Targeting of preexisting and induced breast cancer stem cells with trastuzumab and trastuzumab emtansine (T-DM1)

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The antibody trastuzumab (Herceptin) has substantially improved overall survival for patients with aggressive HER2-positive breast cancer. However, about 70% of all treated patients will experience relapse or disease progression. This may be related to an insufficient targeting of the CD44^high^CD24^low^ breast cancer stem cell subset, which is not only highly resistant to chemotherapy and radiotherapy but also a poor target for trastuzumab due to low HER2 surface expression. Hence, we explored whether the new antibody-drug conjugate T-DM1, which consists of the potent chemotherapeutic DM1 coupled to trastuzumab, could improve the targeting of these tumor-initiating or metastasis-initiating cells. To this aim, primary HER2-overexpressing tumor cells as well as HER2-positive and HER2-negative breast cancer cell lines were treated with T-DM1, and effects on survival, colony formation, gene and protein expression as well as antibody internalization were assessed. This revealed that CD44^high^CD24^low^HER2^low^ stem cell-like breast cancer cells show high endocytic activity and are thus particularly sensitive towards the antibody-drug conjugate T-DM1. Consequently, preexisting CD44^high^CD24^low^ HER2^low^ cancer stem cells were depleted by concentrations of T-DM1 that did not affect the bulk of the tumor cells. Likewise, colony formation was efficiently suppressed. Moreover, when tumor cells were cocultured with natural killer cells, antibody-dependent cell-mediated cytotoxicity was enhanced, and EMT-mediated induction of stem cell-like properties was prevented in differentiated tumor cells. Thus our study reveals an unanticipated targeting of stem cell-like breast cancer cells by T-DM1 that may contribute to the clinical efficacy of this recently approved antibody-drug conjugate.

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The Her-2/neu (c-erbB2, HER2) proto-oncogene is a transmembrane receptor tyrosine kinase that mediates critical cellular functions like growth, differentiation and survival in malignant and normal breast epithelial cells. Homodimerization of HER2 as well as heterodimerization of HER2 with the related receptors HER1/EGFR1/ErbB1, HER3 or HER4 triggers oncogenic signaling. Overexpression of the HER2 gene is associated with an aggressive clinical phenotype, increased disease recurrence and unfavorable prognosis. Although 20–30% of breast cancers show HER2 amplification, genome-wide sequencing has revealed activating HER2 mutations as an alternative mechanism for HER2 activation in breast cancer. Moreover, cancer stem cell (CSCs) were found to overexpress HER2 even in breast cancers not classified as HER2-positive. Thus HER2-directed treatments could thus significantly improve disease-free and overall survival in the adjuvant and metastasized setting. Used as monotherapy, trastuzumab inhibits tumor cell growth. In combination with chemotherapeutics or ionizing radiation, duration of response and progression-free survival are improved.

Mechanistically, trastuzumab does not block only signal transduction via the HER2 receptor and its interaction partners. By binding to HER2-overexpressing target cells, trastuzumab also ‘labels’ tumor cells for recognition by immune effectors like natural killer (NK) cells. These bind antibodies via Fcγ-receptors, which then trigger the so-called ‘antibody-dependent cell-mediated cytotoxicity’ (ADCC). The functional relevance of NK cell recruitment for the activity of trastuzumab is supported by a clinical correlation between NK cell function and therapeutic response, by a loss of activity in Fcγ-receptor IIIA knockout animals and by an improved in vivo effect of trastuzumab upon additional NK cell stimulation. Importantly, the Fcγ-part of trastuzumab, which mediates these immune-stimulatory effects, was retained in trastuzumab emtansine (T-DM1).
In preliminary studies, we have already demonstrated the induction of NK cell–mediated ADCC by trastuzumab in vitro. Moreover, we could provide in vivo evidence that HER2-positive cells surviving an ADCC challenge with NK cells and trastuzumab preferentially show a ‘CSC-like’ phenotype.\(^\text{16}\) CSCs or tumor-initiating or metastasis-initiating cells are, in breast cancer, characterized by a CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) phenotype, expression of ganglioside GD2, aldehyde dehydrogenase 1 (ALDH), high clonogenicity, high tumorigenicity and increased metastatic potential.\(^\text{17–19}\) Current concepts suggest that CSCs often resist cytotoxic treatments and are therefore responsible for recurrence.\(^\text{20,21}\) Moreover, recent data indicate that stress stimuli,\(^\text{22}\) unsuccessful treatments\(^\text{23}\) and inflammation\(^\text{24–26}\) can trigger the conversion of (partly) differentiated cancer cells towards a CSC-like phenotype. The underlying process that is known as ‘epithelial-to-mesenchymal transition’ (EMT) enables transformation of epithelial into highly mobile mesenchymal cells, which is required for embryonic development. In cancer, however, highly mobile cells that have lost their epithelial phenotype may cause metastasis.

A promising new tool for the treatment of HER2-positive breast cancer is the antibody-drug conjugate T-DM1. It consists of the antibody trastuzumab and the potent Maytansine-derivative DM1, which inhibits cell division and induces cell death\(^\text{27}\) by blocking the spindle apparatus.\(^\text{28,29}\) Due to its high toxicity as free drug and low activity at tolerated levels, Maytansine requires a specific targeting to become applicable for antitumor therapy.\(^\text{30–33}\) DM1 was thus chemically linked to trastuzumab with a drug-to-antibody ratio of 3:5:1.\(^\text{31}\) HER2 represents an excellent target for an antibody-drug conjugate as it is highly overexpressed on HER2-positive cancer cells\(^\text{34}\) and also found on CSCs in tumors that do not show general positivity for HER2.\(^\text{8}\) After binding to HER2, T-DM1 is internalized by endocytosis and degraded in lysosomes, causing the release of the active metabolite DM1.\(^\text{35}\)

Importantly, the mechanisms of action of trastuzumab (such as ADCC and the blockade of HER2-specific signaling) are preserved in the T-DM1 conjugate, but the targeted delivery of a highly toxic chemotherapeutic to HER2-positive tumor cells provides an additional benefit that has already yielded impressive clinical results in the first studies.\(^\text{36}\) In this set of experiments, we now investigated whether the new HER2-specific antibody-drug conjugate T-DM1 could also improve the targeting of CSCs.

**Results**

**T-DM1 induces dose- and time-dependent cell death in HER2-positive tumor cells.** To quantify the surface expression of HER2, six breast cancer cell lines (BT-474, SK-BR-3, MCF-7, MDA-MB-231, HCC1806 and HCC1937) were either enzymatically detached with Accutase or mechanically scraped before being stained with trastuzumab followed by a Cy5-conjugated detection antibody. Irrespective of the mode of harvesting, quantification by flow cytometry (Figure 1a) confirmed high levels of HER2 on BT474 and SK-BR-3 cells (commonly classified as HER2\(^{\text{++}}\)) and revealed significant HER2 surface expression on MCF-7 and MDA-MB-231 cells, whereas HCC1806 and HCC1937 cells were negative for HER2. Considering that the common description of MCF-7 and MDA-MB-231 as HER2\(^{\text{++}}\) is based on analysis by immunohistochemistry (compare Table 1), we presume that flow cytometric analysis is more sensitive. In fact, several previous studies could also show that HER2 represents a possible target in MCF-7 cells.\(^\text{6,38}\)

Next, the sensitivity of HER2\(^{\text{high}}\) (SK-BR-3 and BT474), HER2\(^{\text{+}}\) (MCF-7 and MDA-MB-231) and HER2\(^{\text{low}}\) (HCC1806 and HCC1937) breast cancer cell lines towards T-DM1 was tested over 96 h. Light microscopy showed that dead cells round off and detach. Cell density could thus be determined by crystal violet staining and used as a surrogate parameter for cell death (Figure 1b). Data were confirmed by the metabolic WST-1 assay (not shown). Finally, the effect of HER2-specific therapeutics on breast cancer cells was assessed by cell cycle analysis. After staining of the cellular DNA with propidium iodide, cell cycle arrest is typically indicated by an increase of cells in G\(_0\)/G\(_1\) or G2/M-phase, and cell death is evidenced by the appearance of a sub-G\(_0\) fraction (Figure 1c). In all three assays, T-DM1 dose-dependently induced cell death in HER2-expressing breast cancer cells but not in the HER2-negative triple-negative breast cancer cell lines HCC1806 and HCC1937. Likewise, we could not detect any significant effect of T-DM1 on primary lymphocytes, which are HER2-negative (data not shown). This confirmed that cytotoxic effects of T-DM1 are HER2 dependent. Trastuzumab, in contrast, had hardly any influence on cell cycle distribution or cell density in our assays (Figure 1c and data not shown).

**T-DM1 efficiently suppresses clonogenicity of breast cancer cells, even though HER2 surface expression is low on the CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) CSC subset.** Rather than acute cytotoxicity, colony formation is often a better in vitro test for the efficacy of anti-cancer drugs.\(^\text{39}\) Following 48 h of incubation with T-DM1, HER2-positive breast cancer cell lines like SK-BR-3 and BT-474 dose-dependently lost their clonogenic potential (Figure 2a), which suggests a selective depletion of clonogenic cells and thus a
HER2 surface expression on CD44-highCD24-low cells was also shown lower HER2 surface expression on the CD44 high marker for CSCs. Our own previous findings, however, had ranged from 5% to 26% can be used as a surrogate effect of T-DM1. Again, HER2 positivity was a prerequisite for a discernible genic cells rather than the bulk of the tumor cell population.

These values show that T-DM1 preferentially targets clono-

all 6 cell lines (Figure 2c). The striking differences between compared EC50 values for clonogenicity and cytotoxicity for the CD44-highCD24-low subpopulation failed that precluded their use in this assay.

As T-DM1 concentrations \( \leq 100 \text{ng/ml} \) almost abrogated clonogenicity in SK-BR-3, BT-474 and MCF-7 cells, we compared EC50 values for clonogenicity and cytotoxicity for all 6 cell lines (Figure 2c). The striking differences between these values show that T-DM1 preferentially targets clono-

genic cells rather than the bulk of the tumor cell population. Again, HER2 positivity was a prerequisite for a discernible effect of T-DM1.

Importantly, clonogenicity (which, depending on the cell line, ranged from 5% to 26%) can be used as a surrogate marker for CSCs. Our own previous findings, however, had shown lower HER2 surface expression on the CD44-highCD24-low presumed stem-cell-like subset than on their more differentiated counterparts.41 In line with this observation, stem-cell-like CD44-highCD24-low HER2-low cells escaped from targeting with trastuzumab and NK cells.16 The reduced HER2 surface expression on CD44-highCD24-low cells was also now confirmed with primary tumor cells obtained from pleural effusions of five patients with metastasized HER2-positive breast cancer (Figure 2d and data not shown).

Effect of T-DM1 in the presence of NK cells. The therapeutic effect of anti-HER2 antibody treatment was found to correlate with the recruitment of NK cells in to the tumor microenvironment15 and to depend on both innate and adaptive immunity.42 One important mechanism is ADCC,16 which we found to be similar for trastuzumab and T-DM1 (Figure 3a). However, recruitment of immune cells into a tumor and inflammation can also promote EMT, which leads to dedifferentiation of cancer cells towards a CSC-like phenotype.24-26 In fact, coculture of MCF-7 breast cancer cells with NK cells did not induce morphological changes toward an elongated fibroblast-like shape,43,44 thereby indicating the transition from an epithelial to a mesenchymal phenotype (Figure 3b). Cell surface expression of the epithelial adherence factor E-Cadherin also decreased (Figure 3c). Western blotting revealed the induction of ji-Catenin, Vimentin and (non-junctional) Claudin-145 (Figure 3d), which have been described during EMT. The characteristic transcription factors for EMT, Twist, Snail and Slug could, unfortunately, not be visualized by immuno-

blotting that may be due to their low and transient expression in MCF-7 cells. However, qRT-PCR analysis showed an induction of these factors on mRNA level that could be prevented by T-DM1 (Figure 3e). Accordingly, immune-

mediated induction of EMT could be prevented by T-DM1.

Effect of T-DM1 on induced breast CSCs. When T cells or NK cells induce EMT in breast cancer cells, induction of CSC-like properties can be the unwanted consequence. In line with these findings, coculture with NK cells increased the proportion of CD44-highCD24-low cells in the MCF-7 cell line (Figure 4a). As an additional control, use of the ALDEFLUOR reagent system19 confirmed both stem cell induction in coculture with NK cells and the prevention of this effect by T-DM1 (Figure 4b). The enrichment of cells with a CSC signature was reflected by enhanced clonogenicity (Figure 4c).

Both these effects could, again, be prevented by addition of T-DM1 to the cocultures, whereas trastuzumab showed no such effect (Figures 4a–c and data not shown). Similar data were also obtained in other cell lines (shown for SK-BR-3 in Figures 4d and e). Accordingly, T-DM1 seems to be able to block the immune-mediated induction of tumor stem cells.

Maintenance of the CD44-highCD24-low breast CSC phenotype requires autophagy. Our data show that the HER2-specific antibody-drug conjugate T-DM1 exerts strong effects on preexisting (Figure 2b) and induced (Figure 4a) CD44-highCD24-low breast cancer CSCs, even though HER2 surface levels are low on this target cell population (Figure 2d). This apparent contradiction might be resolved by considering the high level of autophagy exerted by these cells. In fact,
Autophagy was reported to be critical for the tumorigenicity of breast cancer stem-like/progenitor cells \(^{46-48}\) and found to positively regulate the CD44\(^{\text{high}}\)CD24\(^{\text{low}}\) phenotype.\(^{49}\) In order to exclude artifacts depending on side effects of autophagy inhibitors, we used three different substances (3-methyl adenine, chloroquine and artesunate), which all
Figure 3  During coculture with NK cells, T-DM1 can induce ADCC in breast cancer cells and inhibit EMT. (a) HCC1806, SK-BR-3 or MCF-7 breast cancer cells were cocultured 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 670 Cell 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. Normal human immunoglobulins G (IgGs) served as a control for the humanized antibody trastuzumab and its conjugate T-DM1 (all used at 50 ng/ml). Data analysis was performed using an unpaired, two-sided Student’s t-test. Results from three independent experiments were analyzed (*P < 0.05, **P < 0.01). (b) MCF-7 breast cancer cells were photographed before (left panel) and after (right panel) 16 h coculture with NK cells to visualize the changes regarding cellular morphology from the epithelial to a fibroblastic shape. (c) Flow cytometry was used to determine E-cadherin surface expression on MCF-7 breast cancer cells before and after 24 h of coculture with NK cells. Although the binding of a PerCP/Cy5.5-labeled isotype control of irrelevant specificity remained unaltered after coculture (green curve), the signals obtained with a similarly labeled anti-E-Cadherin antibody (clone 67A4) before and after coculture are shown in blue (before) and red (after), respectively. SFI values are indicated. (d) MCF-7 breast cancer cells were incubated for 24 h with NK cells, using a tumor cell-to-NK cell ratio of 1:2. After removal of non-adherent (i.e., NK) cells, protein lysates were prepared from the remaining adherent MCF-7 cells and tested for expression of β-catenin, vimentin and claudin by western blotting. β-Actin was used as a loading control. (e) Cocultures were set up as in panel (d). Where indicated, T-DM1 was added at a concentration of 50 ng/ml. After 16 h, NK cells were removed by washing, and RNA was extracted from the remaining adherent MCF-7 cells. The regulation of EMT markers was analyzed by Sybr-Green-based quantitative real-time PCR, using 18S rRNA as internal control.
caused an accumulation of the macroautophagy substrates LC3-II and p62 in MCF-7 cells. Quantification of the obtained western blotting data showed the strongest effect with chloroquine and a somewhat weaker efficiency for 3-methyladenine (Figure 5a). Consequently, 3-methyladenine,50 chloroquine51 and artesunate52 all reduced the size of the CD44highCD24low population (Figure 5b) and the clonogenicity of MCF-7 cells (Figure 5c), up to a complete loss of clonogenicity and of CD44highCD24low cells in the absence of acute cytotoxicity. Using artesunate, similar data were also obtained with primary breast cancer cells from pleural effusions (Figure 5d).

CD44highCD24low breast CSCs show increased autophagy leading to enhanced internalization of HER2 from the cell surface. Interestingly, it has already been hypothesized that autophagy might facilitate resistance against the small molecule HER2 receptor kinase inhibitor Lapatinib in HER2-positive breast cancer cells.53 Thus, we wondered whether the low HER2 surface levels on CD44highCD24low...
Cells might be due to increased internalization of the receptor, which is a phenomenon known to occur in response to receptor activation. In line with this hypothesis, we observed a loss of the CD24<sup>low</sup>HER2<sup>low</sup> subpopulation when autophagy was inhibited. HER2 expression on CD24<sup>high</sup> cells, however, remained unaltered (data not shown). We thus assessed the internalization of the HER2 surface antigen in CD44<sup>high</sup>CD24<sup>high</sup> and CD44<sup>high</sup>CD24<sup>low</sup> cells using trastuzumab labeled with the fluorogenic dye pHrodo. This pH-dependent dye experiences a steep increase in fluorescence upon acidification. As clathrin-coated or other vesicles used for endocytosis are characterized by a low pH, this provided us with a tool to visualize internalization of the conjugated anti-HER2 antibody. Co-staining of trastuzumab-pHrodo-treated cells after 6 h confirmed that >50% of the CD44<sup>high</sup>CD24<sup>low</sup> cells, but only <2% of the more differentiated CD44<sup>high</sup>CD24<sup>high</sup> cells, internalized pHrodo-labeled trastuzumab. Internalization could be prevented by addition of artesunate (Figure 6a). Irrespective of the tumor cell subset, no internalization was observed.

**Figure 5** Enhanced autophagy is an essential requirement for clonogenic CD44<sup>high</sup>CD24<sup>low</sup> stem cell-like breast cancer cells. (a-c) MCF-7 breast cancer cells were incubated with the autophagy inhibitors 3-methyladenine, chloroquine and artesunate for 24 h. (a) Cell lysates were prepared from untreated and treated MCF-7 breast cancer cells and assayed for the expression of LC3-II and p62. β-Actin served as a loading control. Signals were quantified using Image J (U.S. National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/), normalized to β-actin and the relative increase in non-degraded LC3-II and p62 was calculated. (b) The size of the CD44<sup>high</sup>CD24<sup>low</sup> CSC population was analyzed by flow cytometry before and after treatment. (c) MCF-7 breast cancer cells were treated with different concentrations of 3-methyladenine, chloroquine and artesunate for 24 h. Thereafter, the ability of viable cells to form new colonies was analyzed as in Figure 2b (*P < 0.05, **P < 0.01). (d) Primary tumor cells obtained from a pleural effusion of a patient with metastasized HER2-positive breast cancer, treated with different concentrations of artesunate and analyzed by flow cytometry for the presence of a CD44<sup>high</sup>CD24<sup>low</sup> CSC population.
To further confirm that maintenance of the CD44 high CD24 low CSC-like state and internalization of pHrodo-labeled trastuzumab by these cells depends on autophagy, we sought to inhibit autophagy by siRNAs against Beclin and LC3 (Figure 6b). Although downregulation of these essential mediators of autophagy was incomplete, siRNA treatment was still sufficient to reduce the extent of the CD44 high CD24 low population and the percentage of pHrodo-positive cells (Figure 6c). The fact that the remaining CD44 high CD24 low cells showed a hardly reduced uptake of pHrodo might suggest that the loss of the CD24 low signature precedes or coincides with the reduced level of internalization.

Figure 6 Internalization by increased autophagy represents an ‘Achilles’ heel’ of CSCs with regard to T-DM1. (a) The HER2-specific antibody trastuzumab was labeled with the fluorogenic dye pHrodo. pHrodo shows a strong fluorescence in the FL-2H channel once the pH of its surroundings becomes more acidic, for example, upon internalization in endocytotic vesicles. Shown is the fluorescent signal from internalized trastuzumab-pHrodo against CD24 in CD44 high cells, with CD44 high CD24 low cells representing the stem cell-like cancer cell population. Upper left: control MCF-7 cells, upper right: MCF-7 cells were incubated with pHrodo-labeled immunoglobulin G1 (IgG1) isotype control antibodies and counter-stained with anti-CD24-FITC. Lower left: MCF-7 cells were incubated with pHrodo-labeled trastuzumab and stained with anti-CD24-FITC. Lower right: MCF-7 cells were incubated with pHrodo-labeled trastuzumab in the presence of the autophagy inhibitor artesunate (20 μg/ml) and stained with anti-CD24-FITC. Bar chart: Under the various applied conditions, the respective percentage of trastuzumab-pHrodo-internalizing cells was determined and evaluated separately for the CD24 high and CD24 low subsets. (b) Using TransIT TKO transfection reagent, MCF-7 breast cancer cells were transfected with 25 nM of siRNA against Beclin (BECN1 no. s16537, Ambion) or LC3 (no. MAP1LC3A, Ambion) or with a universal negative siRNA control (Sigma). Forty-eight hours later, cells were lysed and the expression of Beclin, LC3 and β-actin was assayed in the various groups by western blotting. (c) MCF-7 cells were transfected with siRNA as in panel (b) before being incubated with trastuzumab-pHrødøa in panel (a). Staining for CD44 and CD24 followed by flow cytometric analysis enabled the quantification of CD44 high CD24 low cells, of trastuzumab-internalising pHrødø cells and of pHrødø CD24 low cells shown in panel (c)
Importantly, as pHrodo and emtansine are similarly small in size, and as they are both coupled to the same large antibody, it may be assumed that T-DM1 will show a similar behavior as this fluorogenic conjugate. We thus propose that CD44\textsuperscript{high}CD24\textsuperscript{low} breast cancer CSCs show a high level of internalization with regard to HER2 and thus evade effector mechanisms, such as ADCC. However, by this same property these cells will preferentially internalize antibody-drug conjugates. This can not only explain the highly efficient targeting of clonogenic CD44\textsuperscript{high}CD24\textsuperscript{low} cells by T-DM1 but also provide a more general rationale for the targeting of breast cancer CSCs with antibody-drug conjugates against HER2 or other oncogenic growth factor receptors.

Discussion

The HER2-specific antibody trastuzumab\textsuperscript{10} has significantly increased overall survival in patients suffering from HER2-positive breast cancer. Moreover, a recent study has also revealed a role for HER2 in the CSC subset of cell lines (including MCF-7) which are not commonly classified as HER2 overexpressing.\textsuperscript{6} Likewise, synergy between trastuzumab and paclitaxel or docetaxel had also been reported in such cell lines.\textsuperscript{38} Accordingly, more patients than previously anticipated might benefit from treatment with anti-HER2 antibodies. Unfortunately, however, the majority of patients treated with trastuzumab experience relapse at some time after treatment. Thus, a better understanding of the mechanism(s) underlying this resistance might pave the way towards more effective strategies. In our previous studies, we could demonstrate \textit{in vitro} that the binding of trastuzumab ‘labels’ HER2-positive breast cancer cells for Fc\gamma-dependent recognition by NK cells,\textsuperscript{16} thereby inducing ADCC.\textsuperscript{12,13} This important immune-stimulatory effect has been preserved in the antibody-drug conjugate T-DM1, which has retained the Fc\gamma-part of trastuzumab (Figure 3a). Also inhibition of HER2 signaling, as evidenced by reduced activation of the phosphatidylinositol 3’-kinase-AKT pathway, is similarly achieved by both trastuzumab and T-DM1 (data not shown). However, T-DM1 combines the mechanisms of action of trastuzumab with the additional specific application of the potent chemotherapeutic DM1\textsuperscript{31,32}.

Unlike trastuzumab, T-DM1 thus induced cell cycle arrest and significant cell death in HER2-positive tumor cells that is likely due to the toxin DM1 contained in T-DM1. EC\textsubscript{50} values over 48 h were mostly >10 \mu g/ml (Figures 1b and c), which might be hard to achieve in patients. Using clinically relevant concentrations (3 \mu g/ml)\textsuperscript{54} of T-DM1, a significant proportion of target cells survived \textit{in vitro} that could point towards chemoresistance. However, HER2-positive tumor cells surviving a treatment with T-DM1 displayed drastically reduced clonogenicity (Figures 2a–c), even at T-DM1 concentrations as low as 50 ng/ml. Likewise, T-DM1 incubation almost abrogated the clonogenicity of sorted CD44\textsuperscript{high}CD24\textsuperscript{low} cells, whereas trastuzumab treatment showed no comparable effect on this highly tumorigenic and poorly immunogenic subset (Figure 2b). This suggests that T-DM1 is far more effective against CSC than trastuzumab. In fact, the major limitation encountered in our previous study was the insufficient targeting of CD44\textsuperscript{high}CD24\textsuperscript{low}/HER2\textsuperscript{low} cells either with trastuzumab alone or in conjunction with NK cells.\textsuperscript{16} Based on the evidence that this subset possesses characteristics of the so-called ‘tumor-initiating’ or ‘metastasis-initiating’ or ‘CSCs’,\textsuperscript{17} a lack of activity against this subpopulation may explain the high rate of recurrence after treatment.

CSCs, however, constitute a rather dynamic subset. We and others have observed\textsuperscript{26} that ‘unproductive’ (i.e., non-lytic) interactions between immune and tumor cells induce EMT in breast cancer cells, which reprograms previously differentiated cancer cells towards a CSC-like phenotype.\textsuperscript{22} Accordingly, tumor cell cultures that had interacted with NK cells showed morphological changes towards a mesenchymal phenotype (Figure 3b), downregulation of the epithelial cell adhesion molecule E-Cadherin (Figure 3c), upregulation of EMT markers (Figures 3d and e), more CD44\textsuperscript{high}CD24\textsuperscript{low} (Figures 4a and d) and ALDH\textsuperscript{+} (Figure 4b) cells and enhanced clonogenicity (Figures 4c and e). Addition of T-DM1 to the coculture, however, prevented this expansion of the CSC population (Figures 3e and 4). This left us with two possible explanations: either tumor cells that had survived the treatment were ‘sufficiently damaged’ to lose all stem cell-like properties or the antibody-drug conjugate might preferentially target highly clonogenic ‘CSCs’ - in spite of their low HER2 surface expression (Figure 2d). This prompted us to investigate the possibility of enhanced internalization by CSCs. In fact, the CSC phenotype has repeatedly been linked to autophagy,\textsuperscript{49,55,56} a tightly regulated catabolic process involving the degradation of a cell’s own components through the lysosomal machinery. On the one hand, autophagy helps to keep up a balance between degradation, synthesis and recycling of cellular products and allows stressed or starving cells to recycle nutrients from unnecessary processes to more essential metabolic tasks. On the other hand, autophagy leads to a constant recycling of cellular components and thus enables longevity\textsuperscript{57} and maintenance of a stem cell-like phenotype not only in hematopoietic\textsuperscript{58} but also in breast cancer CSCs.\textsuperscript{48} In line with these reports, autophagy inhibitors (chloroquine, 3’-methyladenine or arszenate)\textsuperscript{51,59} abrogated CD44\textsuperscript{high}CD24\textsuperscript{low}/HER2\textsuperscript{low} cells and clonogenicity in primary breast cancer cells from pleural effusions and in breast cancer cell lines (Figures 5b and d). Most importantly, however, we measured internalization of trastuzumab by covalently coupling the fluorogenic dye pHrodo to the antibody. This revealed a preferential internalization of the conjugated anti-HER2 antibody by CD44\textsuperscript{high}CD24\textsuperscript{low} cells that could be abrogated by inhibiting autophagy (Figure 6a). Unspecific binding via the pHrodo moiety or the human IgG backbone was excluded by use of the pHrodo-labeled humanized anti-CD20 antibody rituximab. The link between autophagy, CD44\textsuperscript{high}CD24\textsuperscript{low} CSC and internalization of labeled trastuzumab was further confirmed by siRNA-mediated downregulation of LC3 or Beclin (Figure 6b), which resulted in a significantly smaller CD44\textsuperscript{high}CD24\textsuperscript{low} CSC subset and in reduced internalization of pHrodo-labeled trastuzumab. Interestingly, those cells that retained the CD24\textsuperscript{low} phenotype also showed unaltered internalization. We thus propose that the low HER2 (and, most likely also the low CD24) surface expression on CD44\textsuperscript{high}CD24\textsuperscript{low} cells is mainly due to internalization via autophagy. This, once again,
confirms that autophagy enables constant renewal of these cells and may be protective against most therapeutic insults, including immune-mediated destruction via ADCC. However, when it comes to antibody-drug conjugates, this mechanism constitutes an 'Achilles' heel' that may be therapeutically exploited by T-DM1. Considering that the targeting of these cells is thought to be essential for a successful cancer therapy, our in vitro data thus suggest that T-DM1 holds great promise for future treatment of early and metastatic HER2-positive breast cancer. In fact, first results from the EMILIA trial, a phase III study in which 991 women with locally advanced or metastatic, unresectable, trastuzumab- and taxane-treated, HER2-positive breast cancer were enrolled showed longer progression-free survival (9.6 versus 6.4 months) and a superior safety profile for T-DM1 against the combination of capecitabine with lapatinib.60

Further trials (MARIANNE, TH3RESA) are ongoing. From a mechanistic point of view, it is interesting to note that a tubulin inhibitor can prevent the unwanted induction of tumor stem cell characteristics. In line with this notion, pretreatment of cancer cells with paclitaxel (Taxol) likewise prevented the induction of EMT and of stem cell-like features (data not shown). Thus, tubulin-dependent processes seem to have a seminal role for CSC induction. It may thus be speculated whether the induced CD44highCD24low cells actually originate from more differentiated tumor cells via (induced) asymmetric cell divisions. In any case, these data show that the coupling of a potent inhibitor of the mitotic spindle to trastuzumab was a good choice for T-DM1. Moreover, the combination of the anti-proliferative and immune-activating effect of trastuzumab at the cell surface with intracellular delivery of a potent toxin upon endocytosis could serve as a template for further antibody-drug conjugates. Such novel therapeutics might not only be well tolerated but also be particularly effective against CSCs with their high internalization rate.

Materials and Methods

Cell culture and reagents. MCF-7, MDA-MB-231, BT-474, HCC1937, HCC1806 and SK-Br-3 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), authenticated and cultured as indicated by the supplier. Primary tumor cells were obtained from pleural effusions of patients undergoing puncture for the palliative treatment of metastasized breast cancer at our institution. 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-free, D-valine-containing Dulbecco's Modified Eagles Medium supplemented with 2% heat-inactivated FCS (Biochrome, Berlin, Germany), penicillin (100 U/ml) and streptomycin (100 U/ml) (all from PAA, Cölbe, Germany). Non-adherent cells were removed by washing after 24 h. Fibroblast growth was suppressed due to the lack of L-valine. Autophagy inhibitors (3-methyl adenine, artesunate and chloroquine) were obtained from Sigma (Deisenhofen, Germany).

Cytotoxicity and cell cycle assays. Breast cancer cells were treated with different concentrations of T-DM1 and trastuzumab as indicated. Cell density after drug treatment was assessed by crystal violet staining. As this assay is based on the quantification of adherent cells, microscopy was used to confirm that dying cells became round and detached. As an additional control, the metabolic WST-1 assay (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. For cell cycle analysis, cells were detached, fixed and permeabilized for at least 1 h in ice-cold 70% ethanol and washed with PBS before their DNA was stained with propidium iodide (PI). RNA was simultaneously digested with DNase-free RNase A (Carl Roth, Karlsruhe, Germany). PI intercalates into DNA with no sequence preference and thus enables the assessment of the cellular DNA content in the FL-2A channel of the FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Doublets were excluded via the FL-2W channel. Cells containing less than a complete set of chromosomes (i.e., 2n) are considered to be dead.

Clonogenicity assays. Clonogenicity assays are in vitro cell survival assays based on the ability of single cells to establish a new colony. The assay essentially investigates the proportion of cells that can undergo 'unlimited' division. Breast cancer cells were treated as indicated before being seeded at low density on low-attachment plastic material (Greiner BioOne, Frickenhausen, Germany). After 2–3 weeks, colonies were fixed, stained with crystal violet (0.5% w/v) and counted. Stained colonies consisting of at least 50 cells were visible without the use of a microscope.

Flow cytometric analysis of surface expression levels and cell sorting. Cells were either mechanically or enzymatically detached using Accutase (PAA), blocked and stained with 10 μg/ml Trastuzumab or 10 μg/ml T-DM1 (Genentech, Burlingame, CA, USA) followed by a Cy5-conjugated goat anti-human IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) detection antibody. Simultaneously, CD44-RPE (Clone 2B7, BioLegend, San Diego, CA, USA), CD24-FITC (clone SWA-11, kindly provided by Professor Peter Altevogt, German Cancer Research Centre, Heidelberg, Germany) and the life stain 7-aminactinomycin D (Sigma) were applied and analyzed on a FACSCalibur or an Attune flow cytometer (Life Technologies, Darmstadt, Germany). For E-Cadherin surface staining, a PerCP/Cy5.5-labeled anti-human CD334/E-Cadherin antibody (Clone 67A4) and a corresponding isotype control (Clone MOPC-21), both from BioLegend, were used. 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, the stained cells were separated twice on a Digital FACSvantage (BD Biosciences), first in yield and then in purity mode.

ALDH assay. The ALDEFLUOR assay (STEMCELL Technologies, Grenoble, France) enables the identification of stem and progenitor cells based on their high expression of ALDH1. This enzyme converts the fluorogenic substrate BODIPY-aminocadetehyde into a green fluorescent dye that can be visualized by flow cytometry. As the ALDEFLUOR reaction product will leak out of dead cells, only viable cells are identifiable by this assay. Transporter-mediated exclusion of the dye from stem cells is prevented by multi-drug reporter inhibition with a verapamil-containing inhibitor contained in the kit. Tumor cells were treated with different concentrations of drugs for variable intervals of time. Afterwards, the ALDEFLUOR test was conducted according to the manufacturer's instructions.

NK cell preparation and cytotoxicity assays. Peripheral blood lymphocytes were obtained from healthy volunteers by density gradient centrifugation (Biocoll, Biochrom) and cultured for 8–11 days on irradiated (30 Gy) RPMI8866 feeder cells to obtain polyclonal NK cell populations (30). NK cell-mediated lysis of MCF-7 or MDA-MB-231 cells was assessed by modified fluorometric assessment of T lymphocyte antigen specific lysis assays.41 NK cells were thus labeled with eFluor 670 Cell Proliferation Dye (ebioscience, Frankfurt, Germany) while target cells (2 × 10^5/well) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Karlsruhe, Germany). Cocultures were set up using different effector:target ratios, and lytic activity was assessed after 16 h by flow cytometric detection of CFSE^− cells among the eFluor 670-negative target cell population. Values were corrected for spontaneous leakage of CFSE.

Immunoblot analysis. Regulation of EMT-related proteins in MCF-7 cells that had been cocultured with NK cells was analyzed by immunoblot. In short, NK cells were removed by washing. Adherent cells were detached, collected by centrifugation, washed with PBS and lysed in protein lysis buffer containing 50 mM Tris-HCl (tris(hydroxymethyl)aminomethane-hydrochloric acid) pH 8.0 (Sigma), 120 mM sodium chloride, 5 mM EDTA, 2 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM PMSF, 10 mM sodium fluoride (all purchased from Carl Roth, Karlsruhe, Germany), 0.5% Nonidet P-40 and 1 mM sodium orthovanadate (both from Applichem GmbH, Darmstadt, Germany). Proteins were separated on 8–15% polyacrylamide (Carl Roth) gels under reducing conditions, transferred to

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In all, 2\times10^5 breast cancer cells, pHrodo-labeled humanized anti-CD20 antibody Rituximab acidic, for example, upon internalization in endocytotic vesicles. With respect to HER2-specific antibodies were labeled with the fluorogenic 7-AAD. Analysis were performed as described above. Western blotting. Incubation with trastuzumab-pHrodo and subsequent flow cytometric analysis were performed using an unpaired, two-sided Student’s t test.


gene expression, using 10\mu M of Tris-HCL (pH 8.6) with 1.1 mg/ml para-hydroxycoumaric acid (Sigma-Aldrich, Taufkirchen, Germany) and 0.03% hydrogen peroxide (Carl Roth) at a t = 0: 1:0.003 ratio.

qRT-PCR. Total RNA was prepared using TRIFast (Pertlab, Erlangen, Germany) and transcribed with the (Script) cDNA Synthesis kit (Bio-Rad, Munich, Germany). For real-time PCR, cDNA amplification was monitored using the ABSolute QPCR SYBR Green Low Rox mix (ABGene, Epsom, UK) on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). To this aim, 40 cycles of a two-step PCR protocol (95°C for 15s, 60°C for 1 min) were performed. The following primers were used:

18s up: 5'-GGGGTACCATGCAAGAAG-3'; 18s down: 5'-GCTGGAAT TACCGCGGCT-3'; Snail down: 5'-CTGTCACCAACACCAAGAGTC-3'; Snail up: 5'-CCAGCTGCCCTCCAC-3'; Qki-actin down: 5'-AGACGCTGTTGGGCTAGC-3'; Slug up: 5'-GGGGAGAGCCCTTTTGTGGA-3'; Vimentin up: 5'-GAGATAAGTCGCTTGTAAC-3'; Vimentin down: 5'-GCTCTTGTAAGTGTCGACT-3'; Twist down: 5'-GAGCTGGCAGCTTTAAGG-3'; E-Cadherin up: 5'-CCGATGGTGGTGAATG-3'; and E-Cadherin down: 5'-ACAGTAGGCACCATAC-3'. Data analysis was done using the ΔΔCt method for relative quantification. Dissociation curves confirmed the presence of a single specific PCR product.

pHrodo assay. HER2-specific antibodies were labeled with the fluorogenic dye pHrodo (Life Technologies, INVITROGEN CORPORATION, Burlington, ON, Canada) according to the manufacturer’s instructions. pHrodo shows increased fluorescence in the FL-2 channel as the pH of its surroundings becomes more acidic, for example, upon internalization in endocytic vesicles. With respect to breast cancer cells, pHrodo-labeled humanized anti-CD20 antibody Rituximab (Rituxan) was used as a non-binding control.

siRNA transfection. In all, 2x10^5 MCF-7 cells were seeded in a six-well plate. Twenty-four hours later, they were transfected with 25 nM of siRNA against D21H3, anti-human pAKT (clone Ser473), and anti-human AKT (no. 9272). Antibodies against p62 (Poly6477) and against Catenin (Cell Signaling) and homemade enhanced chemiluminescence (ECL) solution.

For real-time PCR, cDNA amplification was monitored using the ABsolute QPCR SYBR Green Low Rox mix (ABGene, Epsom, UK) on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). Analysis of significance was performed using an unpaired, two-sided Student’s t test.

Statistics. Experiments were performed at least three times with similar results, and representative experiments are shown. S.D.s. for flow cytometry data were calculated by using the Summit software (Beckman Coulter, Krefeld, Germany). Analysis of significance was performed using an unpaired, two-sided Student’s t test.

Conflict of Interest
AH participated as a consultant in advisory boards for Roche and received honoraria from Roche. The remaining authors declare no conflict of interest.

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