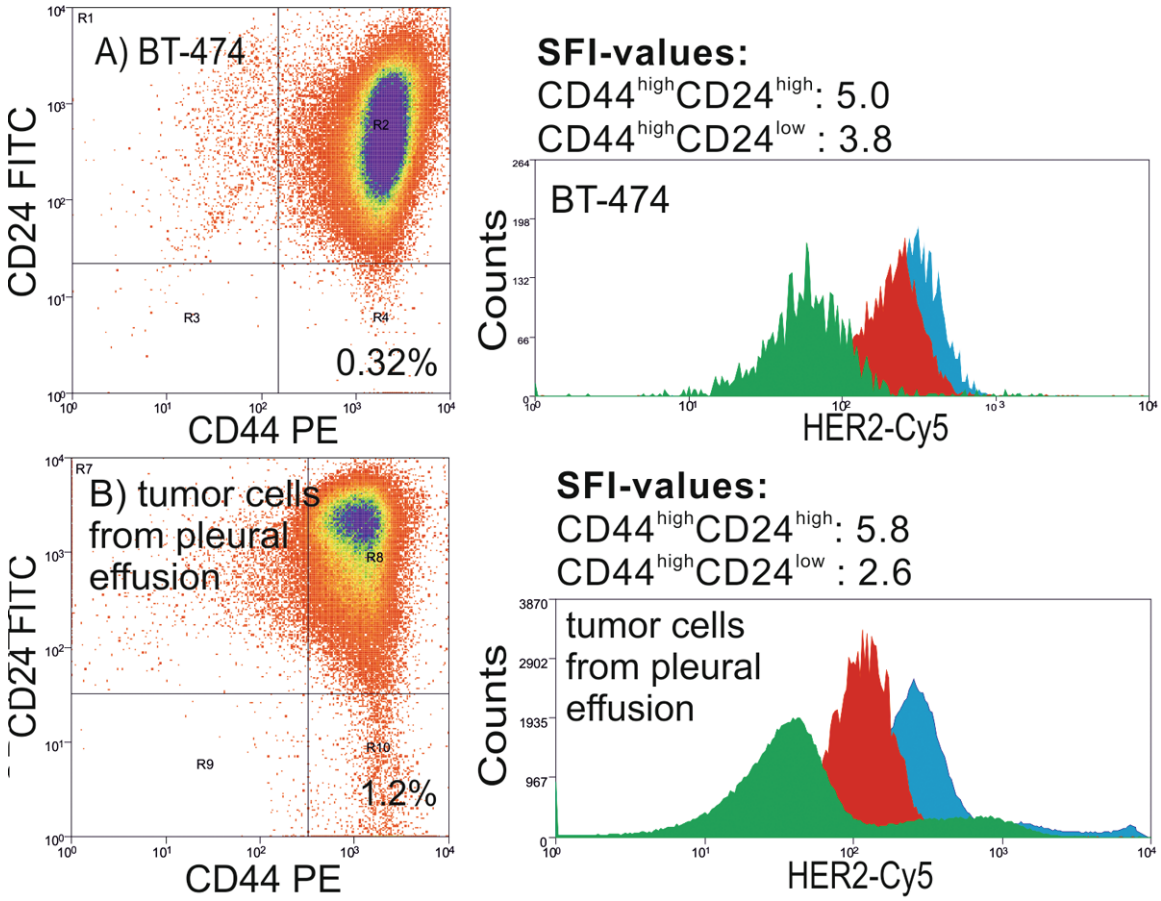


Figure 1. HER2 expression on $CD44^{high}$ $CD24^{low}$ breast cancer stem cells is low. HER2 expression was analyzed by FACS on $CD44^{high}CD24^{high}$ and $CD44^{high}CD24^{low}$ cells from (A) the HER2-positive breast cancer cell line BT-474 and (B) primary breast cancer cells from a pleural effusion from a patient with metastasized HER2-positive breast cancer. Left panels: Stainings for CD44 and CD24. Cells in the lower right quadrant were considered to be $CD44^{high}CD24^{low}$ whereas the upper right quadrant demarks $CD44^{high}CD24^{high}$ cells. HER2/neu-stainings for the respective subsets are shown on the right. Green curve: isotype control antibodies (10 µg/ml); red curve: HER2 expression on $CD44^{high}CD24^{low}$ cells; blue curve: HER2 expression on $CD44^{high}CD24^{high}$ cells (10 µg/ml). Isotype binding was similar for $CD24^{high}$ and $CD24^{low}$ cells. Values for specific fluorescence intensities (SFI) were obtained by dividing the median signal obtained with the specific antibody by the median signal obtained with the corresponding isotype control.

tics, like cisplatin, carboplatin, docetaxel and ionizing radiation, trastuzumab has additive effects and yields improvements in terms of response duration and progression-free survival [8, 9].

In addition to blocking the HER2 receptor, trastuzumab also triggers a mechanism of cell-mediated immunity. When trastuzumab (an IgG1-type antibody) binds to target cells that overexpress the HER2 antigen, effector cells, like natural killer (NK) cells, actively recognize these labeled tumor cells via the Fcγ-part of trastuzumab. The NK cells then induce tumor cell death through ‘‘antibody-dependent cell-mediated cytotoxicity’’ (ADCC) [10-14]. The efficacy of HER2-directed therapies is then further improved by the spontaneous activation of an adaptive anti-tumor immune response which was essential for tumor clearance in mice [15]. A new approach for the treatment of HER2-positive breast cancer is the combined application of two HER2-specific antibodies, trastuzumab and pertuzumab. Pertuzumab is a humanized monoclonal antibody that binds to HER2 near the center of domain II. This inhibits the dimerization of HER2 with other HER family members, thus blocking further signaling processes that are associated with tumor growth and progression. The co-localization of two

HER2 antibodies on distinct epitopes of the HER2 extracellular domain provides more efficient inhibition of tumor growth than one antibody alone [16, 17].

The NEOSPHERE phase II study investigated a novel regimen of applying the combination of pertuzumab and trastuzumab, compared to trastuzumab alone, plus chemotherapy (docetaxel) in the neoadjuvant setting in women with early-stage HER2-positive breast cancer. Their data demonstrated that in that setting the combination of trastuzumab and pertuzumab improved complete remission of pathology in the breast by >1.5 times over that achieved with trastuzumab alone [18]. Based on the crucial role of the immune system for a highly efficient response to anti-HER2 antibodies [15], this may hint at an improved activation of innate and adaptive immune cells.

In preliminary studies, we have successfully demonstrated the importance of NK cell–mediated ADCC for the *in vitro* function of trastuzumab. Moreover, we could provide evidence that a huge proportion of HER2-positive cells that had survived an ADCC challenge with NK cells and trastuzumab showed a "cancer stem cell-like" phenotype [19]. Cancer stem cells (CSC), also termed tumor-initiating or metastasis-initiating cells, had been previously described in mammary cancer [20]. This rare subpopulation which is characterized by a $CD44^{high}CD24^{low}$ phenotype, is held responsible for resistance against different therapeutic approaches and for late recurrence. Therefore, it has become a high priority to target CSCs with different therapeutic tools. In the present study, we investigated the new HER2-specific antibody, pertuzumab, and compared its activity to the combination of trastuzumab and pertuzumab, with particular attention to effects on CSCs.

Materials and methods

Cell culture

MCF-7, MDA-MB-231, BT-474, and SK-BR-3 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as indicated by the supplier. Primary tumor cells were obtained from malignant pleural effusions of patients with metastasized HER2-overexpressing breast cancer. Further investigation of these cells was approved by both the patients and the local ethics committee. Cells were centrifuged, washed with PBS, and transferred to L-valine-deficient Dulbecco's Modified Eagle's Medium, supplemented with D-valine, 2% FCS (Biochrom, Berlin, Germany), penicillin (100 IU/ml), streptomycin (100 IU/ml), and 0.2% sodium pyruvate (all from PAA, Cölbe, Germany). Non-adherent cells were removed after 72 h by washing. Fibroblast growth was suppressed due to the lack of L-valine.

Flow cytometric analysis of surface expression levels and cell sorting

Cells were harvested with Accutase (PAA), blocked with 250 µg/ml human control IgG1 (Beriglobin), and incubated with 5 µg/ml trastuzumab or 5 µg/ml pertuzumab (Genentech, Burlingame, CA, USA). Next, a Cy5-conjugated goat anti-human IgG (Rockland Immunochemicals, Gilbertsville, PA) detection antibody was added. Then, simultaneously, CD44-PE (Clone 2BJ18, BioLegend, San Diego, CA), CD24-FITC (clone SWA-11, kindly provided by Prof. Peter Altevogt, German Cancer Research Centre, Heidelberg), and the viability stain, 7-aminoactinomycin D (Sigma, Deisenhofen, Germany), were applied. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Where appropriate, expression levels are indicated as specific fluorescence intensity values, 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, the stained cells were separated twice with a Digital FACSVantage (BD Biosciences), first in yield mode, then in purity mode.

NK cell preparation and cytotoxicity assays

Peripheral blood lymphocytes were obtained from healthy volunteers and isolated by density gradient centrifugation (Biocoll, Biochrom). Lymphocytes were cultured for 8 to 11 d with irradiated (30 Gy) RPMI 8866 feeder cells to obtain polyclonal NK cell populations. NK cell-mediated lysis of tumor cells was assessed by a modified FATAL assay [21, 22]. Thus, NK cells were labeled with the eFluor® 670 Cell Proliferation Dye (ebioscience, Frankfurt, Germany), and target cells (200 000 per well)

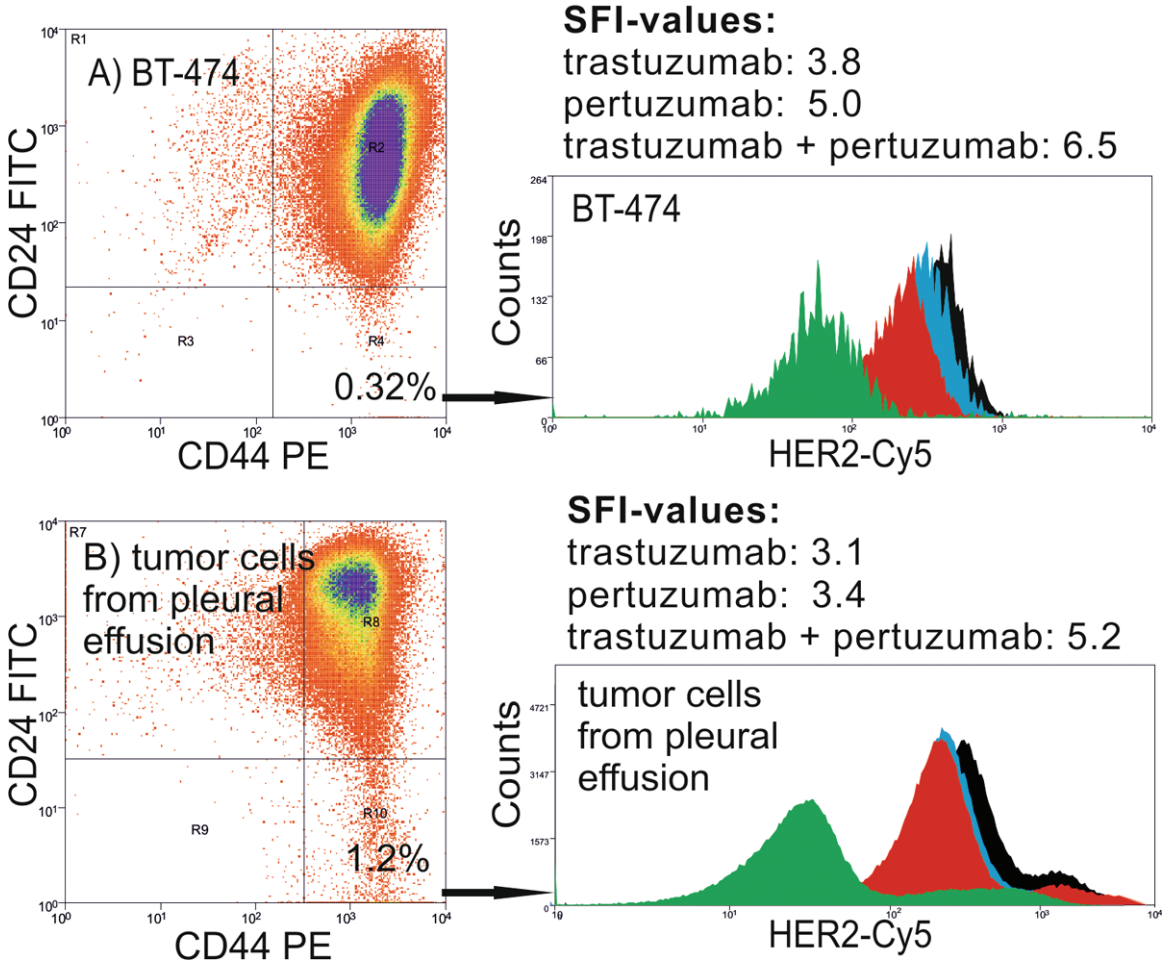


Figure 2. The combination of trastuzumab and pertuzumab leads to increased antibody binding on tumor cells. HER2-specific antibody binding to HER2 positive breast cancer stem cells was quantified by flow cytometry of (A) BT-474 cells and (B) cells from a malignant pleural effusion. Gating for $CD44^{high}CD24^{high}$ and $CD44^{high}CD24^{low}$ cells was performed as in Figure 1, Green curve: isotype control antiboy (10 µg/ml), red curve: trastuzumab (10 µg/ml); blue curve: pertuzumab (10 µg/ml); black curve: combined use of pertuzumab and trastuzumab (each 5 µg/ml). Please note that the data are plotted on a logarithmic scale.

were stained with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Karlsruhe, Germany). Cocultures were set up at different effector: target ratios, and lytic activity was assessed after 16 h by flow cytometric detection of carboxyfluorescein diacetate succinimidyl ester $(CFSE)^{dim}$ cells among the eFluor 670-negative target cell population. Values were corrected for spontaneous leakage of carboxyfluorescein diacetate succinimidyl ester.

Statistics

Experiments were performed at least three times with similar results and representative experiments are shown. Standard deviations for flow cytometry data were calculated using Summit software (Beckman Coulter, Krefeld, Germany). In **Figure 3A**, analysis of significance was performed using an unpaired, two-sided Student's t-test.

Results and discussion

$CD44^{high}CD24^{low}$ breast cancer cells show low HER2 surface expression

HER2 surface expression is known to be heterogeneous, even in HER2-positive breast cancer cell lines. Irrespective of the total amount of HER2, different HER2-positive breast cancer cell lines (SK-BR-3, BT-474, MDA-MB-231) were found to display significantly lower HER2 surface expression levels on the $CD44^{high}CD24^{low}$

(presumed stem cell–like subset) cells than on more differentiated cells. A similar difference between stem cell-like and differentiated cells was observed in primary tumor cells from five patients with metastasized HER2-positive breast cancer, where the cancer cells were obtained from pleural effusions (**Figure 1** and data not shown).

The combination of pertuzumab and trastuzumab increased antibody binding to the surface of HER2-positive tumor cells

Trastuzumab and pertuzumab bind to different epitopes of the HER2 protein (subdomains IV and II, respectively). We compared the amounts of trastuzumab, pertuzumab, or the combination of these HER2-specific antibodies that had bound to targets on tumor cells, SK-BR-3, BT-474, and MDA-MB-231, and to tumor cells from patients with metastasized, HER2-positive breast cancer that had exhibited pleural effusions. We found that the combined application of trastuzumab and pertuzumab increased antibody binding to tumor cells compared to the separate application of trastuzumab or pertuzumab. Moreover, the combined application increased antibody binding to $CD44^{high}$ $CD24^{low}$ tumor stem cells, which showed low HER2 expression about 40% (**Figure 2** and data not shown).

Trastuzumab and pertuzumab enhanced NK-cell-mediated killing (ADCC) of HER2-positive tumor cells

To assess the effect of trastuzumab and pertuzumab on NK cell-mediated lysis of HER2-expressing breast cancer cells, we used a modified FATAL assay [22]. Target cell lysis was determined after 4 h of co-culture with polyclonal NK cells in the presence of trastuzumab, pertuzumab, or the combination of these two HER2-specific antibodies. The ratio of tumor cells to immune cells was 1:2. At high immune to tumor cell ratios, the tumor cells were killed independently of the ADCC effect. However, low immune to tumor cell ratios come closer to physiological conditions [23].

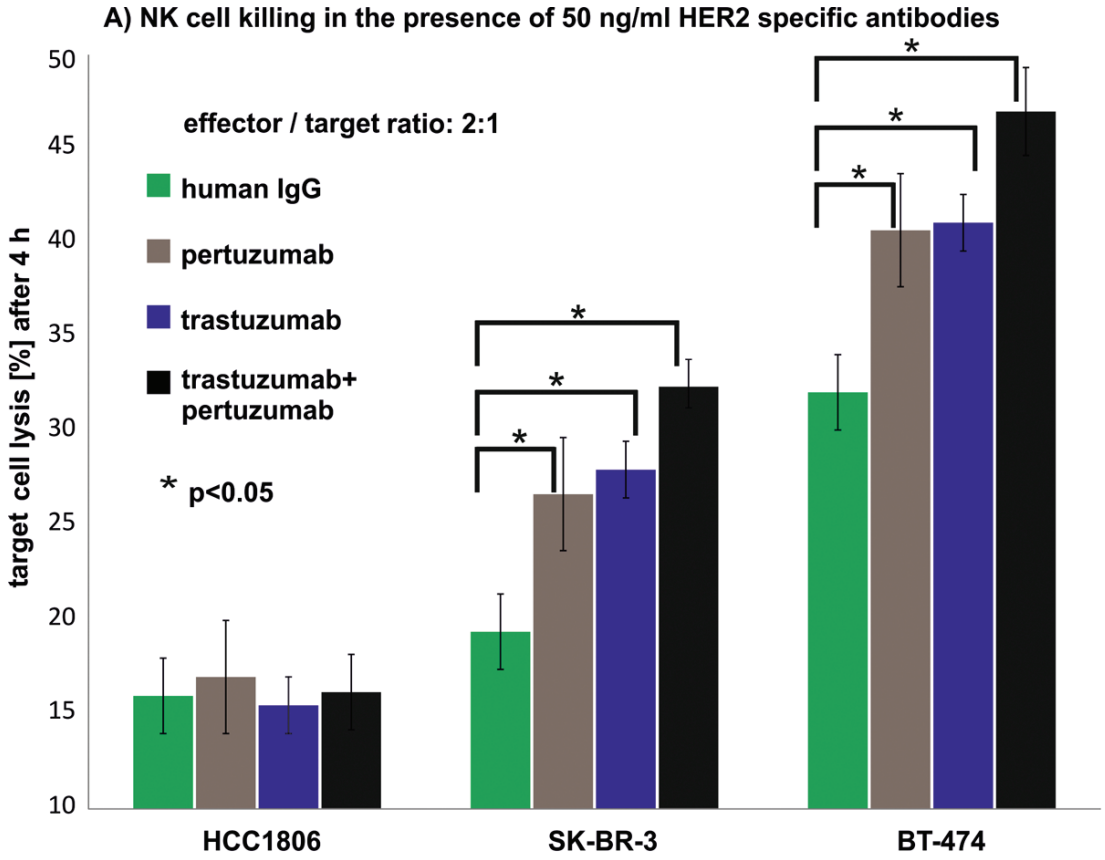
We found that both trastuzumab and pertuzumab enhanced NK-cell-mediated killing of HER2-positive tumor cells. The combined application of these HER2-specific antibodies in the killing assays significantly improved target cell lysis compared to the application of only one antibody; thus, antibody-mediated cytotoxicity was increased (**Figure 3A**). Considering the short time course of these experiments, the effects cannot be explained by growth inhibition.

Importantly, addition of HER2-specific antibodies to the killing assay augmented tumor cell lysis only in tumor cell lines that overexpressed HER2. In HER2-negative cell lines, like HCC1806 or HCC1937, immune cell-mediated lysis could not be improved by the addition of trastuzumab, pertuzumab, or their combination.

Moreover, as in a previous series of experiments (18), HER2-positive breast cancer cells surviving an ADCC challenge with NK cells and trastuzumab or pertuzumab showed an increased population of $CD44^{high}CD24^{low}HER2^{low}$ tumor stem cells. This enrichment of treatment-refractory CSCs could, however, be attenuated when the combination of both HER2-specific antibodies was employed. This suggests that the increased antibody binding to $HER2^{low}$ CSCs which is achieved by the combined use of trastuzumab and pertuzumab (see **Figure 2**) improves the immunotherapeutic targeting of these highly malignant cells during an interaction with natural killer cells (**Figure 3B**). Trastuzumab and pertuzumab without NK cells had no effect on the $CD24^{high}CD24l^{ow}$ population, (data not shown).

Trastuzumab is approved for the treatment of early-stage breast cancer and metastatic breast cancers positive for HER2. It is further approved for application in combination with chemotherapeutic drugs. Trastuzumab has significantly improved the overall survival of patients with HER2-positive breast cancer. However, even when the initial response to the drug is high, tumors can become drug-resistant or show reduced sensitivity, and patients experience relapse after treatment or disease progression. Thus, further development of HER2-specific therapeutics is essential. In particular, pertuzumab is another promising therapeutic that specifically targets the HER2 antigen [8, 24].

This study provided evidence that the combination of trastuzumab and pertuzumab increased total antibody binding to HER2-positive tumor cells *in vitro*. Thus, effects which are mediated


A) NK cell killing in the presence of 50 ng/ml HER2 specific antibodies
target cell lysis [%] after 4 h
effector / target ratio: 2:1
human IgG
pertuzumab
trastuzumab
trastuzumab+ pertuzumab
* p<0.05
HCC1806
SK-BR-3
BT-474

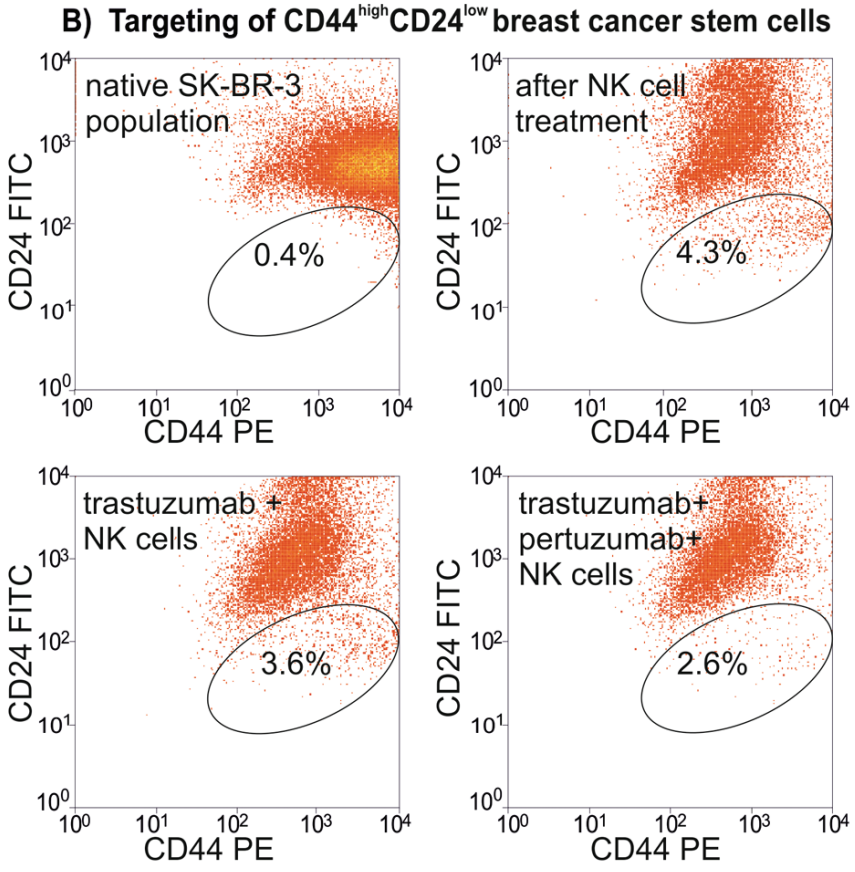

B) Targeting of CD44high CD24low breast cancer stem cells
native SK-BR-3 population
0.4%
after NK cell treatment
4.3%
trastuzumab + NK cells
3.6%
trastuzumab+ pertuzumab+ NK cells
2.6%
CD24 FITC
CD44 PE

Figure 3. Antibody-dependent NK cell killing and targeting of $CD44^{high}CD24^{low}$ cancer stem cells is increased in the combined presence of trastuzumab and pertuzumab. A. Two HER2-positive breast cancer cell lines, BT-474 and SK-BR-3, and the HER2-negative HCC1806 breast cancer cell line, were co-cultured with NK cells for 4 h, at a tumor-to NK cell ratio of 1:2. For better discrimination, immune cells were stained with eFluor® 670Cell Proliferation Dye and tumor cells were stained with CFSE. Target cell lysis of breast cancer cells (as evidenced by CFSE loss) was determined after 4 h by FACS analysis. Antibodies were used at 50ng/ml. Normal human IgGs served as control for the humanized antibodies trastuzumab and pertuzumab. Data analysis was performed using an unpaired, two-sided Student´s t-test. Results from three independent experiments were analyzed, (*$p<0.05$). B. HER2-positive SK-BR-3 breast cancer cells were incubated for 16 h with NK cells at a tumor-to NK cell ratio of 1:2. Among the surviving (i.e. 7-AAD-negative) cells, the $CD24^{high}CD24^{low}$ population was analyzed by FACS immediately after NK cells had been removed by washing. The respective percentages of $CD24^{high}CD24^{low}$ cells are indicated for the native SK-BR-3 population (upper left) and for the SK-BR-3 population after incubation with NK cells (upper right), after incubation with NK cells in the presence of 50 ng/ml trastuzumab (lower left), and after incubation with NK cells in the presence of 50 ng/ml trastuzumab and pertuzumab (lower right).

via the Fc portion of an antibody may be augmented by synergistic application of two different antibodies binding to different epitopes on the same antigen. Moreover, Fuentes *et al*. demonstrated a higher receptor affinity when both trastuzumab and pertuzumab were co-localized on HER2 which further adds to the clinical synergism achieved with the combined application of these two antibodies. In particular, the subdomains I-III of the HER2 receptor became highly plastic in the presence of trastuzumab and showed increased association with pertuzumab [17].

We propose that both inhibition of signals that regulate cell proliferation, differentiation, and apoptosis, and elevated ADCC achieved by the combined binding likely contribute to the synergy shown between trastuzumab with pertuzumab [17, 25]. Consistent with this hypothesis, the level of NK-cell-mediated ADCC was increased by adding a second HER2-specific antibody, particularly when the target cells expressed low to moderate levels of HER2 (i.e., when the available Fcγ receptors on NK cells were not saturated by the amount of anti-HER2 antibody bound to the surface of the tumor cell). Given that HER2 expression is heterogeneous within a single tumor, additional antibody binding might be clinically relevant, particularly for malignancies that do not exhibit uniformly high expression of HER2.

Moreover, although a single antibody may sufficiently liberate the full potential of ADCC effects in *in vitro* assays, antibody penetration may sometimes not be ideal in the tumor tissue. Accordingly, the use of two different antibodies with slightly different physico-chemical properties could be advantageous. Also steric constraints will often inhibit the optimal access of Fc-γ receptor-expressing cells to antibody-loaded tumor cells *in vivo* [26]. In these cases, an additional number of immunoglobulins bound to tumor cells may improve ADCC by offering additional binding sites for Fc-γ-receptors. In fact, we observed synergistic increases in ADCC with antibody concentrations as low as 50 ng/ml; hence, this effect might be highly relevant in situations where antibody penetration is sub-optimal [27].

The importance of the ADCC effect for the anti-tumor activity of trastuzumab was previously demonstrated by Clynes et al. They showed that the efficacy of trastuzumab was attenuated dramatically in knock-out mice that lacked activating FCgRIII receptors [28]. Incidentally, the importance of ADCC for the *in vivo* effect of the combination of trastuzumab and pertuzumab was supported in a recent study [29] which showed that trastuzumab efficacy was improved in mouse models by stimulating CD137 on NK cells. Finally, ADCC-mediated activation of an innate immune response results in cytokine secretion and liberation of antigens which may enable the host to mount a potent adaptive immune response against tumor antigens [15]. Therefore, the ability to strengthen the anti-tumor immune response by increasing ADCC provides a further rationale for the combined administration of two HER2-specific antibodies. Clearly, the available *in vivo* data do not reveal the precise mechanisms underlying the synergy observed with this combination of HER2-directed antibodies. Thus, it remains unclear whether a beneficial effect depends more on the inhibition of HER2-mediated signal transduction or whether the immune-mediated effect predominates. This may also be different for different tumors and for individual cancer patients with different

immune status. Still, as long as the various mechanisms all work towards the elimination of HER2-positive tumor cells, the potential to act via different modes of action only increases the chance of a favorable outcome.

Importantly, immunotherapeutic targeting of $CD44^{high}CD24^{low}HER2^{low}$ cancer stem cells was found to be poor with trastuzumab only [18] and might be improved with the combination of two antibodies. We showed that the level of antibody binding to this subpopulation of aggressive tumor cells was higher when tumor cells were incubated with trastuzumab and pertuzumab compared to binding with one antibody alone. It thus appears likely that the higher antibody load should make these cells more susceptible to immune attack.

Accordingly, the combined trastuzumab and pertuzumab treatment caused elevations in antibody binding in both differentiated tumor cells and stem cells; this observation was confirmed in different HER2-positive cell lines and in tumor cells isolated from pleural effusions of patients with metastasized HER2-positive breast cancer. The positive effect of the combined application of trastuzumab and pertuzumab was also consistent with results from the CLEOPATRA clinical phase II and III study and from the NEOSPHERE study.

In 2010, Genentech initiated a Phase III trial, called Marianne, to evaluate the combination of pertuzumab with the antibody-drug conjugate, T-DM1; T-DM1 comprises trastuzumab conjugated with the tubulin inhibitor, DM1. Based on results from the present study, a positive outcome for this trial arm appears likely.

Acknowledgments

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List of abbreviations

HER2, human epidermal growth factor receptor 2; ADCC, antibody-dependent cell-mediated cytotoxicity; NK cells, natural killer cells; EGFR, epidermal growth factor receptor.

Competing interests

The authors declare that they have no competing interests.

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