# CD4 T-Cell Immune Stimulation of HER2+ Breast Cancer Cells in Response to Trastuzumab *In Vitro*

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# Abstract

**Introduction:** The HER2+ tumor immune microenvironment is composed of macrophages, natural killer cells, and tumor infiltrating lymphocytes, which produce pro-inflammatory cytokines. Determining the effect of T-cells on HER2+ cancer cells during therapy could guide immunogenic therapies that trigger antibody-dependent cellular cytotoxicity. This study utilized longitudinal *in vitro* time-resolved microscopy imaging to measure T-cell influence on trastuzumab in HER2+ breast cancer.

**Methods:** Fluorescently-labeled breast cancer cells (BT474, SKBR3, MDA-MB-453, and MDA-MB-231) were co-cultured with CD4+ T-cells (Jurkat cell line) and longitudinally imaged to quantify cancer cell viability when treated with trastuzumab (10, 25, 50 and 100 □g/mL). The presence and timing of T-cell co-culturing was manipulated to determine immune stimulation of trastuzumab-treated HER2+ breast cancer. HER2 and TNF-□ expression were evaluated with western blot and ELISA, respectively. Significance was calculated using a two-tailed parametric t-test.

**Results:** The viability of HER2+ cancer cells significantly decreased when exposed to 25 □g/mL trastuzumab and T-cells, compared to cancer cells exposed to trastuzumab without T-cells ($p = 0.01$). The presence of T-cells significantly increased TNF-□ expression in trastuzumab-treated cancer cells ($p = 0.02$). Conversely, cancer cells treated with TNF-□ and trastuzumab had a similar decrease in viability as trastuzumab-treated cancer cells co-cultured with T-cells ($p = 0.49$).

**Conclusions:** The presence of T-cells significantly increases the efficacy of targeted therapies and suggests trastuzumab may trigger immune mediated cytotoxicity. TNF-□ expression suggests cytokines may interact with trastuzumab-induced HER2 receptor blockade. Examining molecular mechanisms of breast cancer immune infiltration has the potential to improve response to targeted therapies.


# Introduction

Twenty-five percent of newly diagnosed breast cancer cases will overexpress the human epidermal growth factor receptor 2 (HER2) gene [1]. Preclinical and clinical studies indicate that the immune microenvironment of HER2+ tumors is driven by tumor infiltrating lymphocytes and macrophages, which can produce pro-inflammatory cytokines [2-4]. Immune inflammatory cytokine expression has been demonstrated to influence tumor progression and proliferation, highlighting the interplay between immune interaction, tumor phenotype and progression [2, 3].

Trastuzumab is a clinically approved humanized monoclonal antibody that prevents the dimerization of the HER2 receptor [5, 6]. Trastuzumab is a key component in the treatment of primary and metastatic HER2+ breast cancer, improving progression-free survival [7-10]. Trastuzumab has been observed to exhibit immunogenic qualities through increased granzyme release and natural killer cell activation [11-17]. Lee *et al.* used immunohistochemistry and found patients with increased tumor infiltrating leukocytes (TIL) responded more favorably to trastuzumab, suggesting that TILs may serve as a biomarker to identify which HER2+ breast cancer patients would most benefit from trastuzumab [16].

Moreover, Gagliato *et al*. found that patients with increased TIL were associated with decreased tumor recurrence [14]. Preclinically, trastuzumab has been observed to increase CD11c and F4/80 (markers of dendritic cell and macrophages, respectively) in *in vivo* models of HER2+ breast cancer, highlighting the immunogenic potential of anti-HER2 therapy [15]. Moreover, Fc□R-mediated stimulation of CD4+ T-cells and activation of CD4+ T-cells with HER2-primed dendritic cell vaccines reduced tumor burden through tumor-specific T-cell response [18, 19]. Although clinical studies have shown successful trastuzumab therapy is dependent on immune cell infiltration, there exists a lack of longitudinal studies that examine trastuzumab-induced CD4+ immune interaction with HER2+ breast cancer [20].

Culturing of cancer cells with immune cells, or onco-immune co-culturing, has been used to study immune interactions between cancer cells and tumor associated macrophages (TAMs) [21-23]. Castellaro *et al*. co-cultured MCF-7 breast cancer cells with TAMs and found TAMs promoted cell proliferation and metastasis. Furthermore, Castellaro *et al.* found macrophages increased breast cancer's resistance to tamoxifen, highlighting the interaction between immune cell presence and response to therapy [21]. While onco-immune co-culturing has been studied with cancer cells and TAMs, to our knowledge, the impact of T-cells on HER2+ breast cancer and subsequent longitudinal response to anti-HER2 therapy has not been investigated.

The purpose of this study is to investigate T-cell influence on HER2+ breast cancer in response to anti-HER2 trastuzumab therapy. This study used longitudinal live cell imaging to quantify the effect of immune cell presence on trastuzumab-treated HER2+ breast cancer through *in vitro* co-culturing of CD4+ T-cells and HER2+ cancer cell lines. This data has potential to serve as the foundation for future immune-based enhanced therapeutic response *in vivo*.

# Methods

### Cell culture

HER2+ breast cancer cell lines (BT474, SKBR3, MDA-MB-453), HER2- breast cancer cell line (MDA-MB-231), and CD4+ T-cell line (Jurkat) were obtained from ATCC (Manassas, VA, USA). BT474 cells were grown in improved minimal essential media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% insulin. SKBR3 cells were grown in McCoy's 5A media with 10% FBS and 2 mM L-glutamine. MDA-MB-453 cells were grown in Leibovitz's L-15 media (Sigma, St. Louis, MO, USA) with 10% FBS. MDA-MB-231 cells were grown in Dulbecco's minimal essential media (Gibco, Gaithersburg, MD, USA) with 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Jurkat T-cells were grown in RPMI-1640 media (Gibco, Gaithersburg, MD, USA) with 10% FBS and 2 mM L-glutamine. Co-culturing of cancer cells and T-cells was conducted by suspending cancer cells and T-cells in cancer cell's respective growth medium prior to plating.

### Fluorescence transfection of breast cancer cell lines

The SKBR3 cell line was transfected to express green fluorescent protein (GFP). A GFP plasmid was cloned into a Sleeping Beauty compatible vector (Addgene plasmid #60525). The GFP plasmid was co-transfected with pCMV (CAT) T7-SB100 Sleeping Beauty transposase (Addgene plasmid #34879) with Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA, USA). SKBR3 cells were selectively cultured with McCoy's 5A media supplemented with 10% FBS, 1% L-glutamine and 200 µg/mL geneticin. Fluorescence activated cell sorting was used to separate cells and the highest 25% of GFP expressing cells were used in experiments. The pCMV (CAT)T7-SB100 plasmid and the pSBbie-Neo were gifts from Zsuzsanna Izsvak and Eric Kowarz, respectively [24, 25].

## Live cell imaging and image analysis

Viable cells were engineered to express fluorescence and cancer cell viability was determined by quantifying change in fluorescence signal (see Supplemental Figure 1). *In vitro* experiments examining treatment response in HER2+ breast cancer cell lines, T-cell influence on HER2+ breast cancer response to trastuzumab, and evaluation of timing of T-cell introduction on trastuzumab treated HER2+ breast cancer were carried out for approximately 144 hours on 96-well glass bottom plates (Fisher Scientific. Catalog #165305) with an IncuCyte S3 imaging system (Essen Bioscience, Sartorius, Germany). Preliminary experiments were conducted to determine seeding density of cancer cells to facilitate longitudinal cell growth (BT474: 20,000 cells/well. SKBR3: 7,500 cells/well. MDA-MB-453: 25,000 cells/well. MDA-MB-231: 1,000 cells/well. Jurkat T-cells: 7,000 cells/well). Cell seeding densities that resulted in continuous exponential growth and ~80% confluence at the final imaging timepoint were used. To retain CD4+ T-cells in co-cultured wells during treatment, plates were centrifuged at 500 g for 5 minutes prior to treatment and drug removal. For imaging, phase contrast and fluorescence images were collected every 3-6 hours using 10× magnification (Excitation/emission: 440-480/504-544 nm for green channels and 565-605/625-705 nm for red channels). Phase confluence and fluorescence data was analyzed using the IncuCyte S3 Live-Cell Image Analysis System. Cells were counted by automated image analysis using background subtraction and brightness threshold (2 green calibrated units and 0.8 red calibrated units). Mean values were summarized by averaging replicates at specified timepoints and percent change was determined by $((X_1\text{-}X_0)/X_0)\times 100$, where $X_0$ and $X_1$ represent cell viability at baseline and cell viability at subsequent timepoints, respectively.

### *Evaluation of treatment response to HER2+ breast cancer cell lines in vitro*

To quantify HER2+ breast cancer response to trastuzumab, cell lines were treated with trastuzumab and viability was assessed (Fig.1A). GFP BT474, GFP SKBR3, and FUCCI MDA-MB-453 cells were plated in 96-well plates. On day 1, cells were treated with trastuzumab (10, 25, 50 and 100 µg/mL). On day 2, trastuzumab was removed through media change and cells were longitudinally observed for five additional days (see Fig.2). Percent change in cancer cell viability was normalized to initial confluence. Mean confluence was summarized by averaging confluence at specified timepoints and percent change was determined. Each treatment group has 4-8 replicates.

### *T-cell influence on HER2+ breast cancer's response to trastuzumab*

To test whether T-cell co-culture affects cancer cell response to trastuzumab, HER2+ (BT474, SKBR3 and MDA-MB-453) and HER2- (MDA-MB-231) cells were co-cultured with T-cells on a 96 well plate. On day 1, cells were treated with 25 μg/mL trastuzumab. On day 2, trastuzumab was removed through media renewal and replaced with fresh media (without trastuuzmab). Following trastuzumab treatment, cells were longitudinally observed for five additional days. Changes in cancer cell viability of co-cultured cells was normalized to that of initial cell viability on day 0 and the fold change per replicate was correlated to HER2 western blot expression with a Pearson Correlation Test. Each treatment group has 4-8 replicates.

### *Evaluating timing of T-cell co-culture on HER2+ breast cancer's response to trastuzumab*

To determine whether timing of immune stimulation of HER2+ breast cancer cells in response to trastuzumab impacts longitudinal treatment response, the timing of when T-cells were introduced to BT474 cell culture was examined (Fig.1C). T-cells were co-cultured with BT474 cancer cells either during initial cell seeding on day 0 or day 1. On day 1, groups were treated with trastuzumab (25 μg/mL) and T-cells through media change. On day 2, trastuzumab was removed through media renewal. Following trastuzumab treatment, cells were longitudinally observed for five additional days. Changes in cell viability was normalized to initial cell viability. Each group has 3 replicates.

### *Evaluating TNF-α effect on trastuzumab induced HER2 receptor blockade*

Experiments evaluating tumor necrosis factor-alpha (TNF-α) on trastuzumab induced HER2 receptor blockade were carried out for approximately 120 hours with an EVOS M7000 imaging system (ThermoFisher, Waltham, MA, USA). Phase contrast and fluorescence images were collected every 6 hours using 20× magnification (Excitation/emission: 470/525 nm for green channels). Images were analyzed with MATLAB image analysis code to quantify the number of fluorescent objects per field of view.

To determine whether TNF-α cytokine expression impacts cancer cell viability during trastuzumab induced HER2 receptor blockade, cancer cells were treated with trastuzumab and TNF-α and response was longitudinally monitored (Fig.1D). BT474 and SKBR3 cells were co-cultured with T-cells on a 96 well plate. On day 1, cells were treated with either 1) 25 μg/mL trastuzumab, 2) 100 ng/mL human recombinant TNF-α (R&D Systems. Catalog #: 210-TA-005) or 3) 25 μg/mL trastuzumab + 100 ng/mL TNF-α. On day 2, treatment was removed through media renewal. Following trastuzumab treatment, cells were longitudinally observed for changes in viability for five additional days. Changes in the number of fluorescent objects were used to determine changes in cancer cell viability. Each treatment group has 5-6 replicates.

### HER2 and TNF-α quantification

BT474, SKBR3, MDA-MB-453, and MDA-MB-231 cancer cells were washed with cold PBS and lysed. Lysates were centrifuged and collected for quantification with a Nanodrop 2000c spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA). 20 µg of protein per cell line was run on a NuPAGE Bis-Tris gel and transferred to a PVDF membrane. The membrane was blocked, probed with HRP conjugated mouse anti-human β-actin overnight at 4° C and developed with Amersham ECL western blot detection system (GE healthcare, Buckinghamshire, UK). Membranes were developed and visualized with an SRX-101A Medical Film Processor (Konica Minolta Medical and Graphic, Inc., Shanghai, China). After β-actin was used as a control to confirm consistent protein levels, the membrane was stripped and probed with 1:1000 rabbit anti-human HER2/ErbB2 primary antibody (Cell Signaling Technology, Danvers, MA, USA. Catalog no. #2242) and 1:1000 rabbit anti-human TNF-α primary antibody (Cell Signaling Technology, Danvers, MA, USA. Catalog no. #C25C1) overnight at room temperature. The membrane was washed, incubated with 1:2000 HRP conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology. Catalog no. #7074) for 1 hour at room temperature. The membrane was redeveloped and visualized for protein expression. Bands were analyzed with the Image Studio Lite (LI-COR Biosciences, Lincoln, NE, USA). Individual cell line HER2 and TNF-α expression was normalized to β-actin expression.

### TNF-α expression

BT474, T-cells, and a co-culture of BT474 and T-cells were longitudinally imaged (N = 3 wells per group) to correlate changes in cancer cell viability with TNF-α expression. All experimental groups were either treated with 25 µg/mL trastuzumab or media (control) at $t$ = 24 hours. Treatment was removed at $t$ = 48 hrs. On days 0 and 7, supernatant was collected for ELISA analysis. A TNF-α ELISA kit (LSBio. Catalog No. LS-F2557-1) was used to quantify TNF-α expression. ELISA expression was quantified with a Cytation5 microscope (BioTek Instruments, Winooski, VT). Samples were averaged and normalized to expression on day 0.

### Statistical analysis

Cell viability in co-cultured groups was quantified through longitudinal quantification of cell fluorescence (see Supplemental Figure 1). Groups were summarized by average confluence, average cell number ± standard error of mean (SEM). A Student's t-test was used to assess group differences. Correlation of confluence and fluorescence was analyzed by computing the Pearson correlation coefficient. The Grubbs outlier test was used to eliminate any data points that were statistical outliers. A p-value $< 0.05$ was considered statistically significant. All data and figures were analyzed using GraphPad Prism 7 (La Jolla, CA, USA).

# Results

### Longitudinal *in vitro* imaging reveals trastuzumab effects are saturated at higher concentrations

Fig. 2 displays BT474, SKBR3, and MDA-MB-453 changes in cell viability in response to increasing doses of trastuzumab. BT474, SKBR3, and MDA-MB-453 control groups were observed to have 105.8 ± 20.9%, 446.5 ± 29% and 329 ± 19% increase in cell growth in 7 days, respectively. In groups treated with 10 µg/mL of trastuzumab, a significant decrease in cell growth compared to control on day 7 was only

observed in BT474 cancer cells ($p < 0.01$). In groups treated with 10 μg/mL of trastuzumab, BT474 cancer cells were observed to have a 74.0 ± 3.6% increase in cell growth on day 7 when normalized to baseline cell growth on day 0 ($p < 0.01$), SKBR3 cancer cells were observed to have a 452.5 ± 60.6% increase in cell growth on day 7 ($p = 0.48$), and MDA-MB-453 cancer cells were observed to have 313.4 ± 14% increase in cell growth on day 7 ($p = 0.06$). In groups treated with 25 μg/mL trastuzumab, BT474 cancer cells were observed to have 61.9 ± 4.8% increase in cell growth on day 7 ($p < 0.01$), SKBR3 cancer cells were observed to have 384.8 ± 29.9% increase in cell growth on day 7 ($p = 0.03$), and MDA-MB-453 cancer cells were observed to have 213.3 ± 16% increase in cell growth on day 7 ($p < 0.01$).

BT474 cells treated with 25 μg/mL trastuzumab were observed to have 61.9 ± 4.8% increase in cell growth on day 7, while those treated with 100 μg/mL trastuzumab were observed to have 58.3 ± 5.6% increase in cell growth ($p = 0.38$). SKBR3 cancer cells, cells treated with 25 μg/mL trastuzumab were observed to have 384.8 ± 29.9% cell growth on day 7, while those treated with 100 μg/mL trastuzumab were observed to have 380.5 ± 16% cell growth on day 7 ($p = 0.72$). MDA-MB-453 cancer cells treated with 25 μg/mL trastuzumab were observed to have 213.3 ± 16% cell growth on day 7, while those treated with 100 μg/mL trastuzumab were observed to have 211.2 ± 43% cell growth on day 7 ($p = 0.89$). Trastuzumab doses above 25 μg/mL were statistically similar ($p > 0.05$) showing no additional cytotoxic benefit, therefore 25 μg/mL was used for all future experiments.

### T-cell co-culture increases trastuzumab efficacy in HER2+ breast cancer with respect to HER2 expression

Fig.3 displays BT474, SKBR3, MDA-MB-453 and MDA-MB-231 cell viability in response to trastuzumab (25 μg/mL) in the presence or absence of T-cells. When treated with trastuzumab, BT474 cancer cells demonstrated 100.2 ± 3% increase in cell viability on day 7, while BT474 cancer cells co-cultured with T-cells demonstrated significantly less cell viability, revealing an 81.4 ± 6.9% increase in cell viability on day 7 ($p = 0.01$). When treated with trastuzumab, SKBR3 cancer cells demonstrated 368 ± 49% increase in cell viability on day 7, while SKBR3 cancer cells co-cultured with T-cells demonstrated significantly less cell viability, revealing a 178.6 ± 39% increase in cell viability on day 7 ($p = 0.01$. Fig.3). When treated with trastuzumab, MDA-MB-453 cancer cells demonstrated 154.4 ± 15.7% increase in cell viability on day 7, while MDA-MB-453 cells co-cultured with T-cells demonstrated decreased cell viability trending toward significant, revealing a 134.4 ± 21% increase in cell viability on day 7 ($p = 0.09$). When treated with trastuzumab, MDA-MB-231 cancer cell demonstrated 2162 ± 340% increase in cell viability on day 7, while MDA-MB-231 cancer cells co-cultured with T-cells demonstrated 2342.6 ± 312% increase in cell viability on day 7 ($p = 0.40$). Fig.4 displays HER2 receptor expression in breast cancer cell lines used in this experiment. BT474, SKBR3, MDA-MB-453 and MDA-MB-231 breast cancer cells exhibited 0.49, 0.41, 0.24 and 0.01 fold HER2 expression, respectively, when normalized to β-actin. Decreases in endpoint cancer cell viability in the presence of T-cells and trastuzumab correlates with HER2 receptor expression ($r^2$=0.59, Fig.4D).

Fig.5 displays BT474 cell viability in response to trastuzumab (25 μg/mL) with T-cells co-cultured on day 0 (BT474 co-culture) or on day 1 (BT474 delayed co-culture). When BT474 cells were treated with

trastuzumab on day 1, BT474 cells demonstrated a 100.2% ± 3% increase in cell viability on day 7. When T-cells were introduced to BT474 simultaneously with trastuzumab treatment on day 1, BT474 cells demonstrated a 100.9% ± 6.8% increase in cell viability on day 7, which was statistically similar to BT474 cells treated with trastuzumab ($p = 0.88$). When BT474 cells were cultured with T-cells on day 0 and treated with trastuzumab on day 1, the cells demonstrated an 81.4 ± 7% increase in cell viability on day 7, a significant decrease in viability compared to BT474 cells treated with trastuzumab ($p = 0.01$).

### TNF-α ELISA assay reveals increased expression in T-cells treated with trastuzumab

Fig.6A displays normalized TNF-α expression in 1) BT474 cancer cells, 2) T-cells, and 3) a co-culture of BT474 cancer cells and T-cells treated with trastuzumab. On day 7, BT474 cells, T-cells and co-cultured cells treated with trastuzumab had a 0 ± 20%, 499.8 ± 96%, 143.7 ± 78% increase in TNF-α expression, respectively. On day 7, BT474 cells, T-cells and co-cultured cells treated with a control -45 ± 13%, -78 ± 3%, -42 ± 12% increase in TNF-α expression, respectively. Trastuzumab treated CD4+ T-cells were observed to increase in TNF-α expression compared to CD4+ T-cells treated with a control ($p < 0.01$). Trastuzumab treated cancer cells co-cultured with T-cells had increased TNF-α expression compared to trastuzumab treated cancer cells without T-cells ($p = 0.02$). Increases in TNF-α expression, in cancer cells co-cultured with T-cells when treated with trastuzumab, suggesting TNF-α cytokine expression negatively impacts cell viability during HER2 receptor blockade. Fig.6C displays TNF-α receptor expression in HER2+ cell lines used in this study. BT474, SKBR3, MDA-MB-453 and MDA-MB-231 breast cancer cells exhibited 0.71, 0.21, 0.1 and 0.004 fold TNF-α fold expression when normalized to β-actin expression.

### TNF-α alters treatment response when treated in combination with trastuzumab

Fig.7 displays changes in cell viability in response to trastuzumab and human recombinant TNF-α to determine whether decreases in cell viability in co-cultured groups observed in Fig.3 were the result of TNF-α receptor activation. On day 7, control BT474 cells exhibited a -10.8 ± 30% increase in cell viability. Trastuzumab treated BT474 cells co-cultured with T-cells exhibited a -20.6 ± 13.8% increase in cell viability and BT474 cells treated with trastuzumab and human recombinant TNF-α exhibited a -25.3 ± 13.9% ($p = 0.49$). On day 7, control SKBR3 cells exhibited a 0.6 ± 25.1% increase in cell viability. Trastuzumab treated SKBR3 cells co-cultured with T-cells exhibited a -9 ± 5.4% increase in cell viability and SKBR3 cells treated with trastuzumab and human recombinant TNF-α exhibited a -11 ± 5.6% ($p = 0.48$).

## Discussion

This study seeks to investigate immune stimulation of HER2+ breast cancer in response to anti-HER2 trastuzumab therapy and identify potential mechanisms of enhanced response. Through our experiments, it was observed that immune influence on HER2+ breast cancer in response to anti-HER2 targeted therapy decreased cancer cell viability compared to cancer cells treated with trastuzumab without immune influence. Our data shows immune response to trastuzumab treated HER2+ breast cancer is impacted by CD4+ T-cell stimulation. Delaying the timing of T-cell co-culture by introducing T-

cells 24 hours after HER2+ breast cancer cells were seeded impacted the response of the cells to treatment. This significance was only observed in the cell lines highly overexpressing HER2, such as BT474 and SKBR3 (Fig.4). Slight differences in cancer cells treated with trastuzumab and co-cultured cells treated with trastuzumab were observed in HER2 moderately overexpressing cell line, MDA-MB-453; however, this difference was trending towards statistical significance, suggesting immune stimulation in trastuzumab treated HER2+ breast cancer cells is related to HER2 expression. HER2 overexpression has been noted to increase tumor resistance to hormone-based therapy and certain chemotherapies [26, 27]; however, HER2 expression in relation to immune stimulation and trastuzumab response has not been characterized.

TNF-α serves a dual role in tumor proliferation and apoptosis. TNF-α has been shown to promote destruction of tumor vasculature and synergize with liposome-mediated chemotherapy [28-30]. Donato *et al.* used TNF-sensitive and TNF-resistant MCF-7 cell lines to study TNF-α treatment on poly (ADP-ribose) polymerase (PARP) cleavage and cell death [30]. Increased PARP cleavage and cell death was observed in TNF-sensitive MCF-7 cells, compared to TNF-resistant MCF-7 cells, suggesting TNF treatment increased DNA damage and apoptosis [30]. Conversely, TNF-α has also been observed to promote inflammation and tumor proliferation [31, 32]. Egberts *et al*. used an invasion assay and found that pancreatic cancer cell lines treated with TNF-α were observed to have increased invasive properties and invasion promoting proteins, such as interleukin -8 (IL-8) and matrix metallopeptidase-9 (MMP9) [31]. Similarly, increased expression of TNF-α was proportional to tumor grade and increased expression of TNF-α was observed in invasive breast ductal carcinomas. In our study, *in vitro* ELISA and longitudinal cell imaging suggest TNF-α expression improves efficacy of anti-HER2 therapy. Treatment of cancer cells with TNF-α and trastuzumab resulted in similar decreases in cell viability as trastuzumab-treated cancer cells co-cultured with T-cells in two HER2+ breast cancer cell lines.

TNF-α expression has been studied in breast cancer response to therapy [33-35]. Lee *et al.* observed decreased cell viability in the MCF-7 cell line in response to TNF-α therapy, hypothesizing that TNF-α downregulates estrogen receptor expression [35]. While our study supports the role of TNF-α in decreasing cell viability, two out of three HER2+ cell lines used in our study, SKBR3 and MDA-MB-453, do not overexpress estrogen receptors, suggesting cell viability was not exclusively the result of TNF-α induced estrogen receptor downregulation. Mercogliano *et al.* used plasmid transfection to generate TNF-α overexpressing HER2+ cell lines and found that TNF-α overexpression correlated with trastuzumab resistance [34]. Differences in results from Mercogliano *et al.* and our study could be attributed to treatment incubation time and endpoint analysis. Mercogliano *et al.* measured changes in cell proliferation after 2 days post treatment, whereas our study measured treatment response up to five days post treatment. Moreover, in our study, TNF-α induced changes in cell proliferation occurred approximately 3.5 days after treatment.

While T-cell immune stimulation has been studied in preclinical models of cancer, these studies have focused on changes in CD4+ T-cell viability in the presence of cancer cells [36, 37]. Zhu *et al*. used a MTT glucose metabolism assay to study CD4+ T-cell proliferation when co-cultured with SW480 colon cancer

cells and observed that colorectal cancer cells increased apoptosis in lymphocytes [37]. Youssef *et al*. used Annexin-V staining to determine the impact of cancer cells and oxygenation on T-cell viability and found increased CD4+ T-cell apoptosis when co-cultured with cancer cells in hypoxic conditions, implying cancer cell signaling interaction with immune cells [36]. To our knowledge, no other study has characterized the effect of immune stimulation and anti-HER2 targeted therapies on breast cancer cell viability.

Limitations of this study include the lack of comparative data with CD8+ T-cells. CD8+ cytotoxic T-cells are observed to target cancerous tissue and patients with high CD8+ T-cell infiltration have a significant increase in survival [38]. TALL-104, a CD8+ T-cell cell line, can be used to study CD8+ T-cell influence on trastuzumab treated HER2+ breast cancer [39, 40]. An additional limitation is the lack of *in vivo* data detailing changes in CD4+ T-cell immune infiltration. Future *in vivo* studies may provide additional data and could potentially be simulated through engrafting HER2+ breast cancer in a humanized mouse. Changes in immune infiltration can be determined through tracking of CD4+ T-cell localization with longitudinal noninvasive immuno-PET imaging [41, 42]. Freise *et al*. used a [$^{89}$Zr] anti-CD4 cys-diabody to track CD4+ T-cell localization *in vivo*, with high uptake in the spleen, lymph nodes and thymus [41]. Biological validation of T-cell immune infiltration can be achieved through flow cytometry against peripheral blood [20, 43-45]. Future experiments could investigate the involvement of the Fc-region of CD4+ T-cells and whether co-precipitation experiments can identify interaction between CD4+ T-cells and HER2+ breast cancer cells treated with trastuzumab.

# Conclusion

This study uses *in vitro* longitudinal live cell imaging and fluorescent microscopy to track immune stimulation in trastuzumab treated HER2+ breast cancer. Our data produced preliminary evidence that TNF-α receptor activation could interplay with HER2 receptor activity to affect overall cell viability. Importantly, information from this work can be used to provide insight into clinically relevant TIL in response to successful trastuzumab therapy, and can be used to identify mechanisms of enhanced response.

# Abbreviations

HER2. Human Epidermal Growth Factor Receptor 2

GFP. Green fluorescent protein

TNF-α. Tumor necrosis factor-alpha

TIL. Tumor infiltration leukocytes

ATCC. American Type Culture Collective

PBS. Phosphate buffer saline

ELISA. Enzyme Linked Immunosorbent Assay

MTT. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

MMP-9. Matrix metallopeptidase-9

# Declarations

**Competing interest:**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate:**

N/A

**Consent for publication:**

N/A

**Availability of data and material:**

Datasets generated in this study are available from the corresponding author upon reasonable request.

**Code availability:**

Analysis code used in this study is available from the corresponding author upon request.

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PNS, KJD, GH, TEY, and AGS designed the study. PNS, TRD, and AM performed experimentation. PNS and AGS analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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# Figures

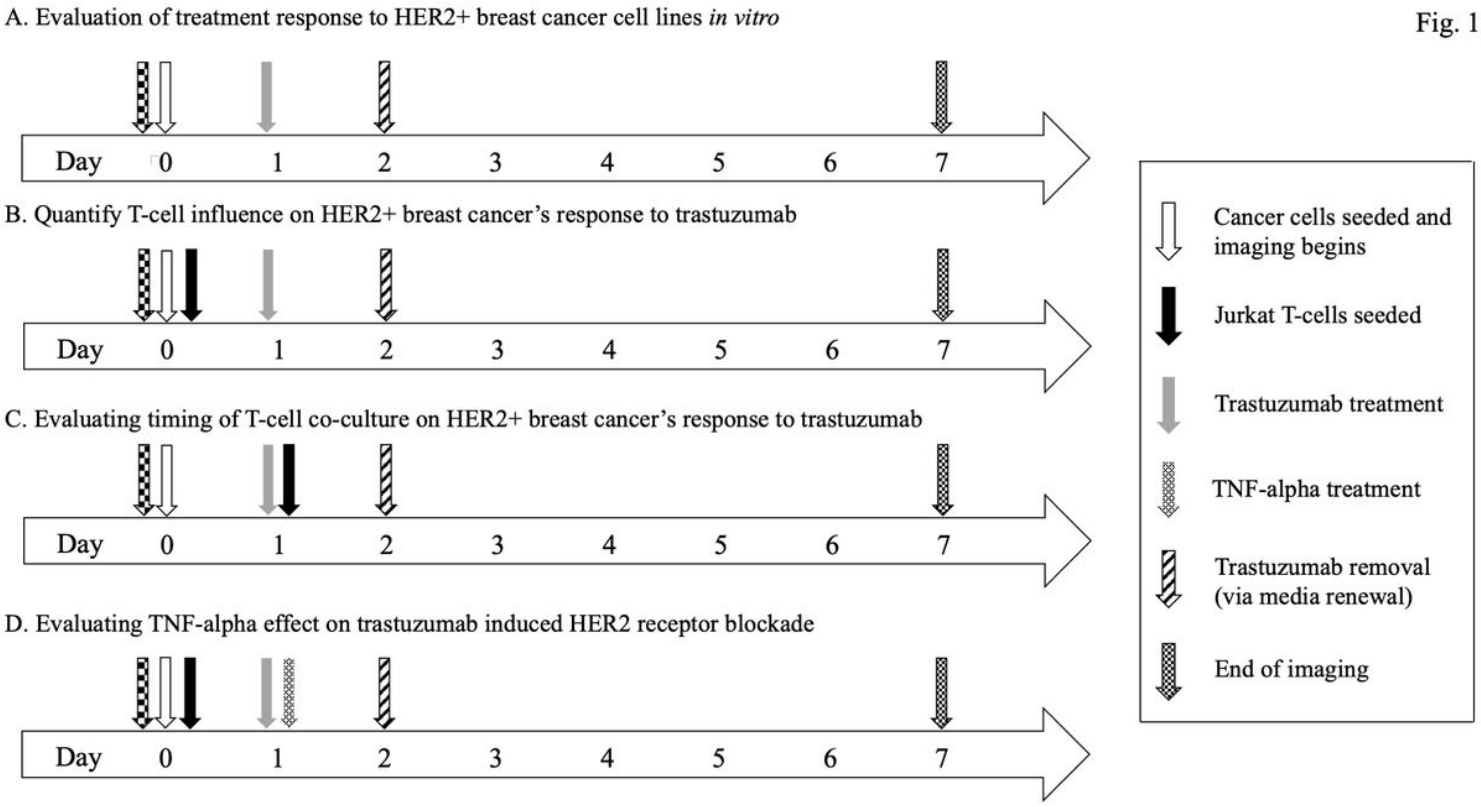
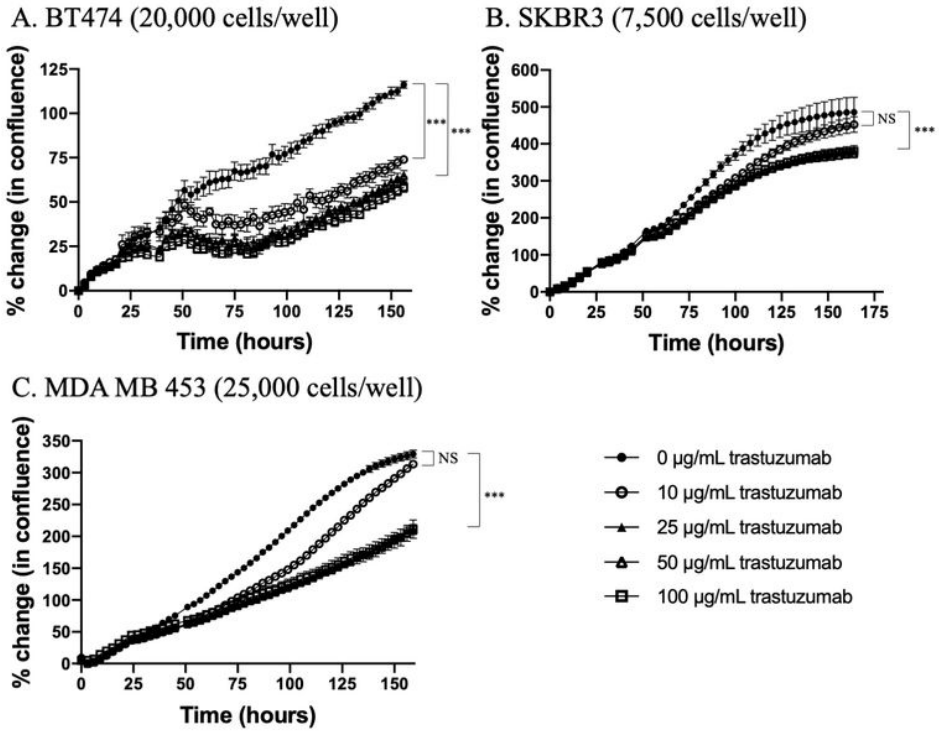



Fig. 1

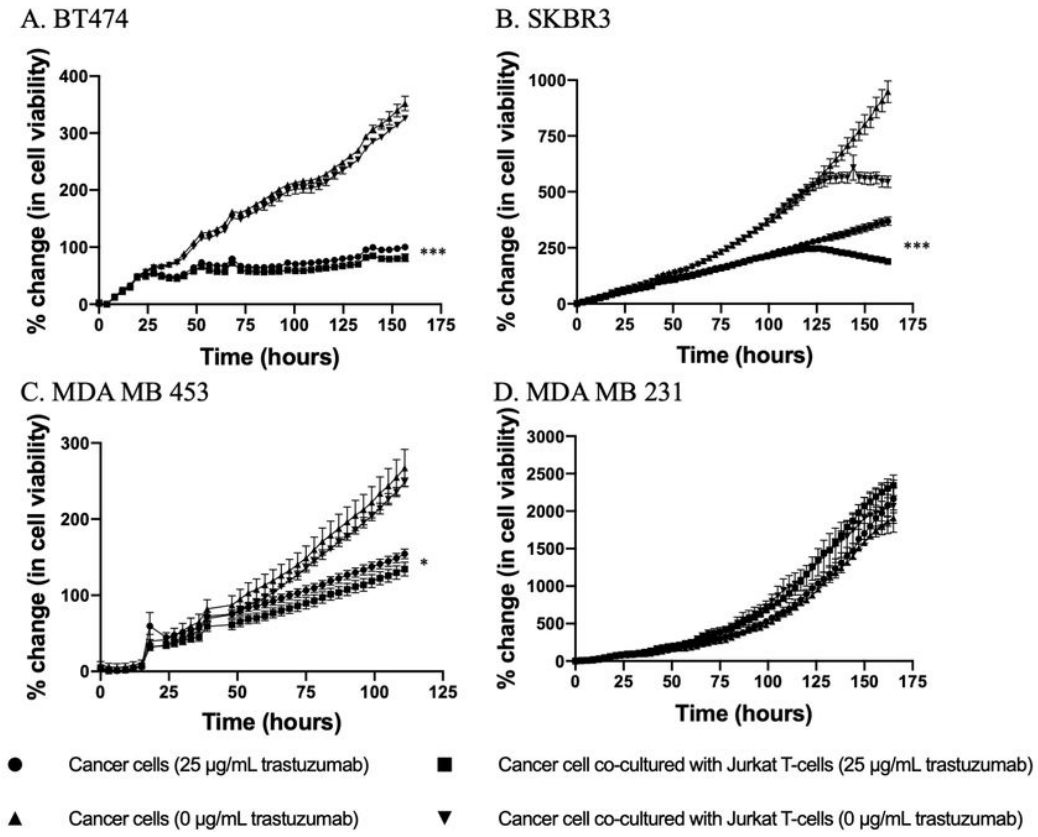
## Figure 1

Timeline of in vitro experiments. A) Treatment response of HER2+ breast cancer cells in response to incremental doses of trastuzumab (0 µg/mL – 100 µg/mL) for 24 hours. B) Quantifying immune influence of CD4+ T-cells on HER2+ breast cancer in response to single agent trastuzumab. C) Evaluating timing of T-cell co-culture on HER2+ breast cancer's response to trastuzumab. CD4+ T-cells were introduced into cell culture at t = 0 hours during initial plating of cells or t = 24 hours during trastuzumab treatment and longitudinal changes in cell viability was assessed. D) Evaluating TNF-□ effect on trastuzumab induced HER2 receptor blockade.



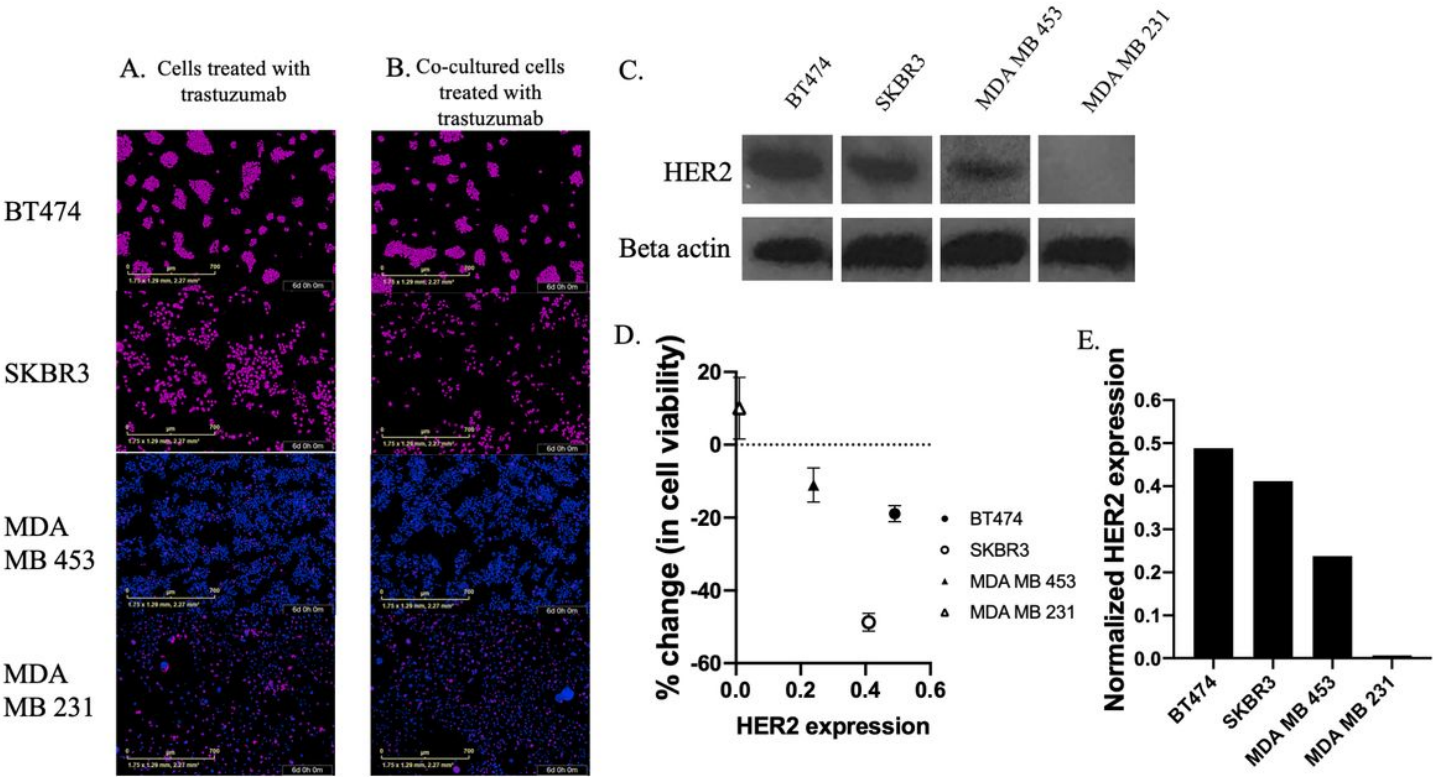

## Figure 2

In vitro treatment response of HER2+ breast cancer to single agent trastuzumab. HER2+ breast cancer cells were treated with incremental doses of single agent trastuzumab therapy and longitudinal changes in cell confluence was observed over 7 days. In comparison to control groups, cancer cells treated with 25 µg/mL, 50 µg/mL and 100 µg/mL were statistically similar ($p = 0.38$, 0.72 and 0.89 in BT474, SKBR3 and MDA MB 453 cell lines, respectively) to one another and 25 µg/mL was used for subsequent experiments.



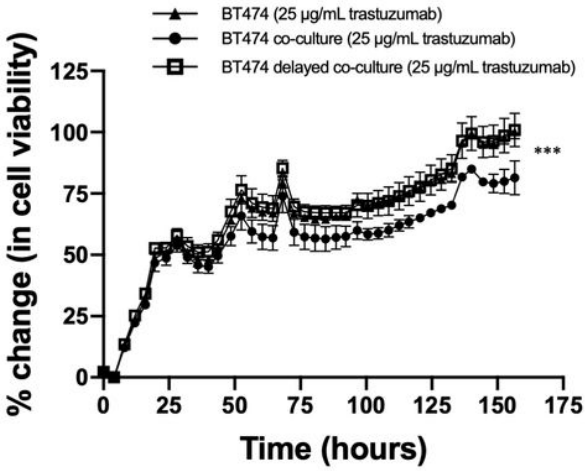

## Figure 3

Immune stimulation of trastuzumab treated HER2+ breast cancer cells. Normalized percent change in confluence of (A) BT474, (B) SKBR3 and (C) MDA-MB-453 and (D) MDA-MB-231 breast cancer cells in response to 25 µg/mL trastuzumab and CD4+ T-cell presence. Significant differences in cell viability between 1) trastuzumab treated cancer cells and 2) trastuzumab treated co-cultured cells was only observed in BT474 and SKBR3 cells ($p = 0.01$). Decreased cell viability between 1) trastuzumab treated cancer cells and 2) trastuzumab treated co-cultured cells was only observed in MDA-MB-453 cells was observed but was only trending toward significance ($p = 0.08$). Error bars are representative of standard error of mean (SEM).



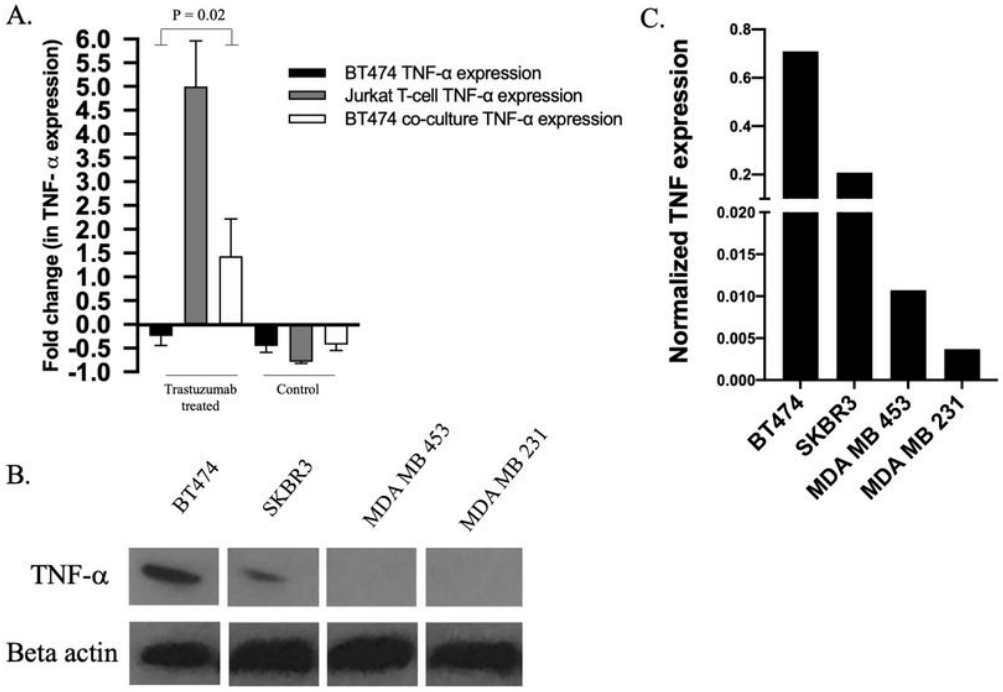

## Figure 4

Representative images of T-cell and cancer cell co-culturing with fluorescence segmentation and HER2 quantification. GFP and RFP segmented images of breast cancer cells treated with 25 □g/mL trastuzumab when cultured without (A) and with (B) CD4+ T-cells. (C) Western blot of HER2 expression in four HER2+ breast cancer cell lines. (D) Correlation of HER2 expression and significance between changes in viability of trastuzumab treated cancer cells and trastuzumab treated co-cultured cells. The fold change in cell viability has a negative correlation to HER2 expression (r2 = 0.59). (E) High HER2 overexpression was observed in BT474 and SKBR3 cell lines. Moderate HER2 overexpression was observed in MDA-MB-453 cell lines. Low HER2 overexpression was observed in MDA-MB-231 cell lines.




## Figure 5

Longitudinal analysis of incubation time on efficacy of CD4+ T-cells to increase efficacy of targeted HER2 antibodies in BT474 cancer cells. To study the impact of T-cells on trastuzumab response in HER2+ breast cancer, T-cells were introduced to cell culture at two different times: t = 0 and t = 24 hours. Significant differences in cell viability were only observed when T-cells were introduced into cell culture at t = 0 hours ($p = 0.01$).




## Figure 6

ELISA analysis of TNF-α expression in trastuzumab treated HER2+ breast cancer. Fold change of TNF-α ELISA analysis from day 0 to 7 reveals an increase in TNF-α expression in trastuzumab treated cells cultured with CD4+ T-cells (p = 0.02). (B) Western blot of TNF-α and β-actin expression in BT474, SKBR3, MDA-MB-453 and MDA-MB-231 cell lines. (C) High TNF-α expression was observed in the BT474 cell line. Moderate TNF-α expression was observed in the SKBR3 cell line. Low TNF-α expression was observed in MDA-MB-453 and MDA-MB-231 cell lines. β-actin was also assayed and used to normalize protein expression.

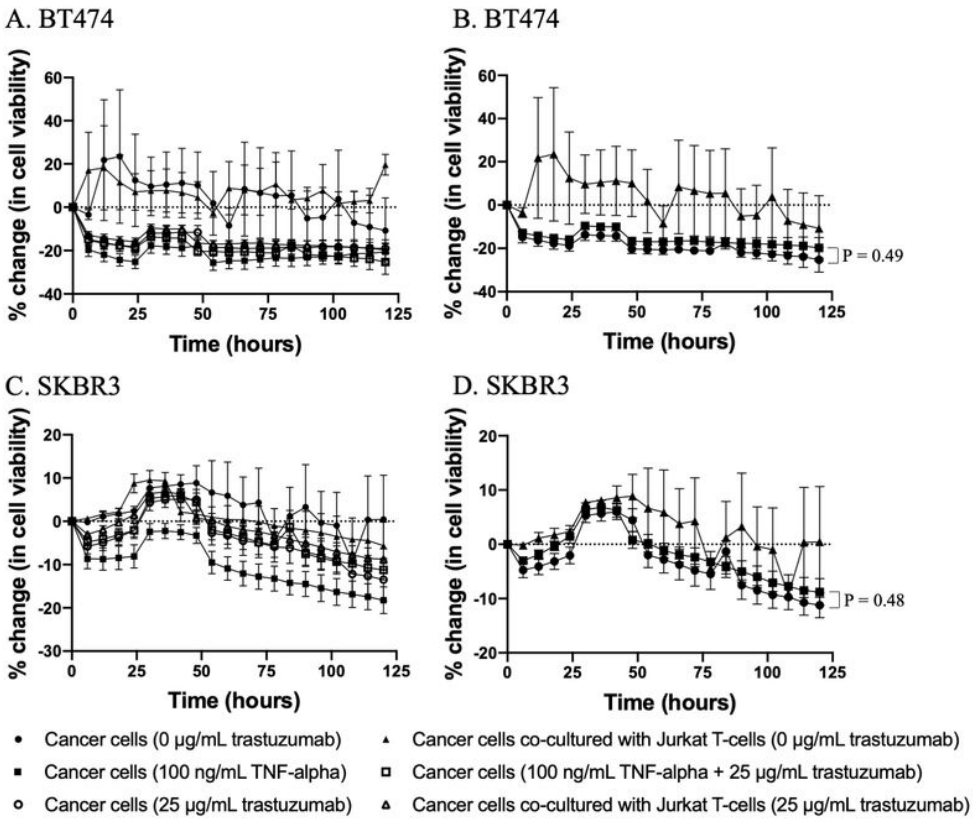



Fig. 7

**Figure 7**

Longitudinal observation of TNF-α and trastuzumab on HER2+ breast cancer. Human recombinant TNF-α and trastuzumab was used to determine if decreased cell viability in co-cultured groups is the result of TNF-α receptor activation in conjunction with trastuzumab induced HER2 receptor blockade in (A) BT474 and (C) SKBR3 cell lines. Two HER2+ cell lines were used and both cell lines had similar responses in cancer cells treated with human recombinant TNF-α and trastuzumab vs trastuzumab treated cancer cells co-cultured with T-cells (p = 0.49 and 0.48, respectively).

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFig.docx
- SupplementalFigure1.tiff