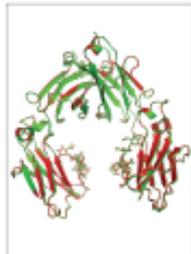


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The level of HER2 expression is a predictor of antibody-HER2 trafficking behavior in cancer cells

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The level of HER2 expression is a predictor of antibody-HER2 trafficking behavior in cancer cells

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Keywords: HER2 degradation, intracellular trafficking

Abbreviations: ADCs, Antibody drug conjugates; ADCC, antibody dependent cell-mediated cytotoxicity; ADCP, antibody dependent cell-mediated phagocytosis.

The receptor tyrosine kinase HER2 is known to play a central role in mitogenic signaling, motivating the development of targeted, HER2-specific therapies. However, despite the longstanding use of antibodies to target HER2, controversies remain concerning antibody/HER2 trafficking behavior in cancer cells. Understanding this behavior has direct relevance to the mechanism of action and effective design of such antibodies. In the current study, we analyzed the intracellular dynamics of trastuzumab, a marketed HER2-targeting antibody, in a panel of breast and prostate cancer cell lines that have a wide range of HER2 expression levels. Our results reveal distinct post-endocytic trafficking behavior of antibody-HER2 complexes in cells with different HER2 expression levels. In particular, HER2-overexpressing cells exhibit efficient HER2 recycling and limited reductions in HER2 levels upon antibody treatment, and consequently display a high level of antibody persistence on their plasma membrane. By contrast, in cells with low HER2 expression, trastuzumab treatment results in rapid antibody clearance from the plasma membrane combined with substantial decreases in HER2 levels and undetectable levels of recycling. A cell line with intermediate levels of HER2 expression exhibits both antibody recycling and clearance from the cell surface. Significantly, these analyses demonstrate that HER2 expression levels, rather than cell origin (breast or prostate), is a determinant of subcellular trafficking properties. Such studies have relevance to optimizing the design of antibodies to target HER2.

Introduction

In breast cancer, overexpression of the receptor tyrosine kinase (RTK) HER2 is observed in 20–30% of patients and is associated with poor prognosis.¹ Monoclonal antibodies such as trastuzumab represent a promising treatment option as they have been shown to be beneficial in a subset of HER2^{hi} breast cancer patients. However, despite considerable interest in the targeting of HER2 with antibodies, there is uncertainty concerning the intracellular trafficking itinerary of trastuzumab and its HER2 target. Understanding these pathways is of direct relevance to elucidating mechanistic aspects of antibody-based HER2-specific therapies.

While a subset of studies report that trastuzumab remains on the cell surface and does not internalize following interaction with HER2,^{2,3} others claim that trastuzumab internalizes^{4,5} and subsequently traffics back to the plasma membrane.⁴ A related unanswered question concerns antibody-induced HER2 degradation; conflicting reports indicate HER2 degradation^{6–9} or a lack

thereof.^{2,4} To further confound these issues, how anti-HER2 antibodies behave in cells that express intermediate or low levels of HER2 (HER2^{int} or HER2^{lo}, respectively), and whether this differs from the behavior in HER2-overexpressing cells has not been investigated. This not only relates to the druggability of HER2, but might also yield insight into factors that contribute to differences in HER2 expression levels.

The discordant results concerning the intracellular fates of anti-HER2 antibodies have implications for their mechanism of action. For instance, antibody-induced HER2 endocytosis and lysosomal degradation is expected to extinguish HER2 signaling. In addition, for antibody-drug conjugates (ADCs), efficient delivery into the endolysosomal pathway is required.¹⁰ By contrast, antibody-HER2 internalization would be expected to negatively affect antibody dependent cell-mediated phagocytosis (ADCP) or antibody dependent cell-mediated cytotoxicity (ADCC), which require antibody persistence on the cell surface.

In addition to HER2-overexpressing cancers, there is interest in targeting HER2 in tumors that express intermediate or low

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levels of HER2, for which recent data support a role for the HER2 signaling axis in tumorigenesis.¹¹⁻¹³ For example, studies have indicated that the heterodimerization of HER2 with HER3, which is one of the most potent activators of the PI3K/Akt pathway known, can play an important role in the pathogenesis of breast and prostate tumors with normal to low HER2 levels.¹¹⁻¹³ This, combined with the variability in HER2 expression due to intratumor heterogeneity,^{14,15} motivates a comparative analysis of anti-HER2 antibody dynamics in cancer cells with a wide range of HER2 expression levels.

In the current study, we performed a quantitative characterization of antibody/HER2 trafficking dynamics in a panel of breast and prostate cancer cell lines. This has been combined with microscopy analyses to define the behavior of the anti-HER2 antibody trastuzumab and HER2 at the level of intracellular trafficking. Our results demonstrate that HER2 can internalize following antibody treatment in all cancer cell lines analyzed. Importantly, both trastuzumab recycling and decreased HER2 levels are observed in HER2^{hi} or HER2^{int} breast cancer cell lines. Unexpectedly, in HER2^{lo} breast and prostate cancer cell lines, the percentage decrease in total HER2 levels is higher than in HER2^{hi}/HER2^{int} cells, with undetectable levels of recycling of internalized trastuzumab combined with efficient entry into degradative, lysosomal compartments. The dynamic behavior of antibody-HER2 complexes in the different cell lines is also consistent with the levels of trastuzumab present on the plasma membrane. In particular, a significant fraction of the antibody persists on the cell surface of HER2^{hi} cells, and this fraction progressively diminishes with decreasing HER2 expression levels. Consequently, the HER2 expression level, rather than cell origin, is a predictor of trafficking behavior. Collectively, these analyses give novel insight into antibody-HER2 dynamics that could have implications for the design of therapeutics.

Results

Variable levels of HER2 following trastuzumab treatment

We first quantitated the levels of HER2 over the course of three days of trastuzumab treatment in a panel of cancer cell lines (Fig. 1). These cell lines have receptor expression levels that range from overexpression of HER2 (HER2^{hi}) to intermediate to low levels of HER2 (HER2^{int} or HER2^{lo}) (Fig. 1A). A flow cytometry-based assay was used to measure the total HER2 levels in cells. Briefly, the cells were treated with trastuzumab for different times and total HER2 levels were quantitated by staining with fluorescently labeled pertuzumab followed by flow cytometry. Pertuzumab and trastuzumab are known to recognize distinct epitopes on domain II and domain IV of the HER2 extracellular domain, respectively.^{16,17} Confirmatory experiments verified that pertuzumab can indeed be used as a noncompeting, staining antibody to label HER2 in cells treated with trastuzumab (Figure S1).

Figure 1B shows the HER2 levels, expressed as a percentage of the steady-state (without antibody) levels, following 24–72 h of trastuzumab treatment in the different cell lines. Interestingly, a

higher percentage of HER2 remains in the HER2^{hi} cells relative to that in HER2^{int/lo} cells. At 72 h, for example, the level of HER2 is reduced by 60–70% in the HER2^{int/lo} cells compared with 10–35% in the HER2^{hi} cells. We verified that this observation is not due to antibody consumption by the HER2^{hi} cells, since the reduction in HER2 levels is similar for a given cell line over a concentration range of 1–100 µg/ml trastuzumab (Figure S2).

We also investigated the reduction in HER2 levels over shorter time scales ranging from 1–48 h of trastuzumab treatment in a subset of cell lines, representing HER2^{hi} (SK-BR-3), HER2^{int} (ZR-75-1) and HER2^{lo} (LAPC-4). Figure 1C shows the decrease in HER2 levels within 1 h of trastuzumab treatment. Further, the HER2 levels continue to decrease until 18 h of trastuzumab treatment in ZR-75-1 and LAPC-4 cell lines. Analyses of the kinetics of the reductions in cell surface HER2 and intracellular HER2 levels showed that at all times post-trastuzumab treatment, cell surface HER2 levels decreased at a higher rate than intracellular HER2 levels (Figure S3). This suggests that HER2 internalization precedes degradation.

Trastuzumab recycling correlates with HER2 expression levels

We next investigated whether internalized trastuzumab is recycled back to the plasma membrane in cells with different HER2 expression levels. We found that trastuzumab recycled in all of the HER2^{hi} (SK-BR-3, BT-474, HCC1419 and HCC1954) and HER2^{int} (BT-483 and ZR-75-1) cell lines in our panel (Fig. 2A). However, the HER2^{lo} cell lines LAPC-4, C4-2B and HCC2185, which have 20-, 25-, 33-fold lower levels of HER2 expression, respectively, than HER2^{hi} HCC1419 cells showed distinct behavior. Specifically, we observed that trastuzumab was not recycled at detectable levels in HER2^{lo} cells (Fig. 2B). Interestingly, the HER2^{int} cell line BT-483 exhibits trastuzumab recycling behavior, but with a lower rate than that in HER2^{hi} cells. For comparative purposes, the recycling behavior of transferrin, a prototypical recycling marker, was similar in HER2^{hi}, HER2^{int} and HER2^{lo} cell lines (Figure S4).

Trastuzumab recycling leads to increased persistence on the cell surface

The differences in trastuzumab recycling behavior in HER2^{hi} and HER2^{int/lo} cells raised the question as to how this affects surface levels of trastuzumab-HER2 complexes, prompting an analysis of trastuzumab persistence on the plasma membrane of cells with different HER2 expression levels (Fig. 3). In all of the cells, trastuzumab levels at the cell surface decrease by ~15% within the first 60 min. However, the behavior at later time points is very different: in the HER2^{hi} SK-BR-3 cell line, a significant fraction of trastuzumab (~75%) remains at the cell surface after 18 h, whereas this fraction is only around 10% in the HER2^{lo} LAPC-4 cell line. Further, in the HER2^{int} ZR-75-1 cell line, which has 2-fold higher HER2 levels than LAPC-4 cells and both recycles and retains trastuzumab within cells, ~40% of trastuzumab remains on the cell surface after 18 h.

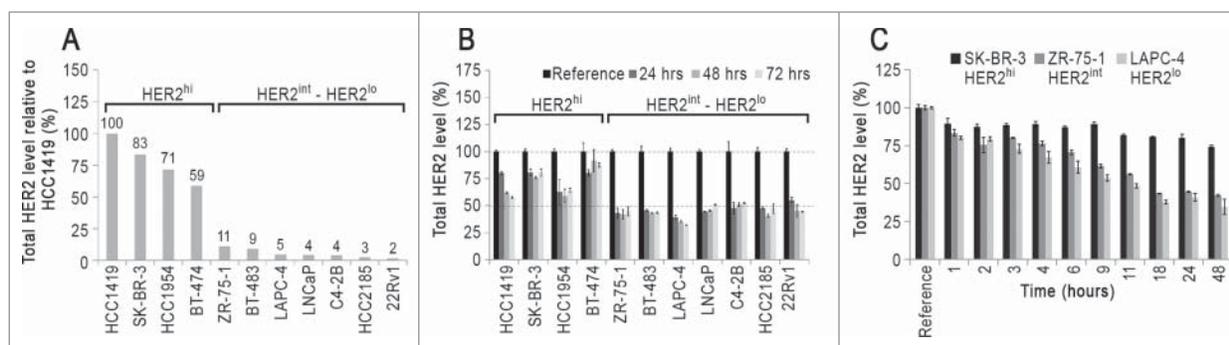


Figure 1. HER2 expression levels and effects of trastuzumab. **(A)** Relative levels of HER2 in the cell line panel used in this study. Cells were fixed, permeabilized and stained with fluorescently labeled trastuzumab and analyzed by flow cytometry. The fluorescence signal for each cell line is normalized with respect to HCC1419 which had the highest signal and is expressed as a percentage. **(B)** The effect of trastuzumab treatment on total HER2 levels. Cells were pulsed for the indicated times with 15 $\mu\text{g/ml}$ trastuzumab, harvested and stained with Alexa 647-labeled pertuzumab to determine total HER2 levels by flow cytometry. For each cell line, the HER2 level in untreated cells is taken as reference, and HER2 levels are expressed as a percentage of reference. **(C)** Kinetics of trastuzumab-mediated reductions in HER2 levels in SK-BR-3, ZR-75-1 and LAPC-4 cells. The cells were treated with 15 $\mu\text{g/ml}$ trastuzumab for the indicated times and at the end of each time point, cells were harvested and total HER2 levels determined as in panel. **(B)** Error bars indicate SD. The experiments are representative of at least two independent experiments.

Importantly, the observed reduction in trastuzumab levels across the different cell lines cannot be accounted for by dissociation of the antibody from HER2 on the cell surface. Specifically, the amount of trastuzumab present in the chase medium collected at the end of each time point is $\leq 5\%$ of the initial amount bound to the cells (data not shown).

Trastuzumab trafficks differently in SK-BR-3 and LAPC-4 cells

The marked differences in the trastuzumab recycling behavior between HER2^{hi} and HER2^{lo} cells prompted us to investigate the intracellular distribution of trastuzumab in these cells. Cells were pulsed with trastuzumab for 15 min and stained with markers for early (transferrin receptor) and late endosomes/lysosomes (LAMP-1) following no chase or a 45 min chase period. In addition, cells were pulse-chased with the fluid phase marker, dextran, to label the luminal compartments of lysosomes. The relatively high HER2 expression in the HER2^{hi} cells resulted in robust plasma membrane staining for trastuzumab, which obscures visualization of the internalized antibody in intracellular compartments (Figure S5). To overcome this, Alexa 488-labeled trastuzumab was used, enabling selective

quenching of surface levels using an anti-Alexa 488 antibody.^{4,18}

Figures 4 and 5 show the intracellular distribution of internalized trastuzumab in SK-BR-3 and LAPC-4 cells. Control experiments with Alexa 488-labeled human anti-HEL IgG1 (isotype control) confirmed that trastuzumab internalization is HER2-mediated and not due to non-specific fluid-phase uptake (Figure S6). Following 15 min of treatment with trastuzumab, the antibody can be detected in the lumina of transferrin receptor-positive endosomes in LAPC-4 cells, whereas in SK-BR-3 cells it is localized on the limiting membranes of these compartments (Figs. 4,5). The difference in endosomal localization of trastuzumab in these cell lines is congruent with the recycling behavior of this antibody in the two cell types. Specifically, luminal delivery of trastuzumab following internalization into early endosomes in LAPC-4 cells is expected to result in trafficking

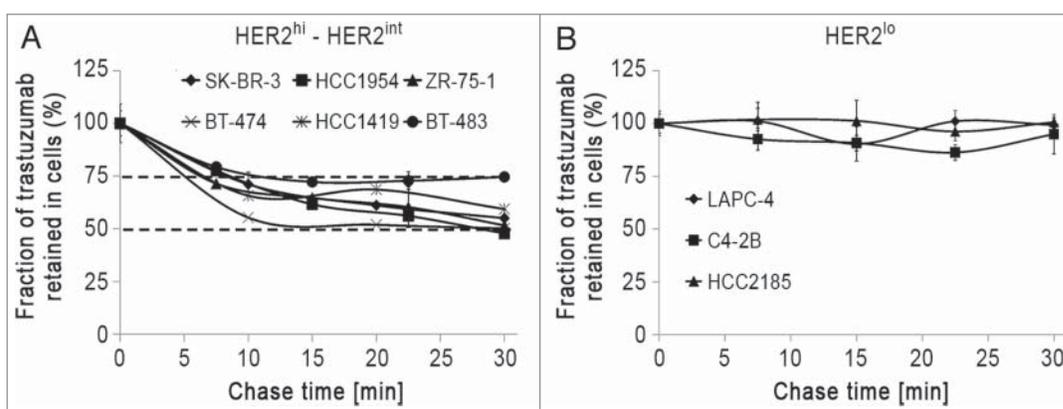


Figure 2. Trastuzumab is differentially recycled in HER2 expressing cell lines. Trastuzumab recycling in HER2^{hi} or HER2^{int} (A) and HER2^{lo} (B) cells are shown. Cells were pulsed with Alexa 488-labeled trastuzumab for 30 min at 37°C. The cells were cooled and incubated with 50 $\mu\text{g/ml}$ anti-Alexa 488 antibody for 30 min to quench the (cell surface) Alexa 488 signal. Cells were chased for the indicated times at 37°C in medium containing 25 $\mu\text{g/ml}$ anti-Alexa 488 antibody. At the end of each time point, the cells were harvested and analyzed by flow cytometry. The fraction retained in cells is calculated by normalizing the fluorescence signal at each time point with the 0 min chase time point signal. Error bars indicate SD. The experiments are representative of at least two independent experiments.

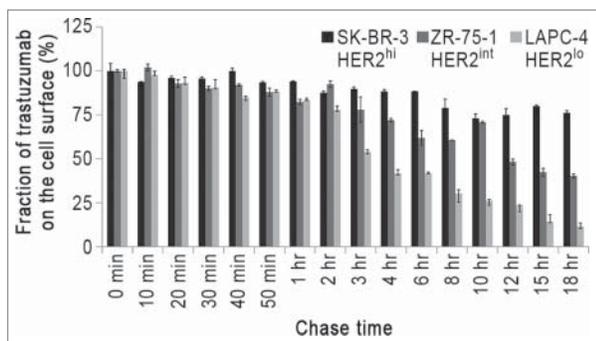


Figure 3. Trastuzumab shows differences in persistence on the cell surface of HER2-expressing cells. Cells were pulsed with 15 $\mu\text{g/ml}$ trastuzumab for 10 min and chased for the indicated times, then harvested, fixed, stained with Alexa 647-labeled anti-human IgG conjugate and analyzed using flow cytometry. The trastuzumab levels are normalized with respect to the 0 min chase time point. Error bars indicate SD. The experiments are representative of at least two independent experiments.

into the late endosomal/lysosomal pathway rather than entry into recycling transport carriers that segregate from endosomes.^{19,20} Importantly, control experiments indicated that the difference in behavior across cell lines is not due to the presence of the quenching antibody (Figure S7).

Consistent with the undetectable levels of recycling of internalized trastuzumab in LAPC-4 cells, following 15 min of antibody treatment and a 45 min chase period, trastuzumab is present in the lumina of LAMP-1⁺ late endosomes/lysosomes (Fig. 5). In SK-BR-3 cells, trastuzumab that is retained in cells following an analogous pulse-chase can be detected on the limiting membranes and lumina of LAMP-1⁺ endosomes (Fig. 4). In earlier studies,²¹ we have demonstrated that recycling receptors can segregate from LAMP-1⁺ late endosomes in cells, suggesting that the membrane associated trastuzumab in SK-BR-3 cells may constitute part of the recycling pool. In both cell types, luminal trastuzumab colocalized with fluorescently labeled dextran, which under the conditions of the experiments, was used as a marker for the lysosomal lumen (Figs. 4,5).

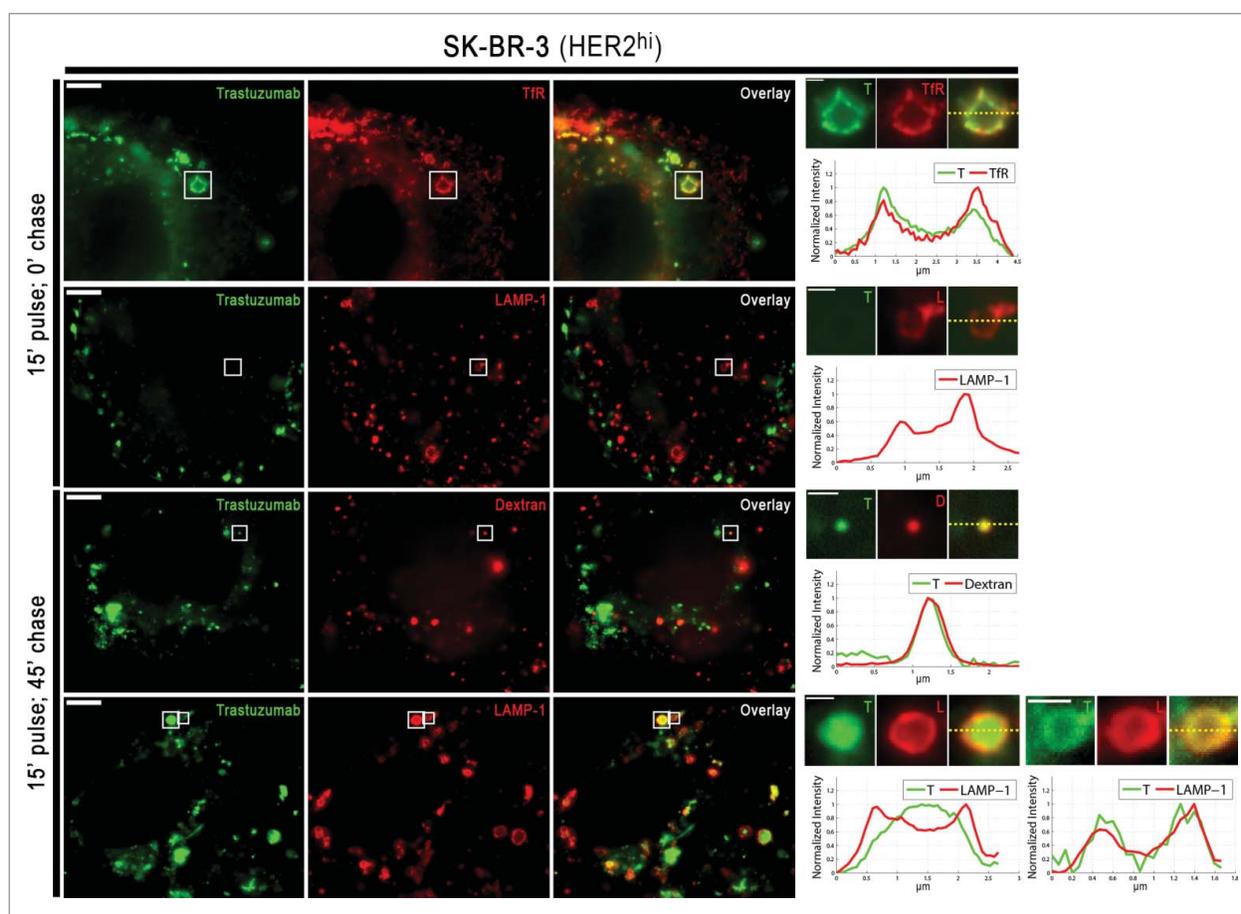


Figure 4. Subcellular trafficking behavior of trastuzumab in SK-BR-3 cells. Cells were pulsed with Alexa 488-labeled trastuzumab for 15 min, washed and chased for either 0 or 45 min. At the end of each time point, the cells were cooled and incubated with 50 $\mu\text{g/ml}$ anti-Alexa 488 antibody to quench the (cell surface) Alexa 488 signal. The cells were fixed, permeabilized and stained for transferrin receptor (Tfr) and LAMP-1. Boxed regions for each set of images are presented on the right hand side of each row as expanded images of individual endosomes/lysosomes. These individual endosomes/lysosomes are shown for trastuzumab (T), Tfr, LAMP-1 (L) or dextran (D). Normalized fluorescence intensities along the yellow dotted lines in the overlays are presented in the fluorescence intensity plots. In all panels, trastuzumab is pseudocolored green and endosomal staining (Tfr, LAMP-1 or Dextran) is pseudocolored red. Scale bars represent 5 μm (large panels) or 1 μm (expanded regions of interest).

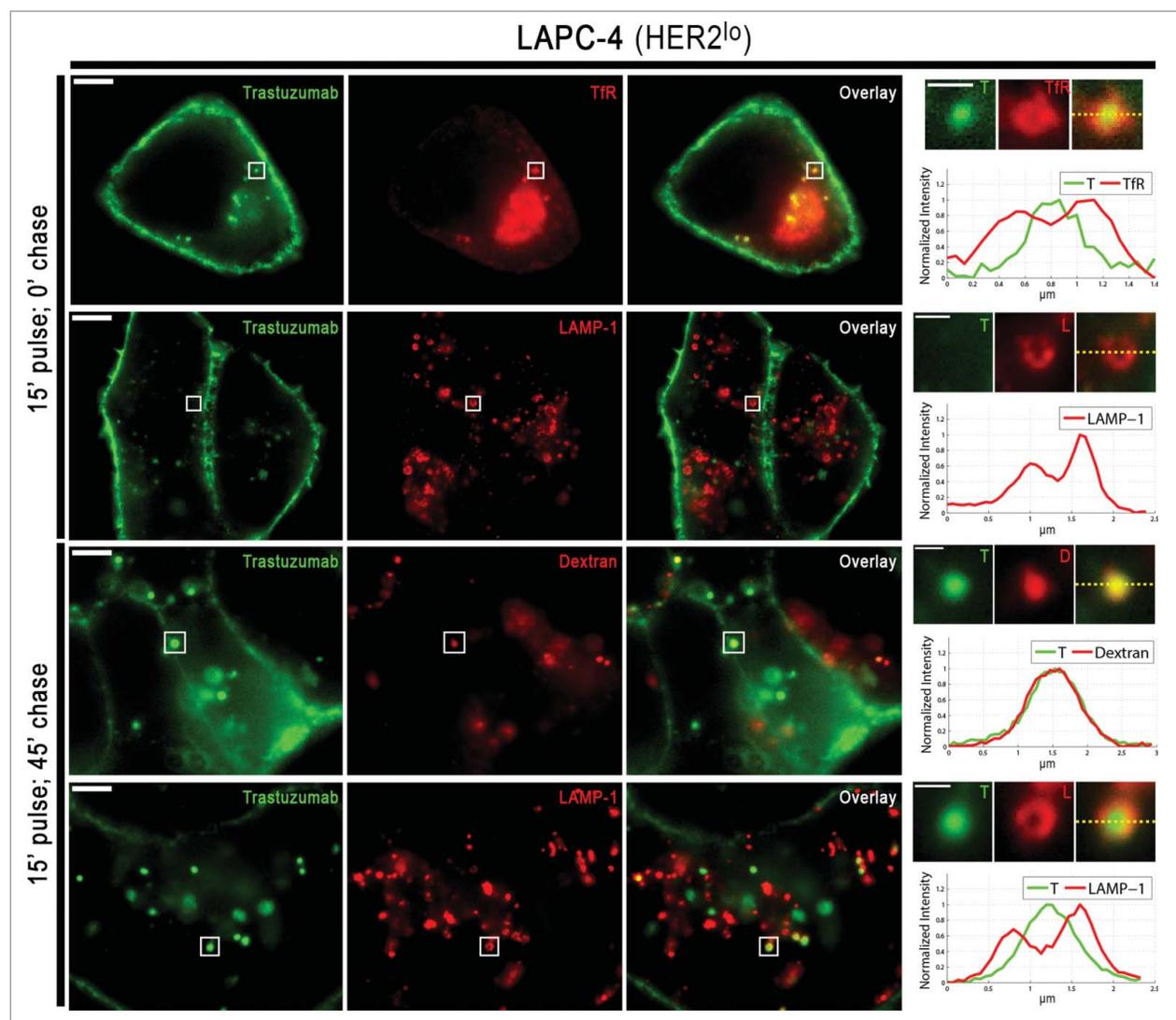


Figure 5. Subcellular trafficking behavior of trastuzumab in LAPC-4 cells. LAPC-4 cells were treated in the same way as **Figure 4**, except that quenching antibody was not used. Boxed regions for each set of images are presented on the right hand side of each row as expanded images of individual endosomes/lysosomes. These individual endosomes/lysosomes are shown for trastuzumab (T), TfR, LAMP-1 (L) or dextran (D). Normalized fluorescence intensities along the yellow dotted lines in the overlays are presented in the fluorescence intensity plots. In all panels, trastuzumab is pseudocolored green and endosomal staining (TfR, LAMP-1 or Dextran) is pseudocolored red. Scale bars represent 5 μm (large panels) or 1 μm (expanded regions of interest).

Discussion

The overexpression of HER2 in malignancies frequently results in an aggressive disease course. This has motivated the development of both single agent and combination therapies directed toward targeting HER2. Further, recent studies have indicated that the HER2 signaling axis can play a role in tumorigenesis in HER2^{int} or HER2^{lo} cells.¹¹⁻¹³ However, despite HER2 being a commonly targeted marker by antibody therapeutics in cancer, the intracellular dynamics of antibody-HER2 complexes is not well defined. In particular, controversy concerning antibody/HER2 behavior in HER2^{hi} cells persists,²⁻⁵ and there is a paucity of data for HER2^{int/lo} cells. Here, we have

systematically analyzed trastuzumab-HER2 trafficking and dynamics in a panel of cell lines with widely varying HER2 expression levels.

In the current study, we demonstrate that trastuzumab-HER2 complexes internalize in all cell lines analyzed, which vary over 50-fold in their expression levels of this receptor. However, following internalization the behavior of trastuzumab-HER2 complexes differs and is dependent on HER2 expression levels. Specifically, in HER2^{hi} cells trastuzumab is efficiently recycled. Nevertheless, a small fraction of antibody-HER2 complexes enters the lysosomal pathway. By contrast, in HER2^{lo} cells, trastuzumab recycling cannot be detected under the conditions of the assay and internalized antibody-HER2 complexes enter the lysosomal pathway.

Trastuzumab-HER2 complexes in HER2^{int} cells demonstrate properties that are between those of HER2^{hi} and HER2^{lo} cells, with both endocytic recycling and an intermediate loss of trastuzumab-HER2 complexes from the cell surface occurring.

Our observations concerning trastuzumab-HER2 dynamics, combined with the microscopy analyses, have led us to propose the following intracellular trafficking model for trastuzumab-HER2 complexes (Fig. 6). In HER2^{hi} cells, internalized trastuzumab trafficks to early endosomes (transferrin receptor-positive) and remains on the limiting membrane, whereas in HER2^{lo} cells the antibody is delivered into the lumen of these compartments within 15 min following uptake. By analogy with earlier studies of the trafficking of the recycling receptor, FcRn,^{19,22} trastuzumab in HER2^{hi} cells is therefore expected to segregate from these endosomes via tubulovesicular transport carriers into the endocytic recycling pathway. The luminal localization of trastuzumab-HER2 complexes in HER2^{lo} cells, which could occur by either multivesicular body formation²³ or HER2 cleavage, results in lysosomal delivery. The molecular mechanisms that lead to these differences in behavior across the different cell lines are not understood and require further analyses. In addition, we also observe that following one hour, trastuzumab can be detected on the limiting membrane of LAMP-1⁺ endosomes in SK-BR-3 cells. In earlier studies, we have shown that FcRn can localize to the limiting membrane and recycle from LAMP-1 positive late endosomes,²¹ suggesting that trastuzumab-HER2 complexes may traffic in an analogous manner in these HER2^{hi} cells.

Importantly, differences in trastuzumab-HER2 trafficking between HER2^{hi} and HER2^{lo} cells are not due to variations

in cancer cell type, since HER2^{lo} cells of both prostate and breast origin show similar characteristics. Nevertheless, efficient HER2 recycling and consequently alterations in the ratio of recycling/degradation could contribute to the maintenance of high surface HER2 levels in HER2^{hi} cells. This could have fundamental relevance to understanding how cells convert to the HER2-addicted, tumorigenic state. An alternative possibility, that is not mutually exclusive, is that HER2 overexpression is initiated by other factors, and the increased numbers of internalized HER2 molecules in HER2-overexpressing cells relative to HER2^{int} or HER2^{lo} cells could directly affect the intracellular trafficking behavior of this potent signaling receptor. Further, although our observations demonstrate differential trafficking of trastuzumab within cells with different levels of HER2 expression, they do not exclude an additional contribution of variations in HER2 biosynthetic rates to the total HER2 levels following trastuzumab exposure.

The internalization resistance of trastuzumab that has been described previously in HER2^{hi} cells using fluorescence microscopy^{2,3} can be explained by our observations of fast endocytic recycling of antibody-HER2 complexes, which when combined with very high levels of HER2 expression on the plasma membrane, obscure the visualization of internalized antibody-receptor complexes in cells. Specifically, to visualize internalized HER2 in these cells, we have found it necessary to quench the signal from fluorescently labeled HER2 on the plasma membrane. More generally, our observations concerning the extent of reduction in HER2 expression levels across different cell lines reflect the

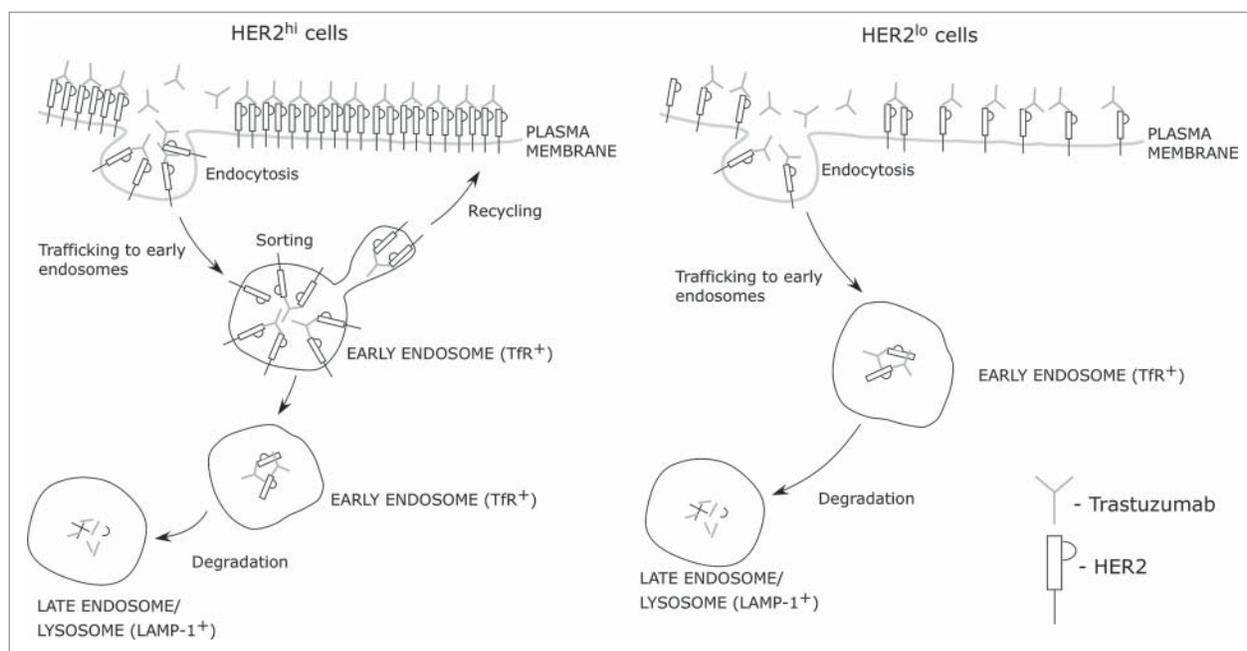


Figure 6. Model for antibody-HER2 trafficking. The schematic shows the intracellular trafficking behavior of antibody-HER2 complexes in cells with different HER2 expression levels.

divergent reports^{5,8} concerning antibody-mediated effects on HER2 in which cell lines with widely varying HER2 expression levels were used.

Prior results concerning the lack of HER2 internalization of trastuzumab in HER2^{hi} SK-BR-3 cells^{2,4,8} appear discrepant given the reported efficacy of trastuzumab as an ADC (trastuzumab emtansine, Kadcyla[®]), which requires the antibody to traffic into the endolysosomal pathway. Although a lower percentage of trastuzumab is internalized in HER2^{hi} cells (SK-BR-3) relative to HER2^{lo} cells (LAPC-4), the absolute numbers of internalized receptors would be much higher in SK-BR-3 cells due to a 16-fold difference in HER2 levels. These observations indicate that ADCs can be used to target receptors that inefficiently enter the endolysosomal pathway if the receptor level is sufficiently high. As a corollary, lower level receptors could be druggable with ADCs if their internalization/lysosomal delivery is high. Collectively, these studies indicate the need for subcellular trafficking studies of receptors to inform the design of ADCs.

Although directing antibody into the endolysosomal pathway is desirable for the effective delivery of ADCs, the relatively fast clearance of trastuzumab from the plasma membrane of HER2^{lo} cells when compared with HER2^{hi} cells would be expected to have a negative effect on antibody effector function. For instance, in prostate cancer HER2 overexpression is rarely observed, and these tumors typically exhibit low surface HER2 levels.²⁴ Thus, antibody-mediated clearance mechanisms such as phagocytosis and ADCC may be significantly abrogated in such tumors. This may also contribute to the disappointing results observed in clinical trials with anti-HER2 antibody treatments in prostate cancer.^{25,26}

In conclusion, our observations provide a quantitative characterization of anti-HER2 antibody dynamics and trafficking in HER2-expressing cancer cells. Significantly, the analyses reveal fundamental differences in subcellular trafficking behavior that are dependent on the expression levels of HER2. In turn, the findings in the current study have relevance to the design and optimization of HER2-directed therapeutics.

Materials and Methods

Antibodies, reagents and cell lines

Trastuzumab (Roche) and pertuzumab (Roche) were obtained from the UT Southwestern Pharmacy. Antibodies were labeled with Alexa Fluor dyes (Life Technologies) using standard protocols. Human IgG1 (isotype control) was purified from the supernatant of a NS0 stable cell line expressing human anti-hen-egg-lysosome (HEL) antibody.²⁷ The following antibodies were used for immunofluorescence staining: rabbit anti-LAMP-1 antibody (Abcam; ab24170), anti-mouse-EEA-1 antibody (BD Biosciences #610456), anti-transferrin receptor antibody (Invitrogen; #13-6800) and mouse anti-LAMP-1 antibody (DSHB – Univ of Iowa; H4A3). All secondary antibody conjugates were purchased from Life Technologies. The cell lines SK-BR-3, BT-474, 22Rv1, LNCaP and ZR-75-1 were obtained from the ATCC and maintained as recommended in ATCC protocols.

The cell lines HCC1419, HCC1954, HCC2185 and BT-483 were generously provided by Drs. Adi Gazdar, John Minna and Kenneth Huffman (University of Texas Southwestern Medical Center at Dallas, Dallas, TX) and maintained in RPMI supplemented with 5% fetal bovine serum. The cell lines C4-2B and LAPC-4 were provided by Dr. N. Sharifi (Lerner Research Institute, Cleveland, OH) and maintained in RPMI and modified IMEM, respectively, supplemented with 10% fetal bovine serum and antibiotics.

Measurement of cell surface, intracellular and total HER2 levels

Cells treated with 15 $\mu\text{g/ml}$ trastuzumab were harvested by trypsinization and fixed with 2% PFA for 20 min at 4°C. To detect cell surface HER2 levels, the cells were stained with 10 $\mu\text{g/ml}$ fluorescently labeled pertuzumab for 30 min at 4°C. To detect total HER2 levels, harvested cells (from separate wells) were fixed as described above, permeabilized with 0.1% saponin for 20 min at 4°C and stained with fluorescently labeled pertuzumab for 30 min at 4°C. The cells were analyzed using flow cytometry (FACSCaliber, Becton Dickinson) to measure the fluorescence signal corresponding to either cell surface or total HER2 levels. Intracellular HER2 levels were determined by calculating the difference between the signal corresponding to total HER2 levels and that of cell surface HER2 levels. For each cell line, all data points are normalized with respect to the signal in untreated cells (labeled as reference) and are expressed as a percentage.

Measurement of trastuzumab levels on the cell surface

Cells were pulsed with 15 $\mu\text{g/ml}$ trastuzumab for 10 min at 37°C, washed three times and chased in medium for different times at 37°C. The cells were harvested, fixed as described above and then stained with 5 $\mu\text{g/ml}$ Alexa 647-labeled anti-human IgG for 30 min at 4°C. The cells were analyzed using flow cytometry (FACSCaliber, Becton Dickinson) to measure the fluorescence signal corresponding to cell surface trastuzumab levels. The fluorescence signal for all of the time points was normalized with respect to the 0 min chase time point and expressed as a percentage.

Trastuzumab and transferrin recycling assay

The recycling experiments were based on fluorescence quenching of Alexa 488 using an anti-Alexa 488 polyclonal antibody (Life Technologies; A11094) and were performed as described previously.^{4,18} Briefly, cells were pulsed with 2 or 10 $\mu\text{g/ml}$ Alexa 488-labeled trastuzumab (degree of labeling = 1) for 30 min at 37°C to allow receptor-mediated endocytosis of the antibodies. The cells were placed on ice, washed three times with ice cold HBSS buffer and incubated on ice for 10 min in HBSS buffer. The cells were then pulsed with 50 $\mu\text{g/ml}$ anti-Alexa 488 antibody for 25 min with agitation to quench the Alexa 488 fluorescence signal on the cell surface. The cells were washed twice with room temperature PBS and chased for different times at 37°C in pre-warmed medium containing 25 $\mu\text{g/ml}$ anti-Alexa 488 antibody to quench the Alexa 488 signal when

the antibody recycles from the cell interior back to the plasma membrane. To assess transferrin recycling, cells (in separate wells) were pulsed with 5 $\mu\text{g/ml}$ of Alexa 647-labeled transferrin and subjected to the same treatment as for trastuzumab-treated cells except that anti-Alexa 488 antibody was not used and also cells were chased in medium with or without 0.25 mg/ml unlabeled transferrin. At the end of each chase phase, the cells were harvested by trypsinization, pelleted by centrifugation, and immediately analyzed using flow cytometry (FACSCalibur, Becton Dickinson) to determine the amount of trastuzumab or transferrin present inside the cells. The fraction of recycled trastuzumab or transferrin was calculated by normalizing the fluorescence signal at each time point with the signal pertaining to the 0 min chase time point and is expressed as a percentage.

Immunofluorescence microscopy

The cells were seeded in glass-bottomed dishes (Mattek Corporation) pre-coated with poly-L-lysine and grown in phenol-red free medium for two days. The cells were pulsed for 15 min with either 5 $\mu\text{g/ml}$ Alexa 488-labeled trastuzumab (SK-BR-3 or LAPC-4 cells) or 10 $\mu\text{g/ml}$ Alexa 555-labeled trastuzumab (LAPC-4 cells) at 37°C and chased for the indicated times. To assess fluid phase uptake, the cells (in separate dishes) were pulsed for 15 min with 5 $\mu\text{g/ml}$ Alexa 488-labeled human anti-HEL IgG (SK-BR-3) or 10 $\mu\text{g/ml}$ Alexa 555-labeled human anti-HEL-IgG (LAPC-4) at 37°C. To quench the signal from the cell surface bound Alexa 488-labeled trastuzumab, cells were washed twice with ice-cold HBSS, incubated on ice for 10 min in ice-cold HBSS and incubated for 30 min on ice in medium containing 50 $\mu\text{g/ml}$ anti-Alexa 488 antibody. Cells were then washed with PBS, fixed with 1.7% paraformaldehyde for 10 min at 37°C, permeabilized with 0.025% saponin for 10 min at room temperature, then stained and counter-stained with primary and secondary antibodies, respectively, each for 25 min at 37°C in 1% BSA in PBS. Cells were stored in 1% BSA in PBS at 37°C and imaged within two days.

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Cells were imaged using a Zeiss Axiovert 200M microscope fitted with a Zeiss 100 \times 1.4NA planapochromat objective and an ORCA CCD camera (Hamamatsu). All image processing was performed in the MIATool software package.²⁸ Image intensities were linearly adjusted for each channel and the individual channels were overlaid to create the color images. The region showing the cell of interest was cropped and exported in uncompressed tiff format. To analyze the location of trastuzumab in endosomes or lysosomes, fluorescence intensity plots were generated by subtracting the background fluorescence intensity from the signal in regions of interest along the displayed dotted yellow lines. The color images were then assembled in Canvas software (ACD Systems) and saved in tiff file format.

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Author Contributions

SR, RJO and ESW designed the project, SR and DK performed the experiments and analyzed the data, and all authors wrote the manuscript.

Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

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