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Trastuzumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) in HER-2-non-amplified breast cancer cell lines

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Background: Antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by CD56 + natural killer (NK) cells may contribute to the activity of trastuzumab in HER-2-amplified tumours. In this study, we investigated the possibility that trastuzumab might induce ADCC against HER-2-non-amplified breast cancer cells.
Methods: Induction of NK cell-mediated ADCC was examined in trastuzumab-treated HER-2-non-amplified breast cancer cell lines. HER-2 protein levels were also determined in tumour and autologous normal tissue samples from patients with HER-2 negative breast cancer.
Results: Trastuzumab induced a significant ADCC response in the HER-2-amplified HCC1954 and SKBR3 cell lines, and in all five of the non-amplified cell lines, which had low levels of detectable HER-2 by western blot (CAL-51, CAMA-1, MCF-7, T47D, and EFM19). Trastuzumab did not induce ADCC in the K562 control cell line or MDA-MB-468, which has very low levels of HER-2 detectable by enzyme-linked immunosorbent assay (ELISA) only. HER-2 protein was detected by ELISA in 14/15 patient tumour samples classified as HER-2-non-amplified. Significantly lower levels of HER-2 were detected in normal autologous tissue compared with tumour samples from the same patients.
Conclusion: Our results suggest that HER-2-non-amplified breast cancer cells, with low but detectable levels of HER-2 protein, can bind trastuzumab and initiate ADCC.
Key words: ADCC, ERBB2, HER-2 low, HER-2-non-amplified breast cancer, trastuzumab

introduction

HER-2 protein overexpression (>30% of tumour cells, 3 + by immunohistochemistry) or gene amplification (FISH HER-2/CEP7 ratio >2.2), or both, occurs in 20%–25% of breast cancers [1, 2], and is associated with a poorer prognosis [3, 4]. Treatment with trastuzumab, a humanised monoclonal antibody directed against the extracellular domain of the HER-2 protein, prolongs the survival of patients with early stage and metastatic cancers whose tumours have HER-2 gene amplification/protein overexpression [1, 5–8].

The major mechanisms of action of trastuzumab are believed to be abrogation of intracellular HER-2 signalling through pathways including PI3K/Akt and Ras/MAPK leading to cell cycle arrest, reduction in angiogenesis, inhibition of extracellular domain cleavage, and antibody-dependent cell-mediated cytotoxicity (ADCC) [9–13].

Trastuzumab is active in combination with chemotherapy in the HER-2-amplified metastatic disease setting [6, 14] but not in HER-2-non-amplified, non-overexpressed metastatic breast cancer [15]. The improvement in patient outcomes in the metastatic setting led to four major studies in patients with

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HER-2-amplified disease in the adjuvant setting, NSABP-B31, NCCTG N9831, HERA, and BCIRG 006 [7, 8, 16]. All four studies showed a benefit for the addition of trastuzumab to chemotherapy in the adjuvant setting of HER-2-amplified disease.

Central review of NSABP-B31 revealed that 174 of the 1787 patients (9.7%) with early-stage breast cancer who were randomised to chemotherapy with or without trastuzumab had cancer which was HER-2 negative by immunohistochemistry (IHC) and FISH [17, 18]. Surprisingly, the benefit of trastuzumab was similar regardless of HER-2 status. The relative risks of disease progression and death among patients receiving trastuzumab and chemotherapy and those receiving chemotherapy alone were 0.34 ($P = 0.014$) and 0.08 ($P = 0.017$), respectively. A recent review of the NCCTG N9831 data also suggested a similar trend that did not reach significance in a smaller number of HER-2 non-amplified, IHC 0/1 + /2 + patients [19].

While this retrospective subset data may be due to chance or to HER-2 testing artefacts [18], another hypothesis is that they might reflect an immunologic effect of trastuzumab on HER-2 non-amplified cancer cells which have low but detectable levels of HER-2 protein. Previous studies have shown evidence of a strong immune effector cell response before chemotherapy treatment in the adjuvant setting [20, 21]. Murine studies have shown that ADCC contributes to the antitumour effects of trastuzumab in HER-2 positive tumours *in vivo* [22, 23]. In two pre-operative clinical studies, trastuzumab treatment was associated with increased tumour infiltration of natural killer (NK) cells [10, 21]. Gennari et al. [21] also reported that patients with objective response to trastuzumab-based treatment had higher numbers of infiltrating leukocytes and higher ADCC activity. In addition to CD16 + CD56 + NK cells, there are a number of other potential effector immune cells that may be involved in trastuzumab-related ADCC, including a T-cell subset (CD3 + CD16 +), CD16 + granulocytes, and CD16 + CD33 + macrophages [24].

To investigate the possibility that trastuzumab might induce ADCC against HER-2 'negative' (non-amplified/low HER-2 expressing) tumours, we examined NK cell-mediated ADCC in trastuzumab-treated, HER-2-amplified and HER-2-non-amplified breast cancer cell lines, covering a wide range of HER-2 protein expression. In addition, in order to assess the differential expression of HER-2 between normal and malignant breast tissue necessary for a selective ADCC response to trastuzumab in HER-2 non-amplified tumour cells, we examined HER-2 protein levels in tumour and normal tissue samples from patients with HER-2 negative breast cancer.

materials and methods

cell lines, cell culture, and reagents

The breast carcinoma cell lines SKBR3 [American Type Culture Collection (ATCC)], HCC1954 (ATCC), T47D [European Collection of Cell Cultures (ECACC)], EFM19 (German Collection of Microorganisms and Cell Cultures, DSMZ), MCF-7 (ATCC), and the leukaemic cell line K562 (ATCC), were maintained in RPMI 1640, 10% fetal calf serum (FCS) at 37° C with 5% CO_2. CAMA-1 (ATCC) was maintained in Minimum Essential Medium, 10% FCS, non-essential amino acids, sodium pyruvate, L-glutamine, and CAL-51 (DSMZ) and MDA-MB-468 (ATCC) in Dulbecco's Modified Eagle Medium, 10% FCS, and 2 mM sodium pyruvate. All FCS used was heat-inactivated. Trastuzumab (Herceptin®; Genentech, Inc., San Francisco, CA) and cetuximab (Erbitux ®, Merck KGaA, Darmstadt, Germany) were obtained from St Vincent's University Hospital, Dublin, Ireland.

immunoblotting

Ten micrograms of protein in loading buffer was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 7.5% gel. Immunoblotting was carried out using mouse monoclonal anti-epidermal growth factor receptor (EGFR) (Lab-Vision, Fremont, CA), mouse monoclonal anti-HER-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and α-tubulin primary antibody and mouse secondary antibody (Sigma–Aldrich, Dublin, Ireland). Luminol detection reagent (Santa Cruz Biotechnology Inc.) was used to visualise the protein bands.

enzyme-linked immunosorbent assay

Quantification of HER-2 was carried out using a HER-2 enzyme-linked immunosorbent assay (ELISA) kit (Merck Chemicals Ltd., Nottingham, UK). Total protein (2.5–10 μg)was used in the analysis of the cell lines and patient samples. SKBR3 was used as the positive control for HER-2 expression and K562 as a negative control. Assays were read on a Labsystems Multiskan Ex at 450 nm with wavelength correction at 570 nm to correct for optical imperfections in the plate.

isolation of NK cells

Approval for obtaining voluntary blood samples was granted by St Vincent's University Hospital Ethics Committee. The NK cells of eight volunteers were utilised in ADCC assays [male $n = 2$, age 25–35 years; female $n = 6$, age 25–35 years ($n = 1$) and 35–45 years ($n = 5$)]. Peripheral blood mononuclear cells (PBMCs) were isolated from blood using Ficoll-Paque plus (GE Healthcare, Chalfont, UK). Positive selection of CD56 + NK cells was achieved utilising CD56 + magnetic beads and a MACS separation column system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Isolation of the CD56 + cells was confirmed using phytoerythrin (PE)-labelled anti-CD56 antibody on a Guava Easycyte flow cytometer at 540 nm (Millipore, Billerica, MA).

antibody-dependent cell-mediated cytotoxicity assays

Antibody-dependent cell toxicity was determined using the Guava Celltoxicity Kit (Millipore) according to the manufacturer's instructions. Percentage of cell death was determined on a Guava Easycyte flow cytometer using Cytosoft™ software (Millipore). K562 cells were utilised as the positive control target cells for NK cell activity [25, 26]. SKBR3 was used as a positive control and MDA-MB-468 as a negative control for trastuzumab-related ADCC. Experiments were repeated for each cell line using NK cells from three separate volunteers. The average activity of the three sets of NK cells used for each cell line is listed in 1.

cell proliferation assays

The anti-proliferative effects of trastuzumab were measured using a 96-well plate acid phosphatase-based proliferation assay, as previously described [27]. A range of trastuzumab concentrations (0.00001–1 μM) was examined over a 5-day period. Acid phosphatase substrate (10 mM) was dissolved in 0.1 M sodium acetate buffer (0.1% Triton X-100, pH 5.5) and

Table 1. Proliferation and percentage increase in ADCC in cell line anel

	SKBR3	HCC1954	CAMA-1	EFM19	T47D	MCF-7	CAL-51	MDA-MB-468	K562
HER-2	+++	+++	+	+	+	+	+	−	−
% Proliferation (100 nM trastuzumab)	58 ± 5**	99 ± 3	97 ± 8	94 ± 1*	76 ± 5*	91 ± 4*	99 ± 1	102 ± 2	ND
% Increase ADCC (200 nM trastuzumab NK : target cell; 1 : 1)[a]	13 ± 4**	29 ± 3**	43 ± 13*	25 ± 7*	21 ± 3*	31 ± 15*	33 ± 8**	0.6 ± 1	0.5 ± 1
% NK activity (against K562)	54 ± 15	67 ± 7	65 ± 12	54 ± 6	54 ± 6	54 ± 6	65 ± 12	61 ± 17	65 ± 7
EGFR	+	+	−	−	+	+	+	+++	−
% Increase ADCC (200 nM cetuximab NK : target cell; 1 : 1)[a]	13 ± 2*	ND	ND	−2 ± 2	ND	ND	25 ± 11*	38 ± 2**	0.2 ± 3

HER-2 and EGFR expression is based on results in 1.

[a]*P* = significant increase in ADCC relative to untreated controls.

*$P < 0.05$, **$P < 0.005$.

ND, not determined, EGFR, epidermal growth factor receptor.

the reaction was stopped by addition of 1 M NaOH. Absorbance was read at 405 nm with wavelength correction at 620 nm. Cell proliferation was determined as a % of control cell growth in the absence of trastuzumab. *P* = significant reduction in proliferation relative to untreated controls.

protein extraction from breast tissues

Breast tissue samples were obtained from the Department of Surgery, St Vincent's University Hospital (Dublin, Ireland), with institutional ethics board approval. Directly following surgical removal and routine pathological evaluation, tissue samples (tumour tissue and visibly normal tissue remote from the tumour) were snap frozen in liquid nitrogen and stored at −80°C. The samples were homogenised using a Mikro-Dismembrator (Braun Biotech International, Melsungen, Germany). Protein was extracted from tissue samples using 200 µl of 50 mM Tris–HCl (pH 7.4) containing protease and phosphatase inhibitors (Roche Diagnostics Ltd., Burgess Hill, UK). This lysate was aspirated through a 19-gauge needle, vortexed for 30 s, and agitated for 20 min at 4°C before the addition of 1% Triton X-100 (Sigma–Aldrich). The samples were agitated for 1 h at 4°C and centrifuged at 13 000 *g* for 20 min at 4°C. The supernatant was removed and transferred to a fresh eppendorf tube.

fluorescence imaging

Cells were seeded at 1×10^5 cells per well on glass chamber slides (NUNC, Roskilde, Denmark). The procedure used to visualise trastuzumab bound to HER-2 was adapted from Wu et al. [28] and the manufacturer's protocol for the use of quantum dot labelling (Invitrogen, Carlsbad, CA). Primary antibody, biotinylated secondary antibody and streptavidin-conjugated qDots were applied in 10% bovine serum albumin/phosphate-buffered saline blocking buffer. DAPI (4′-6-diamidino-2-phenylindole) was applied to all samples. Samples excluding primary antibody were included as controls. qDot 605 fluorescence was examined *in situ* by exciting at 435 nm and visualising the emitted light collected at 605 nm by confocal laser scanning microscopy (Leica TCS AOBS; Leica Microsystems GmbH, Wetzlar, Germany).

statistical analysis of data

Comparative statistical analyses were carried out on the quantitative data generated in the study. Statistical analysis was carried out using Microsoft Excel®. Where appropriate, differences between experimental group mean values were evaluated by two-tailed Student's *t*-tests and considered significant if $P < 0.05$. All error values represent the standard deviation (SD) of the mean of triplicate determinations, unless otherwise stated.

A longer version of the materials and methods section is available as supplemental Materials and Methods at *Annals of Oncology* online.

results

HER-2 expression

HER-2 protein expression was detected by western blot in all cell lines examined except the K562 and MDA-MB-468 cell lines (1A). Analysis of HER-2 levels by ELISA confirmed the western blot results in the two HER-2-amplified [SKBR3 (748.2 ± 296.4 pg/µg), HCC1954 (511.3 ± 80.9 pg/µg)], five HER-2-non-amplified [CAMA-1 (55.1 ± 23.4 pg/µg), EFM19 (43.8 ± 16.7 pg/µg), T47D (43.8 ± 17.2 pg/µg), MCF-7 (17.4 ± 7.7 pg/µg), CAL-51 (50.7 ± 9.6 pg/µg)] breast cancer cell lines, and the HER-2-negative leukaemic cell line K562 (0 pg/µg) (1B). The ELISA detected low HER-2 levels in the MDA-MB-468 breast cancer cells (6.2 ± 1.9 pg/µg), which were not detectable by immunoblotting. The HER-2 amplification status of the cell lines tested is based on previously published data [29].

trastuzumab-induced ADCC

We examined trastuzumab-related CD56 + NK cell-mediated ADCC in each of the cell lines. PBMCs were isolated from the blood of 10 healthy volunteers. The direct cytotoxicity of NK cells against control K562 leukaemia cells ranged from 42% to 89% (ratio 10 : 1, NK : K562), displaying considerable inter-individual variability (supplemental Figure S1, available at *Annals of Oncology* online). NK cells which achieved direct cytotoxicity against K562 cells ranging from 42% to 73% were used to assess trastuzumab-induced ADCC.

The ability of trastuzumab to induce ADCC was assessed in the eight breast cancer cell lines (2) at three NK to target cell ratios and at an NK to target cell ratio of 10 : 1 for K562 (1). The three NK to target cell ratios chosen for each breast cancer cell line were dependent on the susceptibility of each cell line to direct NK cell toxicity. Co-incubation with trastuzumab for 4 h increased ADCC in all cell lines tested (2A, 2B, and 2D–2H), except the two cell lines in which HER-2 was not detectable by western blot, MDA-MB-468 and K562 (2C, 1). The NK:target cell ratio of 1 : 1 was common to the breast

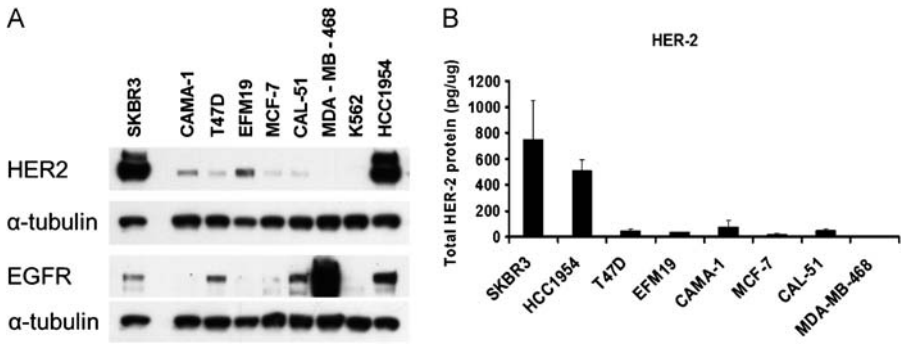

Figure 1. (A) HER-2 and epidermal growth factor receptor (EGFR) expression in the cell line panel determined by western blot and (B) HER-2 expression measured by enzyme-inked immunosorbent assay. All data determined in triplicate ± standard deviation.

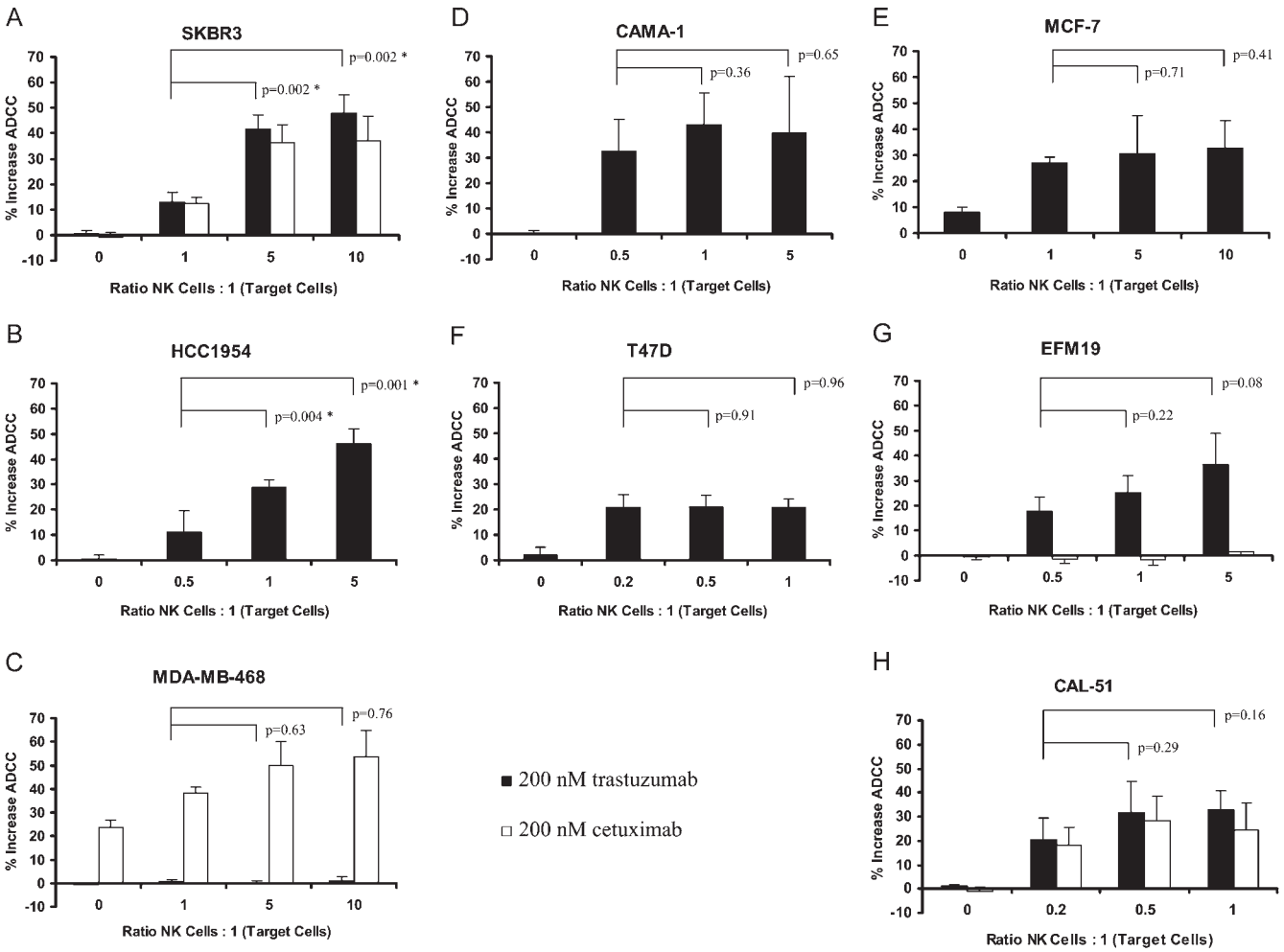

Figure 2. Percentage increase in ADCC following treatment with 200 nM trastuzumab in the (A, B) HER-2 amplified SKBR3, HCC1954, (C–H) HER-2 non-amplified CAMA-1, MCF-7, T47D, EFM19, CAL-51 and MDA-MB-468 cell lines. Percentage increase in ADCC following treatment with 200 nM cetuximab was also determined in the SKBR3, EFM19, CAL-51 and MDA-MB-468 cell lines. All data determined in triplicate ± standard deviation.
*$P < 0.05$.

cancer cell lines tested and the increase in ADCC associated with trastuzumab was significant for all the HER-2 expressing (amplified and non-amplified) cell lines at this ratio. Significant ratio-dependent increases in ADCC were only observed in the HER-2-amplified cell lines, suggesting NK cell number was a limiting factor in the HER-2 amplified but not the HER-2-non-amplified cell lines (2A and 2B). Control ADCC experiments using cetuximab, a monoclonal antibody

Table 2. HER-2 protein levels measured by enzyme-linked immunosorbent assay (ELISA) in patient tumour versus normal autologous tissue

Patient ID	IHC status	Tumour HER-2 (pg/μg) ± SD	Adjacent normal tissue HER-2 (pg/μg) ± SD
1	3+	6328.4 ± 2019.7[a]	0
2	3+	3451.5 ± 271.5	2.7 ± 1.1
3	2+	35.6 ± 5[a]	0
4	2+	15.5 ± 7.3	0
5	1+	23.8 ± 14.9	20.8 ± 18.2
6	1+	25.8 ± 20.9	0
7	1+	36.9 ± 18.3	0
8	1+	12.3 ± 9.9	0
9	1+	32.4 ± 5.9	0
10	1+	38.3 ± 10.7	1 ± 1.8
11	0	61.5 ± 34.2	0
12	0	4.6 ± 3.3	0
13	0	9.9 ± 6.7	0
14	0	3.8 ± 6.1	0
15	0	0	0
16	0	14 ± 0.8	0
17	0	12.1 ± 4.7	2.4 ± 1.2

All data determined in triplicate ± SD.
[a]Data determined in duplicate ± range.
IHC, immunohistochemistry; SD, standard deviation.

to EGFR also capable of eliciting an ADCC response, confirmed that the ADCC response to trastuzumab was specific to cell lines with >6 ng of HER-2 per milligrams of total protein (2). A strong ADCC response to cetuximab in the MDA-MB-468 cells reflected the overexpression of EGFR in this cell line (2).

proliferation studies

The antiproliferative effects and ADCC effects of trastuzumab on cell lines were unrelated (1). Specifically, HER-2 expression was associated with trastuzumab-mediated ADCC in all of the cell lines, but a 5-day treatment with 100 nM trastuzumab without NK cells, led to substantial growth arrest in only the amplified SKBR3 (42% ± 5%) and T47D (24% ± 5%) cell lines (supplemental Figure S2, available at *Annals of Oncology* online). T47D cells are non-amplified according to the ASCO-CAP guidelines which designate tumour cells with greater than six copies of the HER-2 gene as HER-2 positive [2], but have been reported to have twofold to fourfold amplification of HER-2 [29, 30]. A small (<10%) but significant inhibition of proliferation was observed in the non-amplified MCF-7 and EFM19 cell lines. Interestingly, the MCF-7 cell line was the only cell line studied that exhibited cell death in response to trastuzumab treatment alone in the ADCC assays (2E). A similar effect was observed in the MDA-MB-468 cell line in response to cetuximab (2C). Proliferation of the remaining HER-2-non-amplified cell lines, CAMA-1, CAL-51, and MDA-MB-468 and the HER-2-amplified HCC1954 cell line was not affected by the 5-day exposure to trastuzumab. The HCC1954 cell line has previously been reported as insensitive/resistant to trastuzumab *in vitro*, despite amplification of HER-2 [31].

HER-2 levels in HER-2 negative tumours

HER-2 levels were determined by ELISA in samples from tumours classified as HER-2 negative (0/1 + /2 + /HER-2 non-amplified) and autologous adjacent normal tissue (2). Two 3 + samples were also examined. The range of HER-2 expression across the 0, 1 + , and 2 + tumour samples (3.8–61.5 pg/μg) was similar to that of the HER-2-non-amplified cell lines tested (6.2–55.1 pg/μg) (1). 16/17 (94%) of the total tumour samples tested and 14/15 (93%) of the HER-2 negative subset had detectable levels of HER-2. 13/17 (77%) of the corresponding samples of normal breast tissue from the same patients had no detectable HER-2. Three of the four normal samples with HER-2 expression exhibited lower levels than the MDA-MB-468 cell line.

fluorescent imaging of trastuzumab

Trastuzumab bound to surface HER-2 was visualised by fluorescence confocal microscopy. Strong fluorescence was observed in the HER-2-amplified cell lines SKBR3 and HCC1954 (3A and 3B) due to the overexpression of HER-2 in these cell lines. Using the same visualisation parameters, binding of trastuzumab was detected for each of the HER-2-non-amplified cell lines (3C–3H), albeit at lower levels than the HER-2-amplified cell lines (control images—supplemental Figure S3, available at *Annals of Oncology* online).

discussion

Our results show that trastuzumab enhances NK cell-mediated ADCC in HER-2-non-amplified breast cancer cell lines irrespective of the susceptibility of the cell lines to growth inhibition by trastuzumab.

Two studies in the 1990s reported conflicting results regarding the ability of trastuzumab to induce ADCC in low HER-2-expressing breast cancer cell lines [13, 32]. More recently, studies have reported trastuzumab-induced ADCC in cell lines derived from other solid tumour types, including pancreatic, esophageal, and uterine serous papillary adenocarcinoma cell lines, which express low levels of HER-2 [11, 13, 33, 34].

The breast cancer cell lines utilised in this study provide a spectrum of HER-2 protein expression from HER-2-non-amplified to amplified. We observed a significant increase in trastuzumab-related ADCC in the HER-2-amplified and all the non-amplified cell lines tested, except the MDA-MB-468 cell line.

MDA-MB-468 is generally classified as HER-2 negative and consistent with this definition, trastuzumab did not induce an ADCC response in this cell line. Although HER-2 was not detected by western blot, low levels of HER-2 were detected by ELISA and trastuzumab was visualised bound to the surface of MDA-MB-468 cells. This might indicate that a threshold level of HER-2 expression, or a level of HER-2 expression to overcome any innate NK-mediated ADCC inhibition present, is required for initiation of trastuzumab-mediated ADCC. We acknowledge that basal NK-mediated ADCC resistance can vary greatly between tumour cell lines due to a multitude of factors including expression levels of NK modulating molecules

like e-cadherin and major histocompatibility complex Class I or the production of immunosuppressive cytokines [35–37]. The ability of cetuximab to elicit an ADCC response in the MDA-MB-468 cell line suggests that these factors may not be limiting in this instance.

A ratio-dependent increase in ADCC was only observed in the HER-2-amplified cell lines. This is consistent with the ELISA data, showing ~ 10- to 100-fold higher levels of the HER-2 target antigen in the amplified cell lines SKBR3 and HCC1954 compared with the non-amplified cell lines in which antigen : antibody binding was most likely saturated and NK cell number was not a limiting factor.

The proliferation assay results highlight the limited susceptibility of the cell line panel to the direct inhibition of HER-2 signalling compared with ADCC. Four cell lines (SKBR3, T47D, EFM19, and MCF-7) showed significant growth arrest in response to trastuzumab, while seven of the eight breast cancer cell lines tested were sensitive to trastuzumab-induced NK cell-mediated ADCC.

Taken together, these results suggest that breast cancer cells which express low levels of HER-2 are capable of binding sufficient trastuzumab to induce an ADCC response.

While our results are consistent with the findings of Cooley et al. [13], who showed trastuzumab-induced ADCC in four HER-2-non-amplified breast cancer cell lines using a single ratio of NK cells to target cells (10 : 1), our results revealed a strong ADCC response to trastuzumab at much lower NK : target ratios and the need to further investigate the mechanisms underlying the susceptibility of tumour cells to trastuzumab-induced ADCC.

.The clear delineation of HER-2 expression we observed between HER-2 positive and negative tumours and autologous normal tissue is consistent with previous findings that have also shown significant differences between HER-2 expression in tumour and normal tissue [38]. While we acknowledge that the normal breast tissue samples may contain a lower percentage of epithelial cells than the tumour samples, based on the high sensitivity of the ELISA assay (minimum 0.024 pg/μg total protein), if the percentage of epithelial cells in the normal specimens were as low as 10%, the HER-2 protein should still be detectable if the levels were equal to or higher than the tumour tissue. The low levels of HER-2 in normal breast tissue, combined with our ADCC response data and the fluorescence images showing trastuzumab bound to HER-2 non-amplified cells are supportive of the possibility that a tumour-specific trastuzumab-induced ADCC response could be elicited in patients with HER-2-non-amplified breast cancer. These results may also have relevance for the use of anti-HER-2 vaccines in the HER-2-non-amplified disease setting.

Following their subset analysis of NSABP-B31, Paik et al. [17] concluded the current definition of HER-2 overexpression is based on data from advanced disease and may need to be modified for the adjuvant setting. Furthermore, the ability of NK cells to function in ADCC may be reduced in patients with advanced disease as well as post radio-chemotherapy, potentially explaining the lack of benefit for trastuzumab in patients with HER-2 negative metastatic disease [20, 39]. The importance of NK cell function has been highlighted by the observation of a correlation between NK cell function and response to trastuzumab in metastatic breast cancer patients [40]. IgG Fc receptor polymorphisms in leucocytes (including the FcγR IIIa/CD16a expressed by NK cells) have been associated with the clinical efficacy of trastuzumab-based therapy, although these findings have yet to be confirmed [41, 42]. It is also possible that non-immunological mechanisms may be responsible for trastuzumab activity in HER-2-non-amplified disease. Following recent work identifying regulation of HER-2 expression by the Notch signalling pathway as a critical survival pathway, Korkaya and Wicha [43, 44] suggested that HER-2 inhibition by trastuzumab could be beneficial in the absence of HER-2 amplification, by targeting stem cell-like breast cancer cells.

The subset analysis of NSABP-B31 provides the only dataset of patients with HER-2 normal early stage disease, treated with trastuzumab on the basis of random assignment [17, 18, 45]. External review of the HER-2 status of the NSABP-B31 patients is underway as inter-observer variability has been reported in HER-2 testing [18, 46]. Should the subset analysis of the NSABP-B31 trial be confirmed, our results suggest that the hypothesis that trastuzumab might benefit patients with HER-2-non-amplified early-stage breast cancer through an immune-mediated mechanism is a possibility. NSABP-B47 (NCT01275677) is comparing chemotherapy alone to chemotherapy in combination with trastuzumab in patients with low HER-2 expressing, invasive breast cancer and is currently recruiting with low HER-2 expression being defined as an IHC score of 1 + , for which in situ hybridisation is not required, or an IHC score of 2 + which must be accompanied by FISH displaying a HER-2/CEP17 ratio less than two or a HER-2 gene copy less than four per nucleus by FISH/chromogenic in situ hybridisation. Once completed, it will shed further light on the efficacy of trastuzumab in HER-2-non-amplified disease. Further pre-clinical investigation is required to gain a greater understanding of the passive and active immune responses to monoclonal antibody therapies for the treatment of HER-2 negative breast cancers.

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disclosure

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Prognostic significance of L1CAM in ovarian cancer and its role in constitutive NF-κB activation

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Background: Overexpression of L1-cell adhesion molecule (L1CAM) has been observed for various carcinomas and correlates with poor prognosis and late-stage disease. *In vitro*, L1CAM enhances proliferation, cell migration, adhesion and chemoresistance. We tested L1CAM and interleukin-1 beta (IL-1β) expression in tumor samples and ascitic fluid from ovarian carcinoma patients to examine its role as a prognostic marker.
Patients and methods: We investigated tumor samples and ascitic fluid from 232 serous ovarian carcinoma patients for L1CAM by enzyme-linked immunosorbent assay. L1CAM expression was correlated with pathoclinical parameters and patients' outcome. IL-1β levels were measured in tumor cell lysates. Ovarian cancer cell lines were analyzed for the contribution of L1CAM to IL-1β production and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activation.
Results: We observed that L1CAM-expressing tumors show a highly invasive phenotype associated with restricted tumor resectability at primary debulking surgery and increased lymphogenic spread. Soluble L1CAM proved to be a marker for poor progression-free survival and chemoresistance. In ovarian carcinoma cell lines, the specific knock-down of L1CAM reduces IL-1β expression and NF-κB activity.
Conclusions: L1CAM expression contributes to the invasive and metastatic phenotype of serous ovarian carcinoma. L1CAM expression and shedding in the tumor microenvironment could contribute to enhanced invasion and tumor progression through increased IL-1β production and NF-κB activation.
Key words: CD171, cell adhesion, chemoresistance, IL-1β, serous ovarian cancer, soluble L1CAM

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