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Understanding key assay parameters that affect measurements of trastuzumab-mediated ADCC against Her2 positive breast cancer cells

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Abbreviations: ADCC, antibody dependent cell mediated cytotoxicity; HER2, human epithelial receptor 2; SKBR3v, a variant of SKBR3 breast cancer cell line; CPT, cell preparation tube; E/T ratio, effector to target ratio; CI, cell index; MNC, mononuclear cells; AUC-16, area under the curve for 16 hrs after treatment

Use of the antibody trastuzumab to kill HER2$^+$ breast cancer cells is an attractive therapy because of its specificity and minimal adverse effects. However, a large fraction of HER2$^+$ positive patients are or will become resistant to this treatment. No other markers are used to determine sensitivity to trastuzumab other than HER2 status. Using the xCELLigence platform and flow cytometry, we have compared the ability of mononuclear cells (MNCs) from normal and breast cancer patients to kill different breast cancer cell lines in the presence (i.e., ADCC) or absence of trastuzumab. Image analysis and cell separation procedures were used to determine the differential contribution of immune cell subsets to ADCC activity. The assay demonstrated that ADCC activity is dependent on the presence of trastuzumab, the level of HER2 expression on the target, and the ratio of MNCs to tumor cells. There is a wide range of ADCC activity among normal individuals and breast cancer patients for high and low HER2-expressing tumor targets. Fresh MNCs display higher ADCC levels compared with cryopreserved cells. Natural killer cells display the highest ADCC followed by monocytes. T cells and B cells were ineffective in killing. A major mechanism of killing of tumor cells involves insertion of granzyme B and caspase enzymes via the antibody attached MNCs.

Introduction

Personalized medicine offers the best opportunity for successful treatments while minimizing side effects. In cancer patients this tends to be based on the characterization of the tumor and/or the surrounding environment. A good example is the development and use of trastuzumab (i.e., Herceptin) to treat breast cancer patients whose tumors overexpress HER2 on the cell surface. Trastuzumab is a specific monoclonal antibody that recognizes the tumor cell via its antigen (HER2) resulting in the specific killing of the tumor cells.[1,2] There have been some side effects reported for trastuzumab, but clinical data suggests they are minimal.[3] Recent reviews indicate that trastuzumab treatment extends disease free survival in both metastatic and non-metastatic clinical settings.[3-6] This treatment is usually taken for a year and it is fairly expensive. It should also be noted that there are other targeted treatment options for patients that overexpress HER2, including tyrosine kinase inhibitors to the HER2 oncogene.[7]

The actual mechanism for trastuzumab remains controversial. Kute et al.[8] as well as Sliwkowski et al.,[1] Citri et al.,[9] Nahta et al.[10] and Gennari et al.[11] provide reviews indicating the main mechanisms hypothesized to be involved in the killing process. Current thinking has become dominated by two main hypothesized mechanisms. First, there is data indicating that cell signaling is affected by trastuzumab. The binding of trastuzumab to HER2 results in apoptosis, inhibition of cell growth, and even inhibition of angiogenesis. Second, there is data from Clynes et al.,[12] Arnould et al.[13] and Varchetta et al.[14] indicating an immunological pathway whereby trastuzumab targets the tumor cells for elimination via the antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism resulting in the patient's immune system selectively killing the tumor cells. Regardless of the mechanism, only 20 to 30% of patients treated with trastuzumab will have a significant clinical response.[3,4,15] Therefore, it is important to understand the causes of resistance or lack of response in these patients.

We previously reported on the effects of trastuzumab[16] and the development of several cell lines that were resistant to trastuzumab while maintaining HER2 surface expression.[17] Their resistance stemmed from a cell signaling change as previously

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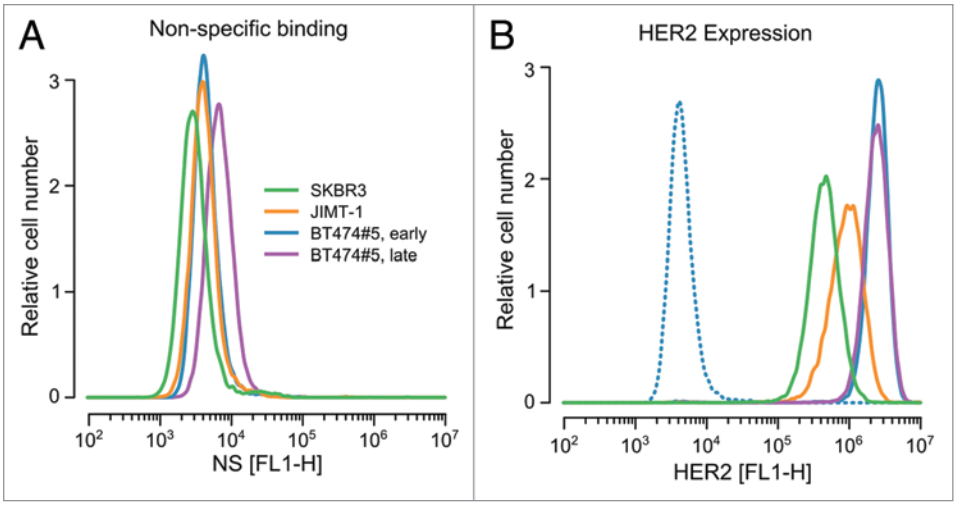


Figure 1. HER2 expression varies among three different breast cancer cell lines. Cell surface levels of HER2 were measured by indirect immunofluorescent staining and flow cytometry. Non-specific binding of the secondary antibody is shown in (**A**). HER2 levels are represented in (**B**) for the cell lines SKBR3 v (green), JIMT-1 (orange), BT474 clone 5 early passage (blue) and BT474 clone 5 later passage (violet). Non-specific staining of BT474 clone 5 early passage also is shown by the dashed line in (**B**).

described.[17] However, it was found that these cell lines, which were resistant, based on in vitro studies, were still sensitive to trastuzumab treatment using an in vivo nude mouse model.[18] In that study, the importance of the immune system was highlighted using in vivo and in vitro model systems where immune cells are present during the treatment.

In this report, we define a method for quantifying a person's ADCC activity utilizing the xCelligence system developed by ACEA Biosciences and Roche Applied Science.[19-21] The purpose of this study was to define key parameters of the assay to determine the feasibility of using it for a future clinical study to determine if efficacy of cell killing from a patient blood sample is a prognostic factor for response to trastuzumab treatment.

Results

Target characterization for the ADCC assay. The expression level of HER2 on three cell lines determined via flow cytometry is provided (**Fig. 1**). The highest level of expression for HER2 is BT474 clone 5 which has previously been shown to be resistant to trastuzumab under in vitro (cell culture as opposed to in nude mice) conditions.[17] This is followed by the JIMT-1 cell line that was isolated from a trastuzumab resistant patient and still displays surface expression of HER2.[22] Finally, an SKBR3 variant (SKBR3v) was identified that has low expression of HER2. The relative expression levels are 100%, 40% and 20% for BT474 clone 5, JIMT-1 and SKBR3v, respectively. Early passages of BT474 clone 5 displayed similar expression levels of HER2 as did later passages indicating this target is stable in its expression of HER2.

The xCELLigence system was used for the measurement of % cell kill of tumor cells by antibody alone, by mononuclear cells alone and by mononuclear cells in the presence of antibody (see material and methods for further details). The initial seeding density of the targets was determined based on the manufacturer's recommendation of a cell index (CI) value of at least 1 to 2 prior to any treatments. Titrations of the targets were performed and 20,000 cells were optimal for BT474 clone 5 while 5,000 cells were optimal for the SKBR3 variant as the initial seeding density.

The effect of trastuzumab alone was determined against the targets by performing a dose response for trastuzumab against BT474 clone 5 and the SKBR3v cell lines. Approximately 24 h after the initial seeding, 100 µl of media was removed and 100 µl media of a serial dilution of trastuzumab was added. These results indicate little change in CI value due to trastuzumab compared with the control (**Fig. 2**). Our studies used 0.1 µg/ml of trastuzumab which may correlate to the level in the tissue. The mean measured level in serum or in cerebrospinal fluid of patients treated with trastuzumab was 20 µg/ml and 0.12 µg/ml respectively.[23] In addition, a low enough concentration was needed so the additive/synergistic effects could be observed within the assay.

Effect of MNCs on BT474 clone 5 with different E/T ratios. The measurement of ADCC activity depends on the ratio of effecter cells to target cells (E/T). In **Figure 3**, increasing numbers of MNC isolated from human blood (effector cells) were co-incubated with BT474 clone 5 cells (target cells). **Figure 3A** demonstrates that as the E/T ratio increases from 0.5 to 6.0 in the absence of 0.1 µg/ml of trastuzumab, there are only modest changes in the CI value compared with the control within the first 16 h after initiation of co-incubation (see **Fig. 3**, arrow between two dashed vertical lines, earliest vertical lines begins co-incubation time with MNC) and this appears to be an increase in CI not a decrease as would be expected for cell killing. At a 6:1 ratio, there is a significant increase in the CI value for unknown reasons and after the first 16 h there is a decrease in CI possibly due to nonantibody dependent cell killing by MNC or loss of cell viability due to excess cells with limited media. In an additional control, MNCs at various concentrations without targets were plated and no change in the CI over a 24 h period was observed (data not shown). Therefore addition of MNC alone does not change the CI values over a 16 h period. **Figure 3B** shows a decrease in cell index over time as the E/T ratio increases in the presence of 0.1 µg/ml of trastuzumab indicating ADCC as the main mechanism of cell killing. Our analysis indicated that monitoring the area under the curve (AUC) for 16 h after treatment was a good indicator for killing activity and this was defined as AUC-16 (see brackets with arrow for time interval). The AUC-16 values for the specific E/T ratios in the absence of trastuzumab range from 24.7 to 22.9 with the control being 20.6. The AUC-16 values for the specific dilutions

in the presence of trastuzumab range from 9.0 to 22.9. The ADCC values, which is defined as the difference in % cell kill when trastuzumab is add, was essentially 0% when trastuzumab was added alone. With increasing E/T ratios of 0.5:1, 1:1, 2:1 and 6:1, the ADCC values were 14.0%, 26.2%, 40.6% and 74.7%, respectively.

ADCC activity against different target tumor cells using MNCs and U937 cells as effectors. **Table 1** shows data from a representative experiment with four different cell line targets and two different effector cells. U937 cells, a monocyte cell line, were used as a positive killer effector control in these studies since they are easy to culture and provide reproducibility between experiments. The AUC-16 values for the four cell lines with media alone ranged from 16.9 to 33.6. The addition of media containing 0.1 μg/ml of trastuzumab to the four cell lines gave an AUC-16 value between 16.9 to 30.9 and a calculated % cell kill ranged from -7.6 to 8.0. By ANOVA, these changes are not statistically significant. U937 cells at an E:T ratio of 6:1 (killer cells:target cell) ranged in % cell kill from -8.5 to 14.9 with only the SKBR-3v target showing a significant difference between the controls. U937 cells at a 6:1 ratio in the presence of 0.1 μg/ml of trastuzumab, displayed a % cell kill that ranged from -0.2 to 50.1 where target BT474 clone 5 gave the highest value followed by values of 23.9 and 17.2 for SKBR3v and JIMT-1 cells respectively, with MCF-7 cells displaying the lowest effect of -0.2. The addition of MNCs at an E:T ratio 6:1 or 2:1 ratios resulted in a wide range of % cell kill where the curves in some cases were higher than the control curve and gave a negative % cell kill while in other cases there was a high positive % cell kill especially in SKBR3v. However, the addition of 0.1 μg/ml of trastuzumab (ADCC effect) demonstrated a clear dose response curve similar to **Figure 2**. The most effective target for ADCC activity at the 6:1 ratio was BT474 clone 5 (84.3% ADCC killing), followed by JIMT-1 cells (33.2% ADCC killing), followed by SKBR3v (21.2% ADCC killing), and MCF-7 (20.9% ADCC killing) which displayed similar results. The most effective target for ADCC activity at 2:1 was again BT474 clone 5 and the other three cell lines displaying minimal ADCC activity. These studies lead us to select BT474 clone 5 and SKBR3v as targets for future work.

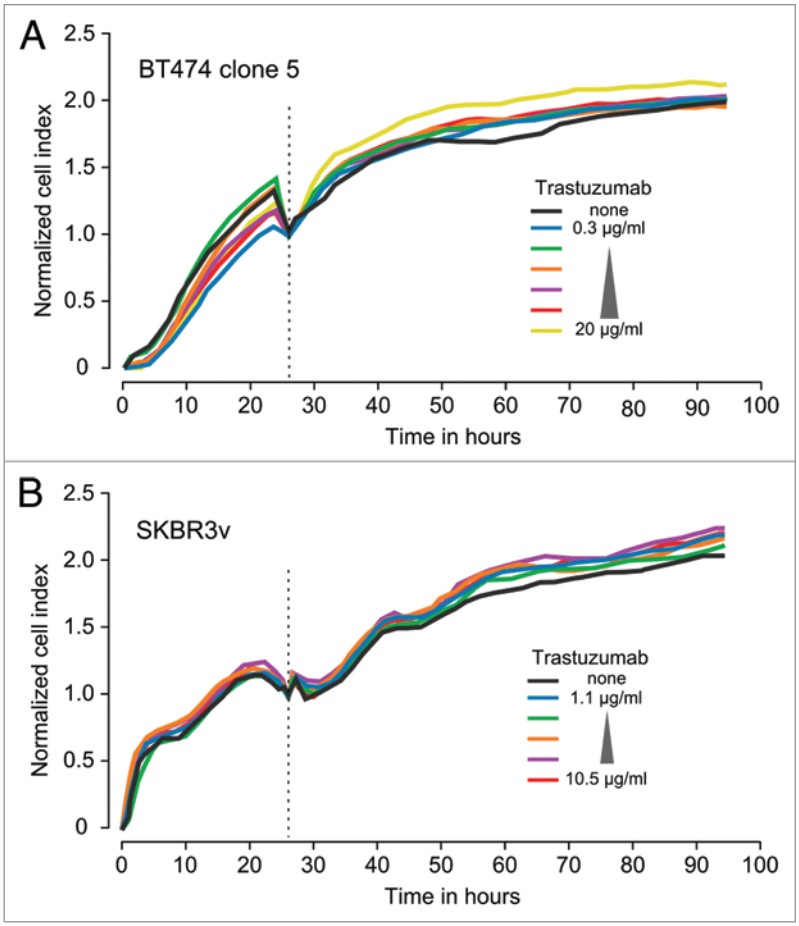


Figure 2. Trastuzumab alone does not affect the normalized cell index for BT474 clone 5 or SKBR3v cells as measured by the xCELLIgence system. BT474 clone 5 cells (**A**) or SKBR3v cells (**B**) were cultured for approximately 26 h and then were treated with increasing concentrations of trastuzumab. The black curve is derived from cells maintained in the absence of trastuzumab while the other colors represent increasing concentrations of trastuzumab as indicated. The cell index (CI) was determined every 15 min over the course of the experiment and normalized to 1 at the time of trastuzumab addition. Curves represent the average of three replicates. Because the standard deviations for each curve overlap, they are not shown in this figure, and growth differences were considered to be insignificant.

Assay variability over time and among different individuals. **Figure 4** shows histograms representing % cell kill and ADCC activity of MNCs from 23 individuals and U937 cells at an E/T ratio of 6:1. Left side parts are in desending order of total killing by effector and trastuzumab together and the right side parts show ADCC based killing alone in the same sequence. The MNCs alone (Effectors alone) from some individuals elicited a "negative" cell killing of BT474 clone 5, which was due to increased cell growth or adherence in the absence of trastuzumab. SKBR3v cells were more readily killed in the absence of trastuzumab, suggesting that SKBR3v cells are more sensitive than the BT474 clone 5 by some non ADCC pathway. Although substantial intra-sample variability was noted in some individuals (e.g., MNC#6 standard deviation), there was no relationship between cell killing ADCC activity and the date of the assay and/or age of the individual. However, additional experimental data are required to confirm these results. We note a wide range and variability of ADCC activity between individuals and between targets. Target cells that have the most HER2 expression (i.e., BT474 clone 5) demonstrated a range of ADCC activity from 21% to 56% with a median value of 41.8% while the range for lower expressing HER2 cells (i.e., SKBR3v) demonstrated a range of -8.4% to 47.3% with a median value of 16.8. During these studies, we also analyzed 6 different breast cancer patients who were candidates for trastuzumab therapy. In these individuals, the range for ADCC activity in BT474

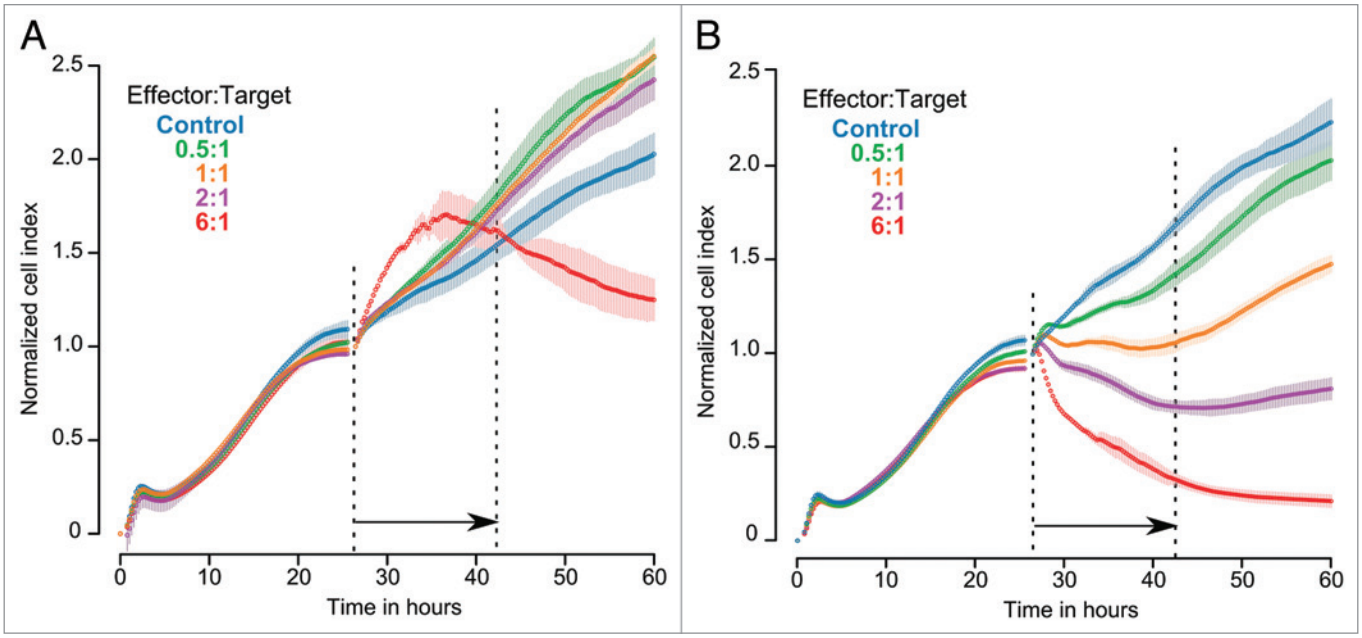


Figure 3. Cell index (CI) values are proportionally reduced with increasing effector to target (E/T) ratios in the presence of trastuzumab. BT474 clone 5 cells were maintained for 26 h and then were treated with media alone (control) or with media plus mononuclear cells isolated from human blood (**A**). Cells were treated in an identical fashion in (**B**) except for the inclusion of 0.1 μg/ml of trastuzumab. Cell index values were normalized at the time of addition. Blue represents growth with no mononuclear cells (control) while green, orange, purple and red represents growth in the presence of MNCs at E/T ratios of 0.5/1, 1/1, 2:1 and 6/1, respectively. The vertical dashed lines indicate the 16 h window of time after treatment used to determine AUC values. Normalized cell index values are plotted in 15 min increments as the average of three replicate with the standard deviation.

clone 5 was 27.2% to 65.5% with a mean of 43.5%, while the range of ADCC activity against SKBR3v was 6.5% to 23.7% with a mean of 17.5%. The ADCC activity against the high HER-2-expressing BT474 clone 5 is significantly elevated compared with the lower expressing SKBR3v for all cancer patients and most normal individuals. The wide range for ADCC measured against both cell lines suggests these targets may permit discrimination among different individuals. The similar results obtained with either continuously cultured U937 cells or U937 cells that were thawed just prior to the analysis demonstrate a stable phenotype for this monocyte cell line indicating a possible use as a standard for our studies between different ADCC killing experimental set ups.

Effector storage characterization (fresh vs. frozen MNCs). To evaluate changes in activity following cryopreservation, cell killing associated with MNCs from seven individuals was analyzed immediately after isolation and after storage for at least seven days in liquid nitrogen. The results using BT474 clone 5 (**Table 2**) show that some ADCC activity was preserved in the cryopreserved samples. However, the ADCC value declined after freezing where the mean value of 58.9% for fresh cells was reduced to 26.7% for frozen cells. The basis for this decline was evaluated by measuring cell killing associated with immune cell subtypes before and after cryopreservation. This analysis showed that NK cell activity was especially susceptible to the freezing and thawing process. Because the monocyte cell line U937 showed no significant change in the ADCC activity (**Fig. 4**) following cryopreservation, these results may suggest that monocytes in general may tolerate cryopreservation.

Underlying biology for the ADCC/assay activity. To elucidate the contribution of the various immune cell subtypes to ADCC activity, the MNCs were fractionated with immunoaffinity (Miltenyi) beads, and the assay was performed at 6:1, 2:1 and 1:1 E/T ratios. The ADCC activity at 6:1 for the MNCs alone was 28.9% while the ADCC activity for the separated NK cells, monocytes, B cells and T cells was 66.9%, 32.8%, 6.9% and 7.3%, respectively (**Fig. 5**). Therefore based on killing efficacy per cell and percent of subtype population it appears that NK cells were the dominant ADCC killing cell type in this sample. If one assumes an additive effect of ADCC activity based on the relative abundance of each subtype in the MNCs, the theoretical ADCC activity for the unfractionated MNCs was predicted to be 19%, which is similar to the observed value of 28.9%. As the ratios for E/T were reduced, the ADCC activities were reduced in a proportional manor in all of the samples (data not shown).

In **Figure 6** the role of the NK cells and monocytes in ADCC activity was confirmed by direct imaging. BT-474 clone #5 cells were treated with MNCs alone at 6:1 or MNCs at 6:1 in the presence of 0.1 μg/ml of trastuzumab. Since the xCELLigence study indicated a specific decrease in cell index after 5 h of exposure to trastuzumab, the co-cultivated cells were analyzed by fluorescence microscopy after staining with an antibody to CD16 to visualize the NK cells and monocytes among the effectors and an antibody to HER2 to visualize the target cells (**Fig. 6**). In the presence of trastuzumab (**Fig. 6B**), MNCs appear more abundantly associated with the tumor cells compared with cultures with only MNCs (**Fig. 6A**). Staining for CD16 indicates that a

Table 1. Cell killing measured by changes in the cell index with four target cell lines and two effector cell populations

Target cell line	Effector population	E:T ratio[a]	Changes in cell index				ADCC effect (%)[d]
			Effector alone		Effector + Trastuzumab		
			AUC-16[b]	Cell killing[c]	AUC-16[b]	Cell killing[c]	
BT474 clone 5	none	NA	22.8	0	24.5	-7.6	-7.6
	U937	6:1	21.7	4.7	11.4	50.1	45.4
	MNC	6:1	11.4	-21	8.3	63.4	84.3
	MNC	2:1	27.9	-22.5	15.2	33.1	55.6
SKBR3v	none	NA	16.9	0	16.9	0.4	0.4
	U937	6:1	14.4	14.9	12.9	23.9	9.0
	MNC	6:1	12.7	25.3	9.1	46.5	21.2
	MNC	2:1	14.6	13.8	14	17.1	3.3
JIMT-1	none	NA	20.7	0	21.8	-5.2	-5.2
	U937	6:1	18.8	9.2	17.1	17.2	8.0
	MNC	6:1	20.2	2.3	13.4	35.5	33.2
	MNC	2:1	20.4	1.4	21.9	-5.7	-7.1
MCF-7	none	NA	33.6	0	30.9	8.0	8.0
	U937	6:1	36.4	-8.5	33.6	-0.2	8.3
	MNC	6:1	39.1	-16.6	32.1	4.3	20.9
	MNC	2:1	42.6	-27.0	41.3	-23.1	3.9

[a]Effector cell to target cell ratio. NA indicates not applicable; [b]area under curve from the start of treatment at 0 h to16 h; [c]100 x [control AUC-16 (no effector) - AUC-16 with effector]/[control AUC-16 (no effector)]; [d]difference between cell killing by effector alone and effector with Trastuzumab (0.1 μg per ml).

Table 2. Changes in the fraction of MNC subtypes and MNC ADCC activity in once-frozen MNCs

Donor	Fresh MNCs					Fresh/Frozen MNCs				
	Lymphocyte subtype (%)				ADCC effect[e]	Lymphocyte subtype (%)				ADCC effect[e]
	NK[a]	B[b]	T[c]	Monocyte[d]		NK[a]	B[b]	T[c]	Monocyte[d]	
MNC#1	28.5	4.2	67.3	26.0	59.7	17.5	14.5	68.0	16.0	45.2
MNC#2	12.0	8.8	79.2	15.8	60.0	9.8	5.4	84.8	21.1	37.7
MNC#3	21.4	22.9	55.7	22.7	73.2	15.1	11.5	73.4	25.3	22.6
MNC#4	17.9	11.3	70.8	18.2	55.2	10.2	11.2	78.6	21.0	30.4
MNC#5	13.5	7.3	79.2	19.5	52.8	5.7	16.0	78.3	10.1	17.8
MNC#6	11.1	5.7	83.2	14.1	67.6	4.7	21.8	73.5	4.9	8.6
MNC#7	10.3	7.7	82.0	14.9	37.8	5.2	16.5	78.3	16.3	24.8
Average (SD)	16.4 (6.7)	9.7 (6.2)	73.9 (9.9)	18.7 (4.4)	58.0 (11.4)	9.7 (5.0)	13.8 (5.2)	76.4 (5.3)	16.4 (7.0)	26.7 (12.3)

[a]Natural killer cells selected as $CD45^+$ $CD3^-$, $CD56^+$ and $CD16^+$; [b]B cells selected as $CD45^+$ and neither T cell nor NK cell; [c]T cells selected as $CD45^+$ and $CD3^+$; [d]monocytes selected as $CD45^+$, $CD14^+$ and $CD3^-$; [e]ADCC effect is the change in cell killing due to Trastuzumab with unfractionated MNCs as described in the Methods section.

significant portion of the MNCs sticking to the HER2-positive tumor cells are NK cells and possibly monocytes.

The PanToxiLux (OncoImmunin, Inc.) assay (see material and methods for further details) was used to determine if granzyme B and or upstream caspase activity are involved in the killing of tumor cells in this assay. This assay measures the ability of granzyme B and upstream caspases to be transported from effector cells into the tumor targets. Such a mechanism is possible when the effector cell attaches to the tumor cell through an antigen-antibody complex such as the FCγ receptor-trastuzumab complex. An example of how this assay is performed and analyzed is shown in **Figure 7**. One observes in **Figure 7A** the red fluorescent target cells (R1) and **Figure 7C** the non-red effector cells. In **Figure 7B**, one observes that mixing of the red tumor cells with the non-red effector cells in the presence of a specific non-fluorescent enzyme substrate, PanCyToxiLux, results in two distinct clusters of cells that are red and non-red. If trastuzumab is added to target cells, effector cells and substrate (**Fig. 7C**), the target cells move from red only (R1) to cells having both red and green fluorescence (R2) because the enzymes activate this

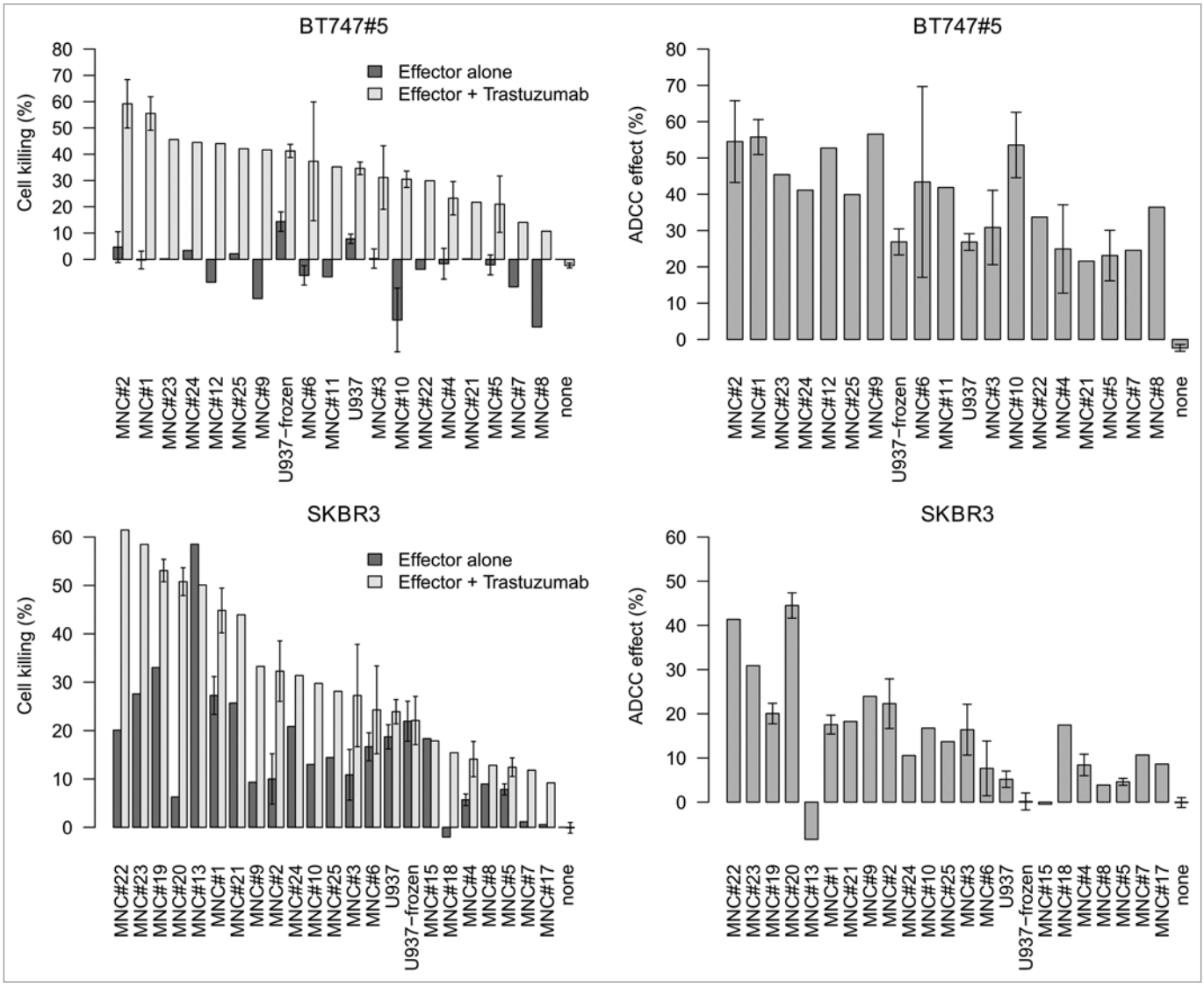


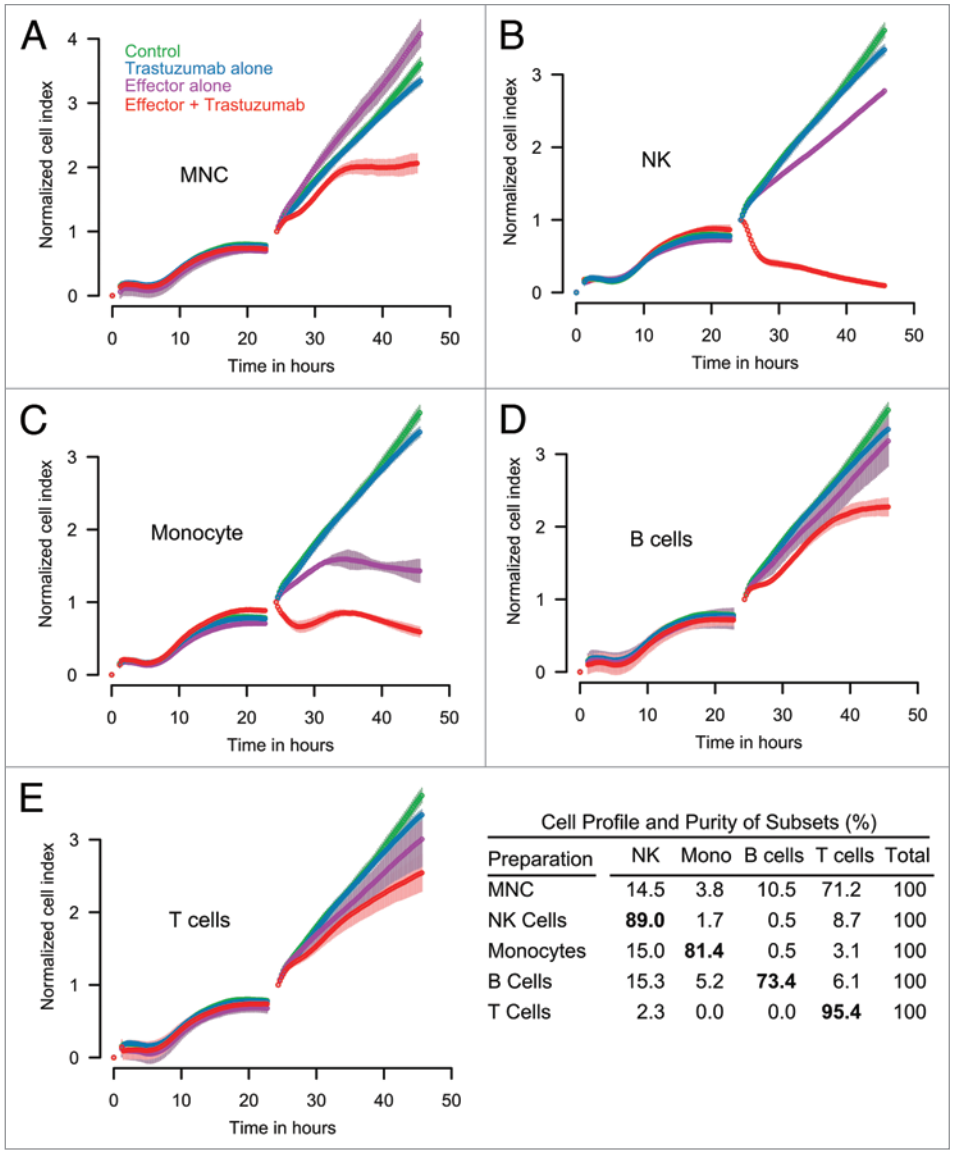
Figure 4. ADCC activity varies widely among 23 individuals and target cell type. Mononuclear cells from 23 individuals or U937 cells were applied to BT474 clone 5 target cells (top parts) or SKBR3v cells (bottom parts). All E/T ratios were 6:1. Samples replicated three or more times are presented as the mean and standard deviation. The light bars and dark bars represent percent cell kill mediated by effectors with and without trastuzumab, respectively. The ADCC activity represents the difference in percent cell kill due to the addition of 0.1 ug/ml of trastuzumab and is represented in the charts of histograms to the right in the same sequence order of samples.

substrate and now make it fluoresce green. In this study, 31% of the target cells have been activated via the transported enzymes. **Table 3** provides a quantitative description of an increase in the percent activation in granzyme B and upstream caspase activity when effector cells are added in the presence of trastuzumab with no increase seen when effector cells are added in the absence of trastuzumab. In addition, there is less activation when SKBR3v is used as the target. This data confirms that ADCC specific killing appears to depend upon granzyme B and caspase activity.

Discussion

This study describes a method for the analysis and quantification of ADCC killing by trastuzumab using an xCELLigence instrument and a PanToxiLux assay. Similar results were observed using the two different methods to measure ADCC activity. These data suggest that granzyme B and upstream caspases are involved in the killing of the tumor cells. The advantage of the xCELLigence system is it measures the rate of killing, is mostly reproducible, and can be standardized with the main disadvantage residing in the equipment cost. The advantage of the PanToxiLux assay is it is less expensive if not purchasing a flow cytometer, it can be done rapidly, and it measures specific enzyme activity. In addition, frozen down labeled target cells can also be used for this assay (data not included). The disadvantage is that not all effector cells will use these enzymes as a mechanism of action and the standardization may be difficult.

Four breast cancer cell lines were used in the studies to analyze ADCC killing with multiple MNC preparations and a control U937 cell line. Our data show that a low, physiologic dose of (0.1 μg/ml) of trastuzumab has little, if any, effect on CI values for any of the cell lines when compared with the untreated target cells. Even when the trastuzumab was increased to 20 ug/ml, there was no change in the CI values over time for BT474 clone 5 and SKBR3v cells. It was previously demonstrated that BT474 clone 5 is resistant to trastuzumab based on in vitro studies but still overexpresses HER2 and is still sensitive to trastuzumab under in vivo conditions.[17,18] These cells were used as targets to minimize the intracellular cell signaling component of trastuzumab killing.[18] It should be noted that the parental BT474 cell line is sensitive to killing by trastuzumab as measured by cell counting, flow cytometry, animal studies and xCELLigence profiles.[16-18] The SKBR3 cells used in this study have been defined as a variant since these cells no longer overexpress HER2 at similar levels to BT474. Several investigators have measured SKBR3 for HER2 expression levels and found them to be similar to BT474. The cell line described here, SKBR3v, expresses only 20% of the HER2 levels observed in BT474 cells based on flow cytometry measurements. These cell lines remained stable based on flow cytometry expression of HER2 and therefore were considered good targets for representing high and low expression of HER2.



Cell Profile and Purity of Subsets (%)

Preparation	NK	Mono	B cells	T cells	Total
MNC	14.5	3.8	10.5	71.2	100
NK Cells	**89.0**	1.7	0.5	8.7	100
Monocytes	15.0	**81.4**	0.5	3.1	100
B Cells	15.3	5.2	**73.4**	6.1	100
T Cells	2.3	0.0	0.0	**95.4**	100

Figure 5. Natural killer cells exhibit the greatest ADCC activity among sub-populations of mononuclear cells. MNCs were tested for ADCC killing effect (**A**) or were separated into sub-populations and then tested. (**B**) Shows NK cells, (**C**) shows monocytes, (**D**) shows B cells and (**E**) shows T cells. Green lines represent the control, blue show 0.1 ug/ml of trastuzumab alone, violet shows MNCs or sub-populations at E/T of 6 to 1, and red shows MNCs or sub-populations at E/T of 6 to 1 in the presence of 0.1 ug/ml of trastuzumab. The flow cytometry results measuring the distribution of immune subtypes among purified cells and MNCs are given in the Table insert at the bottom right.

In this study, the ADCC activity is dependent on the E:T ratios (**Fig. 1**), is reproducible over multiple samples from the same individual against two targets and does not display changes over time. In order to be clinically meaningful, there needs to be significant variation for the ADCC activity among individuals which indeed was observed. Furthermore, it is clear that the expression of HER2 affects the ability of the MNCs to kill in the presence of trastuzumab. The range and median or mean values of MNCs for ADCC activity overlap between normal individuals and breast cancer patients who are HER2 positive and will be treated with trastuzumab. This range is very broad and we hope our clinical study comparing trastuzumab response to ADCC activity will be correlated in our future studies. Our attempts to use cryopreserved MNCs indicated that the ADCC activity is greatly diminished compared with the fresh MNCs. The loss of ADCC activity following cryopreservation correlated with the loss of NK cells during the cryopreservation process, which seems to be a likely reason for decrease in ADCC activity.

Since this a biological assay, it is important to have a reproducible positive control for ADCC killing. Our use of U937 cells provides a reproducible effector cell control that acts differently in the two targets. In the BT474 clone 5, the addition of U937 cells alone has minimal effect, but when trastuzumab is added, there is a significant decrease in cell index. In SKBR3v, one observes a relative decrease in cell index when U937 cells

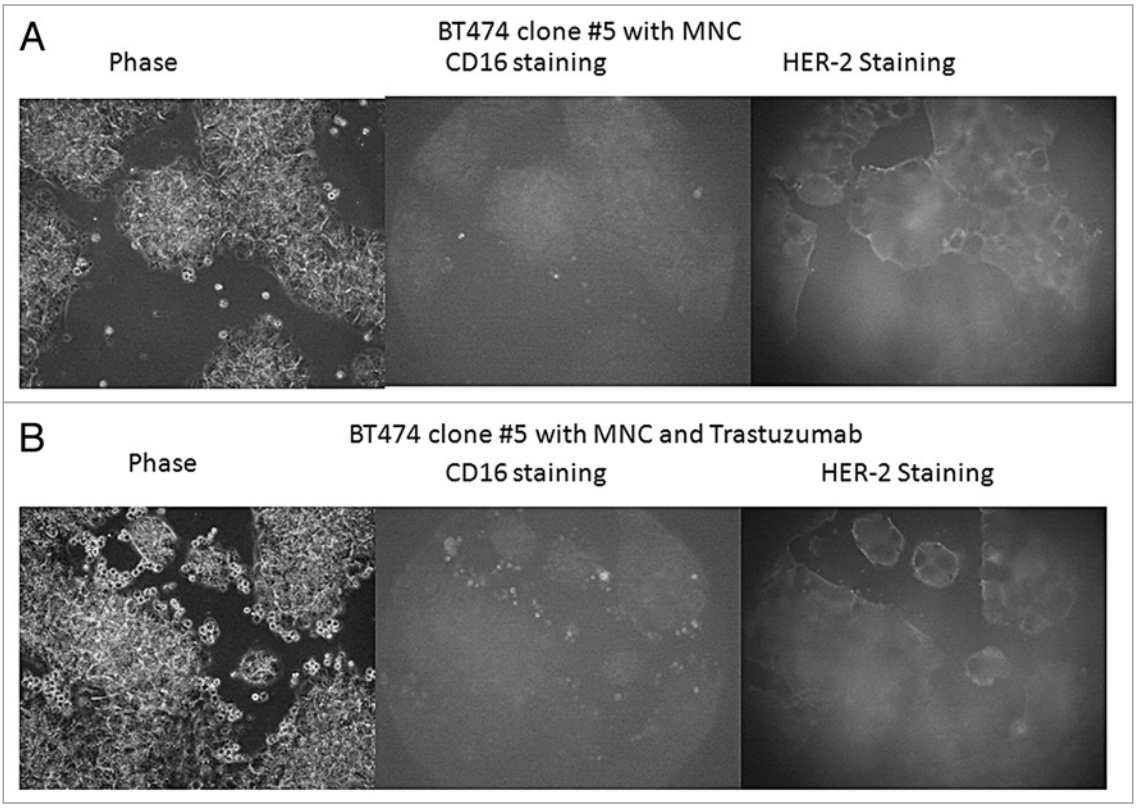


Figure 6. Trastuzumab increases the number of CD16$^+$ cells associated with BT474 clone 5 cells during ADCC. BT474 clone 5 cells were treated with MNCs in the absence (**A**) or presence of 0.1 μg/ml of trastuzumab (**B**) in a 6-well plate for analysis by immunofluorescence microscopy or in a 96-well plate for analysis by the xCELLIgence system. After significant ADCC activity was detected by the xCELLigence system, cells in the 6-well plate were analyzed by immunofluorescence microscopy. Phase contrast images are shown in the left parts, staining for CD16 in the middle part and staining for HER2 in the right parts.

are added alone but there is little further decrease in the presence of trastuzumab. It also should be noted that this cell line can be frozen and thawed immediately prior to use with similar results.

These studies suggest that the major immune cell subtype contributing to the ADCC effect are NK cells. This is based on measuring ADCC activity following MNC fractionation with Miltenyi immunoaffinity beads and by image analysis. This is in agreement with other investigators[24] that have demonstrated that the Fcγ receptor is critical for the trastuzumab to interact with the effector cell and bring the effector cell into a "kiss of death" relationship with the target. The Costa group initiated a neoadjuvant study on women with primary breast cancer being treated with trastuzumab given 4 weeks prior to surgery.[11] Comparison of responding vs. non-responding patients, indicated no change in serum or tissue levels of trastuzumab, no change in HER2 expression in the tumors, no change in blood vessel diameter, and no change in proliferation as measured by K_i-67 staining. They did demonstrate a relationship of response to an increase in infiltrating leukocytes and a higher ADCC activity. Varchetta et al. have further shown that the ADCC activity for trastuzumab was related to number of NK cells present.[25]

Additional studies are needed to compare the relationship of trastuzumab response in breast cancer patients with the assay described in this study. It would be of great value to determine if patients with low ADCC activity (lower tertile) do not respond as well to trastuzumab as those patients with a high degree of ADCC activity (upper tertile) based on this assay. The patients with low activity might then be candidates for other treatment such as lapatinib, a tyrosine kinase inhibitor, or T-DM1, a trastuzumab convalantly bound to maytansine which is a microtubule-disrupting drug. These compounds target the HER2 positive cells but are not thought to use the immune system to kill tumor cells. Another question of importance is whether this ADCC activity is affected by chemotherapy or other treatments (surgery, etc.) the patient is undergoing. If the patient displays depressed ADCC due to chemotherapy, then it may be clinically beneficial to wait until the patient's activity is high before treating with trastuzumab. Although these studies were limited to trastuzumab only, similar studies could be performed with any of the other monoclonal antibody treatments since any monoclonal antibody therapy has a high likelihood of displaying a similar mechanism of action.

Materials and Methods

Cell lines and HER2 antibodies. BT474, SKBR3 and U937 were obtained from American Type Tissue Culture Collection

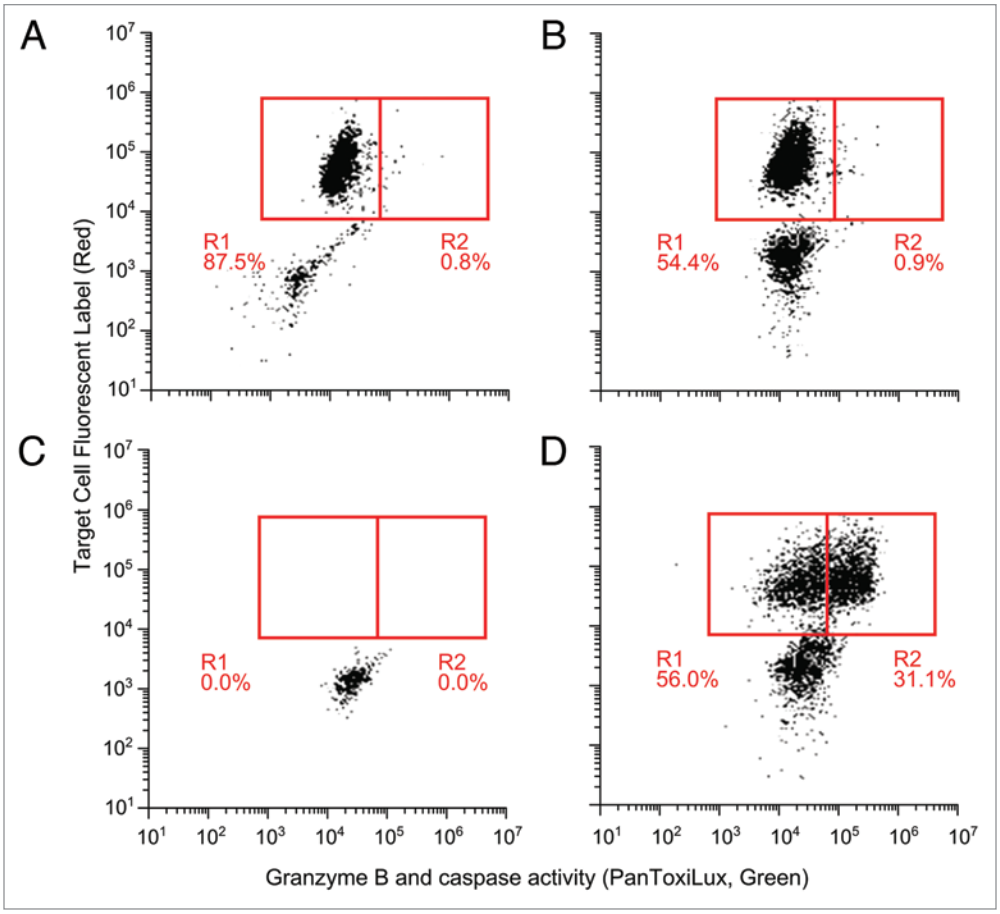


Figure 7. The "kiss of death" assay shows increased granzyme B and caspase activity in target tumor cells in the presence of trastuzumab. Target cells labeled with the fluorescent dye, TFL4, alone (**A**) or effector cells not labeled with TFL4 (**C**) were analyzed by flow cytometry. TFL4-labeled target cells were mixed with effector cells at an E/T ratio of 14:1 alone (**B**) or with 0.1 μg/ml of trastuzumab (**D**). Values indicated for region R1 indicate the percentage of target cells with no green fluorescence (indicating no granzyme B and caspase activity). Values associated with region R2 identify the percentage of target cells (red fluorescence) with granzyme B/caspase activity (green fluorescence).

(ATTCC, Manassas, VA USA) and maintained according to the recommendations of the ATCC. The resistant BT-474 cell clone 5 was obtained from selection after treatment with trastuzumab for greater than 2 weeks and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA USA) with penicillin/streptomycin, 5% L-glutamine and 10% FBS.[17] The SKBR3 variant (SKBR3v) of the parental cell line with lower HER-2 expression was obtained during culturing of the cells and was maintained in the same media as the BT474 clone 5. The JIMT-1 cell line was a gift from Dr. Janos Szollosi and was grown in DMEM/F12 with penicillin/streptomycin, 5% l-glutamine and 10% FBS. Trastuzumab (Genentech, South San Francisco, CA USA) was obtained from the Wake Forest University North Carolina Baptist Hospital pharmacy.

HER2 expression. The expression of HER2 was determined by flow cytometry using indirect fluorescent staining methods.[18] 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. The percent of cells with specific staining and the intensity of staining were determined by Accuri-6 C Software.

Isolation and characterization of mononuclear cells (MNCs). Blood was collected from individuals that provided consent and all protocols were approved by the institutional review board. The MNCs were isolated using the cell preparation tube (CPT) protocol as defined by Becton Dickinson Inc. After washing with media to remove the sodium citrate, the cells were counted and diluted to the recommended concentrations after counting with a hemocytometer. The effector to target (E/T) ratios ranged from 1:2 to 6:1 based on the target cells initial seeding. Where reported, the excess cells were analyzed for lymphocyte subpopulations and monocyte concentrations using flow cytometry (see below). Where indicated, the MNCs were frozen down using a standardized protocol (90% fetal bovine serum, 10% DMSO) by freezing them initially at -80°C before being transferred to liquid nitrogen for long-term storage. When used, these MNCs were then thawed and washed to remove DMSO prior to use in the studies.

Measurements via xCELLigence system. The xCelligence system measures real time proliferation or cell death in each well of a specialized 96 well plate. This plate has electrodes at the bottom of each well, which measures cell adherance. The

Table 3. Activation of granzyme B and caspase activity in two target cell lines by MNCs at different effector: target ratios

Effector	Target	E/T ratio[a]	Subs[b]	Trast[c]	Target cell status		
					Activated[d]	Non-activated[e]	% activated[f]
none	BT474 clone 5	NA	no	no	2	2026	0.1
none	BT474 clone 5	NA	yes	no	23	2615	0.9
Donor MNCs	none	NA	no	no	0	1	0
Donor MNCs	none	NA	yes	no	0	0	0
Donor MNCs	BT474 clone 5	14:1	yes	no	47	2852	1.6
Donor MNCs	BT474 clone 5	14:1	yes	yes	1323	1057	55.6
Donor MNCs	BT474 clone 5	6:1	yes	no	337	5586	5.7
Donor MNCs	BT474 clone 5	6:1	yes	yes	1235	2696	31.4
none	SKBR3v	NA	no	no	0	7038	0
none	SKBR3v	NA	yes	no	59	3804	1.5
Donor MNCs	none	NA	no	no	0	27	0
Donor MNCs	none	NA	yes	no	0	1	0
Donor MNCs	SKBR3v	14:1	yes	no	0	1730	0
Donor MNCs	SKBR3v	14:1	yes	yes	362	1495	19.5
Donor MNCs	SKBR3v	6:1	yes	no	33	2569	1.3
Donor MNCs	SKBR3v	6:1	yes	yes	212	3376	5.9

[a]Effector to target ratio, NA indicates not applicable; [b]target cells labeled with fluorescent substrate for granzyme B and caspases (yes); [c]Trastuzumab added to 0.1 μg per ml (yes) or no addition (no); [d]number of labeled target cells with high fluorescence activity for granzyme B and caspases (R1 in Fig. 7); [e]number of labeled target cells with low fluorescence for granzyme B and caspase activity (R2 in Fig. 7); [f]100* fraction of activated cells (100 x activated/non-activated).

more cells that are attached to the bottom of the well the higher the resulting impedance or cell index. After adding target cells to each well, they are grown for approximately 24 h before the addition of any treatments. The three cell lines were harvested and transferred into a 96-well E-Plate, which contains electrodes across the bottom of each well. Using the xCELLigence platform (Roche Applied Science), a cell index (CI) value was obtained which reflects the number of cells attached to the bottom of the plate and or the degree of attachment to the bottom of the well.[19] In addition to measuring cell growth and cell death[20] this instrument has been previously used to evaluate ADCC activity.[18,21,26]

For these experiments, the number of cells added to each well varied from 5,000 to 20,000 per well in a total volume of 200 μl of media. After sufficient growth under cell incubation conditions of approximately 24 h, 100 μl of media was removed and 100 ul containing different agents were added to each well. Specifically, wells were given 100 μl of media, 100 μl of effector cells, or 100 ul of effector cells containing 0.2 μg/ml of trastuzumab. The effector to target (E/T) ratio was defined as the number of effector cells added divided by the number of cells plated at the start of the study. The CI was measured every 15 min for at least 24 h after the treatment addition. The perturbations due to these additions were directly reflected by changes in the CI value over time. Each treatment was performed in triplicate with average and standard deviation obtained by the software provided with the instrument. The raw CI curves were then normalized to a CI value of 1.0 at the start of the treatment phase in order to correct for differences in pipetting and growth variability among individual wells. The quantitative effect was determined by measuring the area under the growth curves from the application of treatment through the 16 h of monitoring (AUC-16). The percent inhibition or cell kill (% cell kill) was determined by dividing the difference between the control and treated AUC-16 by the AUC-16 of the control and multiplying by 100. The ADCC activity level was defined as the difference in % cell kill in the presence and absence of trastuzumab.

Separation of lymphocytes subtypes for analysis. Using Miltenyi beads (Miltenyi Biotec Inc.) and the manufacturer's protocol, specific immune cell sub-populations were isolated and then measured for ADCC activity. Briefly, MNCs were isolated as described above and then treated with $CD56^+$ magnetic beads and passed through a column that was attached to a magnetic platform. The flow through was saved for further separation and defined as non-NK cells while the NK cells ($CD56^+$) remained on the column. After removing from magnet platform, the purified NK cells were removed by adding defined media, counted and used for the ADCC assay. The non-NK fraction was subsequently treated in a similar manor at different times with magnetic beads containing CD14, CD19 and CD3 antibodies and monocytes, B cells and T cells were harvested, respectively. The unsorted MNBCs and all isolated sub-populations were then incubated with target cells at E/T ratios of 6:1, 2:1 and 1:1 using the above procedure. These effector cells populations were also analyzed for NK, monocyte, B cell and T cells subsets for purity using the flow cytometry procedure as described above.

Image analysis of ADCC activity. BT474 clone 5 cells were seeded in 6-well plates and grown until islands of cells were

observed. These cells were then treated with effector cells at an E/T ratio of 6:1 in the presence or absence of trastuzumab. A parallel study was performed using the xCELLigence assay to determine when to stop the image experiment. After 4 h of treatment when a significant decrease in the CI was observed in the xCELLigence assay due to the treatment of effectors and trastuzumab, the 6-well plate was washed and then stained for CD16 (NK cells), or stained for HER2 using the indirect procedure as described above. Image analysis was then performed via phase contrast, CD16 expression and HER2 expression.

Flow cytometry assay. The Pantoxilux assay (Oncoimmunin, Inc.), a flow cytometry-based assay was used to determine which enzymes from the effector cells are involved in the killing assay.[27] The tumor cells are first labeled with a red fluorescent probe, TFL4, and washed in PBS. These tagged target cells were then mixed with media or media containing effector cells at different E/T ratios calculated as described above. After centrifugation, the cell pellet was mixed with media containing a specific non-fluorescent enzyme substrate, PanCyToxiLux, in the presence and absence of 1 μg/ml of trastuzumab. After a 1 min centrifugation step, the mixture was incubated for 30 min at 37°C. Upon interaction of the effector cell with the target cell, specific enzymes, granzyme B and upstream caspases are transferred to the target and the non-labeled enzyme substrate in the target cells is converted into a green fluorescent probe After one wash step, the cells are analyzed for two color expression using an Accuri-6C flow cytometer. A shift to more green fluorescence in the target cells as seen in **Figure 7D** where the red target cells move from R1 to R2, would indicate that these specific enzymes only found in effector cells are transported to the target cell where they activate the fluorescence of the enzyme substrates. The percent activation is equal to the number of fluorescent green target cells divided by the total number of target cells after multiplication by 100.

Statistical analysis. The variation in cell killing and ADCC associated with MNCs from different individuals was determined in experiments performed over the course of 18 mo. AUC-16 and ADCC values from multiple determinations were presented as an average with the standard deviation if more than three samples were analyzed for that donor or effector cell line. Pairwise differences in ADCC activity between treatments and individuals were analyzed by the two sample t-test (for normally distributed data) or the Wilcoxon Mann-Whitney test. One way analysis of variance was used to compare variation among related treatments. p values less than 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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