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ORIGINAL ARTICLE

Breast tumor cells isolated from in vitro resistance to trastuzumab remain sensitive to trastuzumab anti-tumor effects in vivo and to ADCC killing

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Abstract An understanding of model systems of trastuzumab (Herceptin) resistance is of great importance since the humanized monoclonal antibody is now used as first line therapy with paclitaxel in patients with metastatic Her2 overexpressing breast cancer, and the majority of their tumors has innate resistance or develops acquired resistance to the treatment. Previously, we selected trastuzumab-resistant clonal cell lines in vitro from trastuzumab-sensitive parental BT-474 cells and showed that cloned trastuzumab-resistant cell lines maintain similar levels of the extracellular Her2 receptor, bind trastuzumab as efficiently as the parental cells, but continue to grow in the presence of trastuzumab and display cell cycle profiles and growth rates comparable to parental cells grown in the absence of trastuzumab (Kute et al. in Cytometry A 57:86–93, 2004). We now show that trastuzumab-resistant and trastuzumab-sensitive cells both surprisingly display trastuzumab-mediated growth inhibition in athymic nude mice. This demonstrates that resistance developed in vitro is not predictive of resistance in vivo. The observation that in vitro resistant cells are sensitive to trastuzumab in vivo could be explained by antibody dependant cellular cytotoxicity (ADCC). Therefore, both parental and trastuzumab-resistant cells were assayed for ADCC in real time on electroplates with and without trastuzumab in the presence of a natural killer cell line (NK-92), and granulocyte or mononuclear cellular fractions isolated from human peripheral blood. Mononuclear cells and NK-92 cells were more effective in killing both parental and trastuzumab-resistant cells in the presence of trastuzumab. Both trastuzumab-resistant cells and trastuzumab-sensitive cells showed similar susceptibility to ADCC despite displaying divergent growth responses to trastuzumab. The granulocyte fraction was able to kill these cells with equal efficacy in the presence or absence of trastuzumab. These results support a model of trastuzumab tumor cell killing in vivo mediated primarily by ADCC from the mononuclear fraction of innate immune cells and suggest that in the clinical setting not only should changes in signaling transduction pathways be studied in acquired tumor resistance to trastuzumab, but also mechanisms by which tumors impede immune function should be evaluated.

Keywords ADCC · Trastuzumab · Herceptin · Breast cancer · Resistance · BT-474

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Introduction

Trastuzumab is a humanized monoclonal antibody (mAb) obtained from a mouse mAb against the tyrosine kinase receptor, Her2 [4]. It is approved as a first line therapy in combination with chemotherapy for metastatic breast cancer patients whose tumors overexpress Her2 [23]. In addition, trastuzumab is approved in the adjuvant setting being shown responsible for a reduction in the risk of recurrence for women with operable Her2+/node-positive breast cancers when combined with standard postoperative chemotherapy [20]. Despite trastuzumab's anti-tumor efficacy, a significant group of Her2 positive tumors is innately resistant

or develop acquired resistance to the mAb treatment through unknown mechanisms. Sixty to seventy percent of metastatic patients with Her2 positive tumors appear intrinsically resistant to trastuzumab as a sole therapy [2, 29]. The Winer group measured expression of Her2 before and after treatment with trastuzumab in 32 patients. Eighty-three percent of these patients still had Her2 positive tumors while the remaining patients had either no measurable tumor to analyze or the tumor became Her2 negative [3]. Furthermore, a cell line developed from a patient resistant to trastuzumab treatment was found to maintain high levels of Her2 expression with no identifiable mutations [27]. This would indicate that loss of the expression of the trastuzumab target is not a major reason for trastuzumab resistance. An understanding of the key mechanisms that enable tumor cells to develop trastuzumab resistance is likely to lead to improved outcomes for patients on trastuzumab therapy.

In vitro studies have indicated that trastuzumab primarily induces a G1 phase accumulation of cells via signal transduction perturbations [14–16, 31], with some increase in the induction of apoptosis [15, 31]. However, additional studies in vivo have suggested alternative mechanisms of action for trastuzumab, such as the inhibition of angiogenesis [12] and or increased susceptibility to killing by the innate immune cells through antibody dependant cellular cytotoxicity (ADCC) [7]. These in vivo mechanisms could be independent or potentially dependent upon trastuzumab's simultaneous ability to inhibit signal transduction.

Antibody dependant cellular cytotoxicity relies on the Fc constant regions of the trastuzumab antibody to engage cells of the innate immune system such as NK cells, monocytes and macrophages. Once engaged, the innate effector cells secrete perforin and other factors that destroy the tumor cells.

To better understand the mechanisms of tumor cell killing and resistance to trastuzumab, our laboratories collaborated to select clones from BT-474 cells that grew in the presence of trastuzumab [14]. The treatment of parental BT-474 cells with trastuzumab results in an accumulation of cells in the G1 phase of the cell cycle [14], and an increase in background apoptosis resulting in a near static equilibrium (T. Kute and J.P. Vaughn, unpublished observations). Furthermore, trastuzumab binding to these sensitive cells results in a translocation of a cyclin dependent kinase inhibitor (p27) into the nucleus which halts cell proliferation. Although our resistant clones retain over-expression of Her2 similar to what is seen in patients treated with trastuzumab [3], trastuzumab treatment does not cause p27 translocation into the nucleus nor inhibition of growth in these resistant clones [14]. In this study, we test whether cells resistant to growth inhibition by trastuzumab signal transduction also show that resistant phenotype in vivo in the nude mouse. If inhibition of the signal transduction pathway is essential for the trastuzumab in vivo killing mechanism, then resistant cells will still grow effectively in the nude mouse under trastuzumab treatment. However, if ADCC is sufficient for trastuzumab killing, the resistant cells should be killed with similar efficacy as non-resistant parental cells. Our results indicate the latter hypothesis is probable because trastuzumab is effective in the nude mouse model and efficiently stimulates ADCC in both resistant and sensitive cells. These novel findings would suggest that studies of trastuzumab resistance in the clinic should investigate how tumor cells might evade immunosurveillance.

Materials and methods

Cell lines and HER2 antibodies

The human breast cancer cell line, BT-474, was obtained from the American Type Tissue Culture Collection (ATTCC, Manassas, VA, USA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with penicillin/streptomycin, 5% L-glutamine and 10% FBS. The resistant BT-474 cells were obtained from selection after treatment with trastuzumab for greater than 2 weeks [14]. Both cell lines had similar growth curves and similar aneuploid status based on DNA index. The NK-92 cell line was obtained from the ATTCC and maintained in an alpha minimum essential medium supplemented as recommended by ATCC. Trastuzumab (Genentech, South San Francisco, CA, USA) was obtained from the Wake Forest University North Carolina Baptist Hospital pharmacy, and pertuzumab, an antibody also specific for Her2, was a gift from Genentech.

Her2 expression

The expression of Her2 was determined by flow cytometry using indirect fluorescent staining methods. The cells were harvested by trypsin and EDTA and then washed with PBS containing 1% BSA (PBS-A). In the primary reaction, the cells were treated at 4°C for 30 min with either no antibody or 10 μg/ml of the designated antibody to Her2. After washing the cells with PBS-A to remove the non-bound primary antibody, the cells were treated with a 1:50 dilution of goat anti-human IgG-PE-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After washing with PBS-A, the cells were resuspended in PBS-A for flow cytometry which was performed using a BD-FACSAria instrument (BD Biosciences, San Jose, CA, USA). Non-specific binding was defined by a similar protocol but only the goat anti human IgG-PE was added. The

cell population was gated on forward scatter and side scatter. The relative fluorescence was determined by excitation at 488 nm and using 530/30 nm band pass filters to measure PE fluorescence. Percent of cells with specific staining and the intensity of staining were determined by the Becton Dickinson FACS Diva Software.

Animal studies

The trastuzumab-sensitive and trastuzumab-resistant cells were grown to greater than 60% confluency and harvested by the standard procedure. The animals were pretreated with estrogen pellets (Innovative Research of America, Sarasota, FL, USA) since these cells contain estrogen receptors. Into the lower mammary fat pad of each animal, 150 µl of 1–2 million cells in a 50% Matrigel and 50% media were injected. The tumors were monitored for growth. Upon reaching at least 50 mm^3, the animals were randomized by size and some were given intraperitoneal injections of trastuzumab (10 mg/kg) at 21 days with subsequent treatments every 7–10 days. The size of the tumors were determined by measuring the width and length of the tumor and using the equation; size = $(width^2 \times length)/2$. There were five animals in each arm of the treatments. These experiments were performed in duplicate. "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws (e.g., the current version of the German Law on the Protection of Animals) where applicable.

Growth curves via RT-CES system

The trastuzumab-sensitive and trastuzumab-resistant cells were harvested and transferred into a 96-well E-Plate, which contain electrodes across the bottom of each well that measure cell index based on impedance using the RT-CES system (ACEA Biosciences, San Diego, CA, USA. This technology has recently been acquired by Roche Applied Science and is being marketed under the name Xcelligence System). Cell index correlates with the number of cells attached to the bottom of the plate [24]. There have been prior reports that have characterized the use of the RT-CES system to measure dynamic changes in the number of live, adherent cells and how it compares to the MTT assay [30]. There have also been prior reports regarding the use of the RT-CES to evaluate cytotoxicity using immune cells as the effector cells and how they compare to the MTT and thymidine incorporation assays [10, 24, 32]. It has also been briefly reported that the RT-CES system can be used to evaluate ADCC [10, 32]. For the experiments reported here, the number of cells added to each well varied from 5,000 to 20,000 per well with a total volume of 100 µl of media. After sufficient growth which was based on the cell index value, the cells were treated with 100 µl of media alone or with 100 µl of media with different concentrations of trastuzumab added. The cell index was measured every 30 min until the cells became confluent. The degree of cell inhibition is directly related to the change in cell index over time. Each treatment was performed in triplicate with average and standard deviation obtained by the software provided by the instrument. The quantitative effect was determined by measurement of area under the growth curves (AUC) from application of treatment through the 72-h of monitoring. In this analysis, the area under the growth curve for either the control or treated cells were determined. The percent inhibition or killing values (% cell kill) was determined by dividing the difference between the control and treated AUC by the AUC of the control and multiplied by 100. These experiments were done multiple times with similar results.

The analysis of variance was used to assess differences between groups; to test for a difference in the rate of change, the interaction between group and dose was analyzed. To test for differences between doses and groups, unadjusted pairwise comparisons were done.

Measurement of ADCC by RT-CES system

The cells were plated on the 96 well E-Plate as described above and allowed to grow for 24–48 h. The cells were treated with media alone, with the natural killer cell line (NK-92) at an effector to target ratio of 1:1, or with a similar ratio of NK-92 cells containing 0.1 µg/ml of trastuzumab. The cell index values were monitored similar to the growth curves as described above. Each treatment phase was performed in triplicate with the average and standard deviation obtained from the provided program. The quantitative effect was determined as described above by determining the % cell kill. Further studies on ADCC killing were performed using different concentrations of mononuclear and polymorphonuclear (PMN) cells that were obtained from normal individuals of various ages and two breast cancer patients who were exposed to trastuzumab. All of these individuals were informed of the study and gave their consent. The mononuclear and PMN cells were separated by standard procedures as previously described [19] and were consistently equal to or greater than 95% pure as determined by microscopic differential counting. These ADCC killing studies using mononuclear or PMN cells at different effector to target ratios were completed in a similar manner as those performed using the NK-92 cell line.

Results

Previous results from our laboratory and others have demonstrated that both trastuzumab-sensitive BT-474 cells and

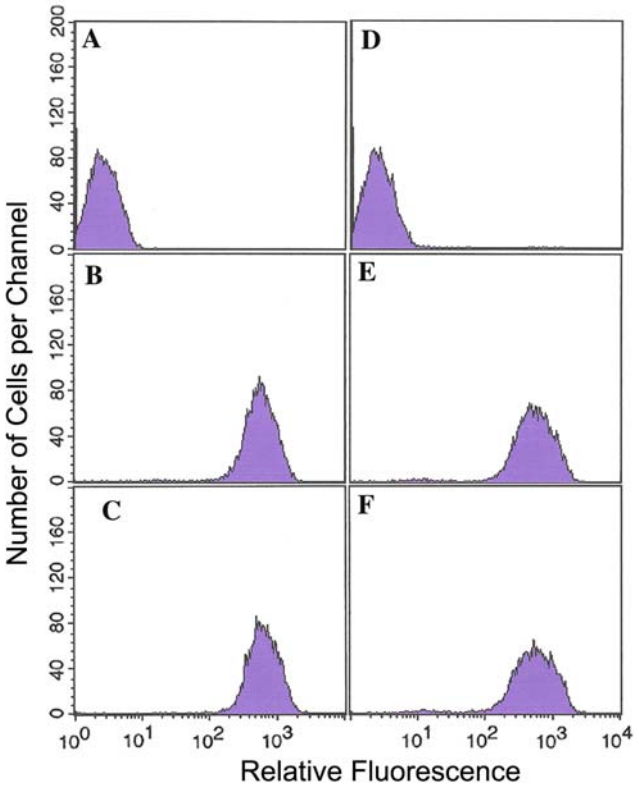

Fig. 1 HER2 is maintained at similar levels in parental BT-474 cells and in a trastuzumab resistant clonal cell line. Flow Cytometry expression of HER2 on BT-474 cells and on a BT-474 trastuzumab resistant clone. *Panel* **a**–**c** BT-474 cells treated with no primary antibody (**a**), 10 μg/ml of trastuzumab (**b**) or 10 μg/ml of pertuzumab (**c**). **a**, **b**, **c** were then treated with goat anti human IGG-PE labeled. *Panel* **d**–**f** BT-474 trastuzumab-resistant clone treated with no primary antibody (**d**), 10 μg/ml of trastuzumab (**e**) or 10 μg/ml of pertuzumab (**f**). **d**, **e**, **f** were then treated with goat anti human IGG-PE labeled

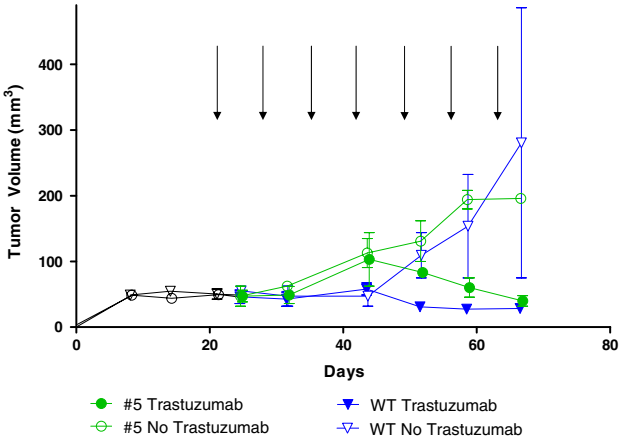

Fig. 2 Both parental BT-474 cells and in vitro selected trastuzumab resistant cells are growth inhibited in nude mice treated with trastuzumab. Growth of BT-474 cells and BT-474 trastuzumab resistant clone in athymic nude mice. Animals were given a subcutaneous injection of either cell line. Tumors were allowed to grow until they reached approximately 50–60 mm^3. Animals were divided equally based on size into treated or non-treated groups. On day 21, the animals were treated with an i.p. injection (*arrows*) of trastuzumab at 10 mg/kg. The animals were given subsequent trastuzumab treatments (10 mg/kg) every 7–10 days (*arrows*). Tumor growth was followed for an additional 45 days. *Open inverted triangle* indicates BT-474 no trastuzumab treatment, *open circle* indicates BT-474 Resistant Clone no trastuzumab, *dark filled inverted triangle* indicates BT-474 plus trastuzumab, *dark filled circle* indicates BT-474 Resistant Clone plus trastuzumab

resistant BT-474 clones express high levels of Her2 on their cell surfaces [1, 14, 15]. Figure 1 confirms that both the sensitive and trastuzumab-resistant cells used in the present study contain the Her2 antigen capable of binding either trastuzumab or pertuzumab via an indirect immunofluorescence assay using flow cytometry. The expression is high in both cell lines using the two different antibodies. There is no significant difference between the expression levels in the cell lines studied. Observations under a fluorescent microscope are also identical and demonstrate membrane fluorescence (data not shown).

Athymic mouse studies were performed to determine if in vitro trastuzumab resistance would be directly predictive of in vivo trastuzumab resistance. If in vitro resistance is not transferable to in vivo conditions, this would suggest that signal transduction inhibition is not critical for in vivo cell killing. Figure 2 demonstrates that both the resistant and sensitive cell lines produce tumors in the athymic nude mouse model in the absence of trastuzumab. When trastuzumab was given after the development of a measurable tumor on days 21–57, neither tumor showed any significant growth compared to the non-treated controls. By day 50 a significant difference is seen between the non-trastuzumab treated and trastuzumab treated tumors, but little difference is seen between resistant and sensitive parental tumor cells. This experiment was repeated and gave similar results (data not shown). Immunohistochemistry analysis of the remaining tissue at the end of the study confirmed that all tumors maintained comparable levels of Her2 overexpression (data not shown).

In order to further confirm that these cells retained their sensitivities or resistance to trastuzumab, a novel real-time growth curve assay was performed in the absence and presence of different concentrations of trastuzumab using the RT-CES system (see "Materials and methods"). This system measures cell index by electrical impedance across the bottom of each well of a 96-well E-Plate and increase of impedance is a direct measurement of cell growth. In some cases, the addition of reagents can cause a rapid increase in the cell index and this has been demonstrated to be caused by changes in temperature or changes in the media. In Fig. 3a–d the two cell lines grown for 120 h in the RT-CES system demonstrate an increase in the cell index as a function of time when grown in media alone. If increasing amounts of trastuzumab were added to the trastuzumab-sensitive cell line, there was a dose-related loss of the cell

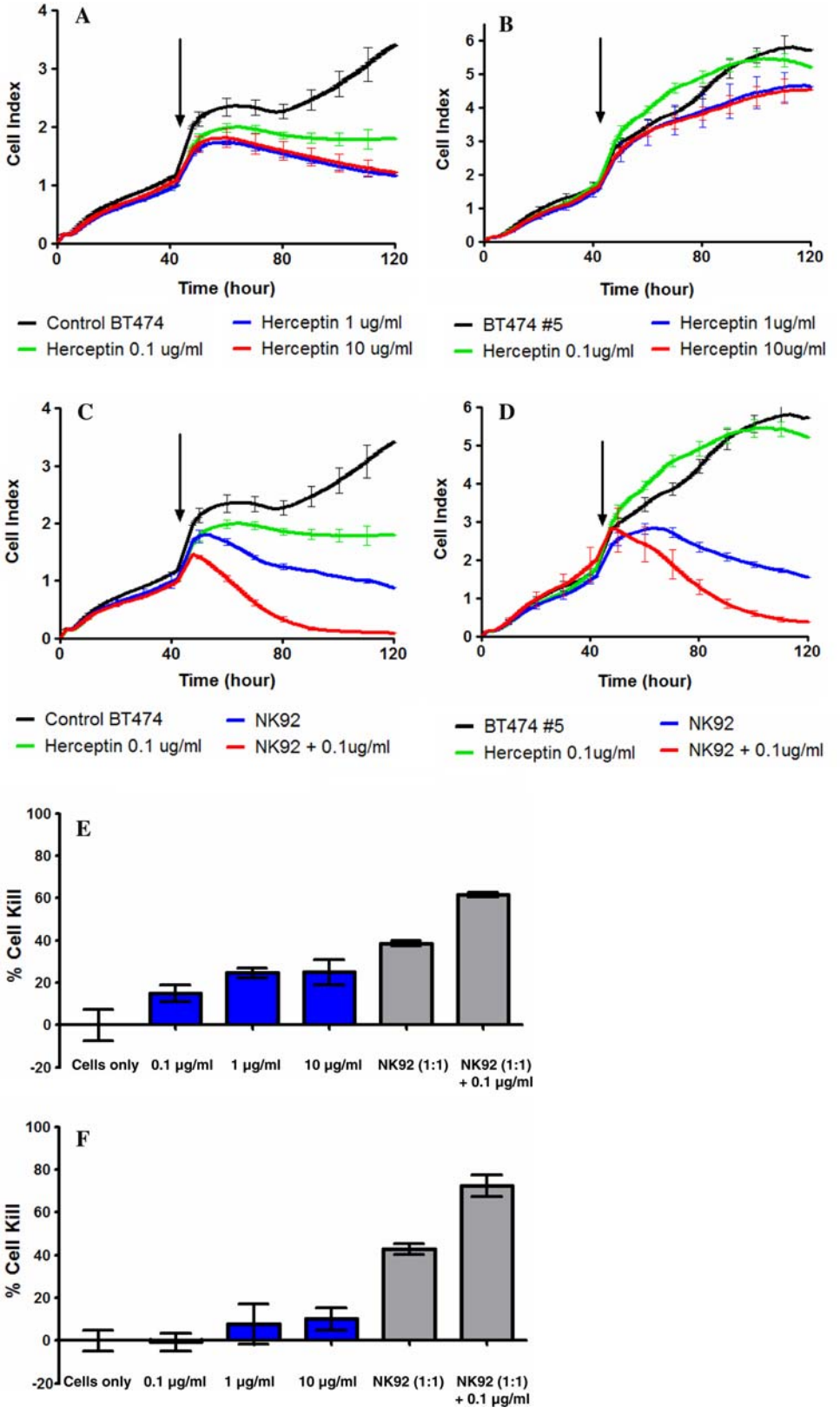

Fig. 3 Trastuzumab growth inhibition curves for BT-474 cells and BT-474 trastuzumab-resistant clone under various conditions using the RT-CES system. BT-474 cells (*panel* **a**) or BT-474 trastuzumab-resistant clone (*panel* **b**) were plated onto a 96-well E-Plate and allowed to grow for 48 h. Cells were grown in media alone (*black*) or were treated with trastuzumab (see *arrow*) at 10 µg/ml (*red*), 1 µg/ml (*blue*), or 0.1 µg/ml (*green*). BT-474 cells (*panel* **c**) or BT-474 trastuzumab-resistant clone (*panel* **d**) were grown similar to *panel* **a** and **b** but were treated with 0.1 µg/ml trastuzumab (*red*), NK-92 cells at a 1:1 effector to target ratio (*green*), or trastuzumab (0.1 µg/ml) and NK-92 cells at an effector to target ratio of 1:1 (*blue*). Cell indices of triplicate wells were measured every 30 min. Quantitation of these results by measuring the change in area *under the curves* in relationship to the non-treated controls are presented in *panel* **e** and **f** for the sensitive and resistant cell lines, respectively

index value that appears to reach maximal growth inhibition by 1 µg/ml of trastuzumab (Fig. 3a). A significant ($P = 0.0001$) decrease in percent cell growth relative to the non-treated control was observed in a dose-dependent manor for the sensitive cells (Fig. 3e). This result obtained by the RT-CES system is similar to the dose–response

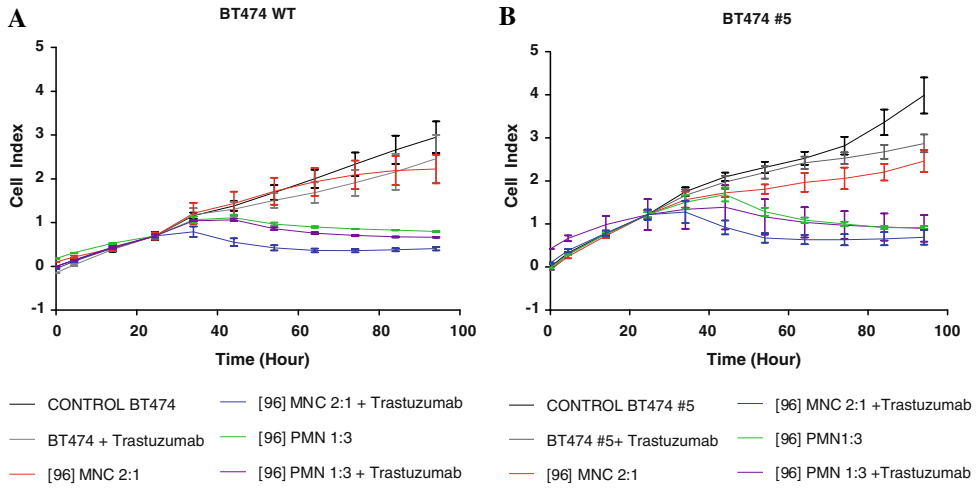

Fig. 4 Parental BT-474 and trastuzumab-resistant cells are killed by ADCC with near equal efficacy. ADCC killing curves using human white blood cells against BT-474 and BT-474 trastuzumab-resistant clone cells measured using the RT-CES system. BT474 cells (*panel* **a**) or BT474 trastuzumab-resistant clones (*panel* **b**) were plated onto a 96-well E-Plate and allowed to grow for 24 h prior to treatment. The cells were grown in media alone (*black*) or treated with isolated mononuclear cells at an effector to target ratio of 2:1 (*blue*, *red*) or granulocytes at an effector to target ratio of 1:3 (*green*, *purple*). Treatment with trastuzumab (0.1 μg/ml) alone (*gray*) or in combination of effector cells (*blue* = MNC) or (*purple* = PMN) is shown with *dashed lines*. Cell indices of triplicate wells were measured every 30 min

curves previously reported [14]. The low dose growth sensitivity of 0.1 μg/ml was not always observed and this sensitivity varied with the assay. In contrast to the trastuzumab-sensitive parental cell line, Fig. 3b demonstrates that the trastuzumab-resistant cell line does not show a dose–response curve similar to the parental cells ($P = 0.0007$). There is, however, a significant but small difference in cell growth inhibition at the higher concentrations ($P = 0.007$ and $P = 0.0009$) compared to the non-treated cells (Fig. 3f). These results performed by this novel procedure (E-Plate) confirm previous flow cytometric cell cycle profiles indicating that while trastuzumab treatment diminished S-phase activity in parental BT-474 cells, it had virtually no inhibition of S-phase activity in BT-474 resistant clones [14].

Using the RT-CES system, one can also measure ADCC activity. The treatment of the sensitive and resistant cell lines with a natural killer cell line (NK-92) alone results in diminished growth and a large cytotoxic effect in Fig. 3c and d, respectively. It should be noted that the addition of non-adhering cells such as NK-92 does not result in an increase in the cell index [10, 32]. The quantitative results demonstrate a 42.7 and 46.5% killing by NK-92 cells in the trastuzumab-sensitive and trastuzumab-resistant cells, respectively (Fig. 3e, f). This was not significant between the groups. If one adds the NK-92 cells and 0.1 μg/ml of trastuzumab, a significant increase ($P = 0.0001$) in killing of both cell lines is observed (Fig. 3e, f). This killing is synergistic in both cells. This effect clearly suggests that ADCC killing occurs in vitro for both the sensitive and resistant cell lines with similar efficacy. Another antibody to Her2, pertuzumab, produced similar results (data not shown). These results would indicate that the killing pathway is independent of the antibody used in the ADCC killing assay. In addition, the use of trastuzumab against a non-Her2 expressing tumor line (HeLa) shows no ADCC killing (data not shown).

We further investigated the issue of trastuzumab-mediated ADCC activity using human white blood cells (WBC) instead of the NK-92 cell line. In these studies, WBCs were obtained from 14 individuals, two of which had a history of breast cancer with one currently on trastuzumab therapy and one that had previously had trastuzumab therapy. The age range was 25–65, and 20% were male individuals. WBCs were separated into PMN granulocytes (neutrophils and eosinophils) and mononuclear cells (lymphocytes, NK cells and monocytes) as discussed in "Materials and methods". These cells were used in the RT-CES system similar to the use of the natural killer cell line (NK-92). Mononuclear cells were capable of some killing, but the addition of a very low, non-toxic dose of trastuzumab (0.1 μg/ml) resulted in a dramatic and significant increase in the killing of both the trastuzumab-sensitive BT474 cells (Fig. 4a) and the trastuzumab-resistant cell line (Fig. 4b). When granulocytes were tested, a clear killing effect was measured, but the addition of trastuzumab did not change the killing profile and no ADCC was observed. This figure is a representative experiment for individual no. 96 which demonstrates the sensitivity of the cell killing assay.

Figure 5 provides a summary of ADCC killing of parental BT-474 as defined by AUC calculations by either mononuclear cells or granulocytes for 14 individuals. The

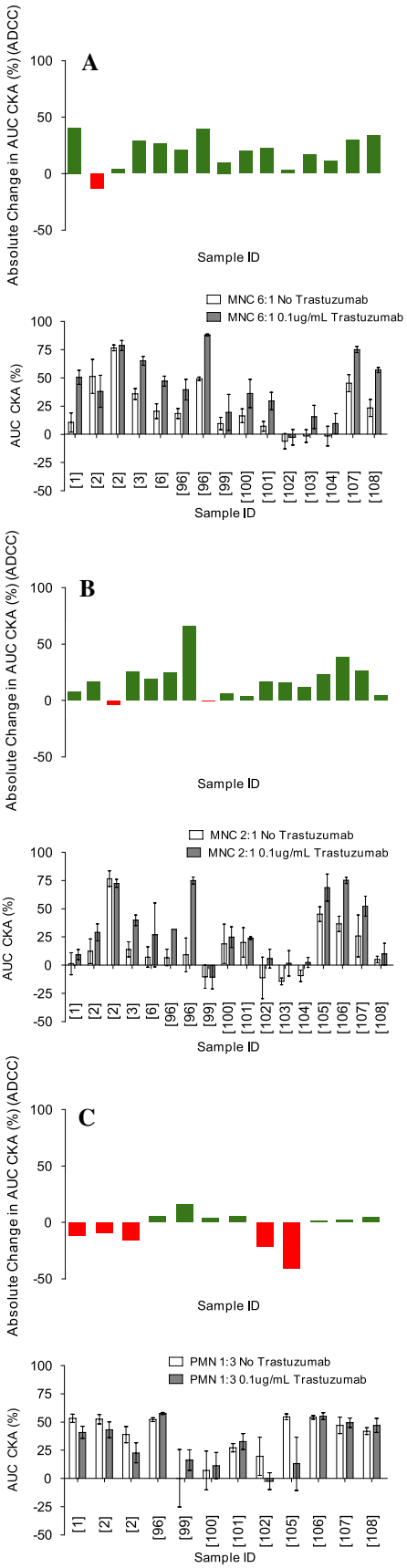

Fig. 5 Mononuclear cells and granulocytes isolated from 14 individuals show large variability of tratuzumab ADCC activity. Summary of ADCC killing by mononuclear cells or granulocytes from 14 individuals. BT-474 cells were added to a 96-well E-Plate and allowed to grow for 24–48 h. Blood collected from 14 different individuals was separated into mononuclear (*MNC*) or granulocyte (*PMN*) cell fractions. BT-474 cells were treated with media alone, trastuzumab (0.1 μg/ml), effector cells+/− trastuzumab. **a** MNC at 6:1 effector to target ratio without (*open bar*) or with trastuzumab (*filled bar*) (0.1 μg/ml). **b** MNC at 2:1 effector to target ratio without (*open bar*) or with trastuzumab (*filled bar*) (0.1 μg/ml). **c** PMN at 1:3 effector to target without (*open bar*) or with trastuzumab (*filled bar*) (0.1 μg/ml). The *upper part of each figure* gives the AUC difference due to the addition of trastuzumab (*ADCC*). The cell indices were monitored in triplicate and the average curves analyzed for relative area *under the curve* for cell killing activity as described in "Materials and methods"

standard errors for the AUC values are based on triplicate assays. Two of the individuals were done twice and two of individuals had been treated with trastuzumab. The range of cell killing due to the effector cell addition alone or with effector and trastuzumab is very different between individuals. The upper figures for A and B indicate that the difference due to trastuzumab in the mononuclear addition (i.e., ADCC activity) is also highly variable. These differences are highly significant ($P < 0.0001$). However, the addition of trastuzumab to the PMN fraction (Figure C upper) did not improve the cell killing effect ($P = 0.7615$). Interestingly, the individual previously (no. 104) and the individual currently treated with trastuzumab display two of the lowest base effector mediated cell killing measurements and also less pronounced ADCC activity at the 6:1 ratio of MNC.

Conclusion

In this study we show that in vitro selected trastuzumab-resistant BT-474 cells are sensitive to trastuzumab treatment in the nude mouse model. Surprisingly, in vivo trastuzumab inhibited both resistant cells and the sensitive parental BT-474 cell lines grown in the nude mouse with indistinguishable efficacy. Previously we have shown that resistant clones selected from parental BT-474 cells in vitro are very similar to the parental line, in that they maintain similar overexpression of Her2 expression, bind trastuzumab avidly and maintain a similar aneuploid status as measured by DNA index [14]. Our previous results showed that parental BT-474 cells are blocked in the G1 phase of the cell cycle and undergo a higher frequency of apoptosis with as little as 1 μg/ml (J.P. Vaughn and T. Kute, unpublished observations). However, in vitro selected resistant cell lines demonstrate none of these effects under the same conditions and proliferate in the presence of trastuzumab

with growth rates similar to untreated parental cells [14]. Yet, this clear growth advantage of the resistant BT-474 cell line in vitro does not translate into increased tumor growth in mice in the presence of trastuzumab.

Since both resistant and sensitive BT-474 tumor cells maintain similar Her2 expression levels (Fig. 1), one hypothesis that could explain our results is that tumor inhibition in vivo is primarily mediated through ADCC activity dependent on the amount of target Her2. If this is the case, one would expect resistant cells and sensitive BT-474 cells to be equally susceptible to ADCC. To test this supposition, we used a novel and efficient cell killing assay to measure the ability of innate immune cells to kill both trastuzumab-resistant and sensitive cells. We conclude that both cell lines can be killed with near equal efficiency by natural killer cells (NK-92) and both cell lines have similar increased sensitivity to trastuzumab ADCC mediated by NK-92 cells. In addition, our data suggests that both mononuclear cells and PMN cells can kill both kinds of tumor cells with similar efficiency, but ADCC occurs exclusively when trastuzumab is added to the mononuclear cells. The cell killing assays also gave similar results when pertuzumab was used instead of trastuzumab (data not shown). Therefore, we conclude that the similar capability of innate immune cells to utilize ADCC against both trastuzumab-resistant BT-474 tumor cells and parental BT-474 cells is compatible with the hypothesis that ADCC is the primary mechanism of tumor inhibition in the nude mouse model.

There are five mechanisms reported in the literature that may explain the acquired resistance to trastuzumab. These include: loss of expression of Her2 on the cell surface or mutation at the trastuzumab epitope [8, 11], excess mucin molecules (i.e., Muc-4) on the cell surface that prevent trastuzumab from binding [17, 21], loss of signaling alterations in the cell when trastuzumab binds [6, 14, 16, 18, 31], prevention of Her2 degradation by trastuzumab as seen in the extracellular domain levels in serum [13], and finally alterations in the immune system that suppresses the efficacy of trastuzumab occurring through the ADCC mechanism [7, 9, 28]. All of these resistance strategies have been demonstrated to result in trastuzumab resistance in vitro or in vivo. Down-regulation of Her2 or mutation of the trastuzumab epitope can be observed in vitro [11], but does not appear commonly in clinical samples [3, 9]. At this time, it is not clear which other resistant pathways are most significant or if more than one interact commonly to produce enhanced resistance. However, our data would suggest that loss of cell signal transduction inhibition is not critical for the development of resistance to trastuzumab in vivo.

Two previous in vivo studies have mutated the Fc region of trastuzumab and shown a dependence of tumor inhibition on that region that engages ADCC [1, 7]. However, these previous experiments showing trastuzumab-mediated ADCC occurred with co-inhibition of signal transduction, the effects of which have been shown to inhibit cell growth and weaken tumor cells. It was not known if efficient ADCC could occur without co-inhibition of signal transduction by trastuzumab. Our in vitro selected resistant cells allow us to test the efficacy of trastuzumab ADCC alone without the cytotoxic contribution of the inhibition of signal transduction. Our results suggest that inhibition of signal transduction may be of minimal contribution to the ability of trastuzumab to inhibit tumor growth and that ADCC can occur efficiently in tumors that are not growth inhibited. Her2 antibodies have been screened at great cost specifically to perturb signal transduction in vitro. Our results suggest that this may not be necessary. In fact it would seem logical that screening antibodies for efficacy of ADCC may be a better indicator of mAb performance in vivo.

To our knowledge only one other trastuzumab resistant cell line has been studied both in vitro and in vivo. Unlike the selection of our in vitro resistant clones, the breast cancer cell line JIMT-1 was isolated in vivo from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab [27]. Interestingly, when tumors were established in a nude mouse, and later challenged with trastuzumab (similar to our approach Fig. 2), the JIMT-1 cell line was shown to be resistant. This is clearly different from our observations. Overexpression of Muc-4 was found in JIMT-1 cells and suggested responsible for this observed in vitro and in vivo resistance [17]. However in a recent study, it was shown that co-injection of JIMT-1 cells and trastuzumab resulted in growth inhibition of the tumor [1]. This in vivo growth inhibition was suggested to be ADCC mediated because F(ab)2 capable of perturbing signal transduction could not inhibit tumor growth in vivo [1]. Our conclusions of the importance of ADCC are complementary to the JIMT-1 work but differ in cell type used, mechanism of in vitro resistance, and the methods used in the studies.

An important clinical pilot study has directly implicated ADCC in trastuzumab response of patients. The Costa group initiated a neoadjuvant study on women with primary breast cancer being treated with trastuzumab given 4 weeks prior to surgery [9]. The group's findings showed that all patients had high levels of trastuzumab in the serum and tissues. Comparison of pre-treatment and post-treatment samples showed no down regulation of Her2 expression in the tumor cells, no change in blood vessel diameter, no change in proliferation as measured by Ki67 staining, but the presence of a strong lymphoid cell infiltrate was observed in the post-treated tumor sample [9] In the study's limited number of responding patients, a correlation was found between trastuzumab response and ADCC killing [9]. A more recent

study further linked ADCC activity with clinical responses to trastuzumab based on flow cytometry analysis. Trastuzumab response was correlated with increased populations of NK cells [28].

Of patients with metastatic breast cancer that have Her2 overexpression only 12–34% benefit from trastuzumab alone suggesting the majority of tumors have unexplained "innate resistance" to trastuzumab [3, 29]. Since our results have shown that ADCC killing efficacy varies greatly in the population (Fig. 5), people who have poor pretreatment ADCC efficacy, as monitored from blood, might well be the patients that fail to respond to trastuzumab. It is interesting to note that two individuals who were on or had received trastuzumab prior to this study had very low killing effects. Currently, we are developing a prospective pilot study to determine if lack of efficient ADCC activity predicts lack of trastuzumab initial response or could predict the beginning of relapse for those patients who have had initial benefit from trastuzumab.

Our results support ADCC as the major mechanism of trastuzumab efficacy and the clinical implications are important. Few patients have a complete tumor response with trastuzumab/paclitaxel before relapse. Instead, in most patients, trastuzumab/paclitaxel inhibits disease progression for a period of time and then relapse occurs. This acquired resistance could be explained by failure of ADCC rather than selection of a rare resistant tumor phenotype. In such a model, the supply of appropriate cells enabling ADCC may become exhausted during the course of treatment. The ADCC model of trastuzumab raises questions concerning clinical intervention. Could one supply additional mononuclear cells with trastuzumab in an adaptive immunotherapy approach? Approaches such as macrophage adaptive immunotherapy alone have not yielded promising results as a cancer therapy in general, but the delivery of the effective killing cell plus the targeting mechanism (trastuzumab) together may offer a better approach. Could the population of cells that mediate ADCC be increased or stimulated by a cytokine therapy with trastuzumab/paclitaxol to improve outcome? Increasing the immune function by the combination of IL-2 with trastuzumab was attempted by Repka et al. by co-administering trastuzumab and IL-2 for 7 weeks, but no significant benefit was seen [22]. However, more work needs to be done with trastuzumab in this area especially considering that GM-CSF has been documented to stimulate rituximab immunotherapy in B-cell lymphomas [5]. It should be noted that the use of multiple antibodies also has been shown to increase the killing of breast cancer cell lines under in vitro and in vivo conditions [25, 26]. Collectively, these results suggest that the use of different antibodies as well as monitoring patients' ADCC activity may enhance mAb-based treatments for breast cancer. Better characterization of the patient's immune function might predict the response to mAb treatments.

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