CD4⁺ T-Helper Type 1 Cytokines and Trastuzumab Facilitate CD8⁺ T-cell Targeting of HER2/neu-Expressing Cancers

Jashodeep Datta¹, Shuwen Xu¹, Cinthia Rosemblit¹, Jenessa B. Smith², Jessica A. Cintolo¹, Daniel J. Powell Jr², and Brian J. Czerniecki¹,³

Abstract

Vaccination strategies incorporating the immunodominant HLA-A2-restricted HER2/neu-derived peptide 369–377 (HER2369–377) are increasingly utilized in HER2/neu-expressing cancer patients. The failure of postvaccination HER2369–377-specific CD8⁺ T cells to recognize HLA-A2⁺/HER2/neu-expressing cells in vitro, however, has been attributed to impaired MHC class I/HLA-A2 presentation observed in HER2/neu-overexpressing tumors. We reconcile this controversy by demonstrating that HER2369–377 is directly recognized by high functional-avidity HER2369–377-specific CD8⁺ T cells—either genetically modified to express a novel HER2369–377 TCR or sensitized using HER2369–377-pulsed type 1–polarized dendritic cells (DC1)—on class I–abundant HER2low but not class I–deficient HER2high, cancer cells. Importantly, a critical cooperation between CD4⁺ T-helper type-1 (Th1) cytokines IFNγ/TNFα and HER2/neu-targeted antibody trastuzumab is necessary to restore class I expression in HER2high cancers, thereby facilitating recognition and lysis of these cells by HER2369–377-specific CD8⁺ T cells. Concomitant induction of PD-L1 on HER2/neu-expressing cells by IFNγ/TNFα and trastuzumab, however, has minimal impact on DC1-sensitized HER2369–377-CD8⁺ T-cell-mediated cytotoxicity. Although activation of EGFR and HER3 signaling significantly abrogates IFNγ/TNFα and trastuzumab-induced class I restoration, EGFR/HER3 receptor blockade rescues class I expression and ensuing HER2369–377 CD8⁺ cytotoxicity of HER2/neu-expressing cells. Thus, combinations of CD4⁺ Th1 immune interventions and multivalent targeting of HER family members may be required for optimal anti-HER2/neu CD8⁺ T-cell-directed immunotherapy. Cancer Immunol Res; 3(5): 455–63. ©2015 AACR.

Introduction

HER 2/neu (HER2) is amplified in a number of solid malignancies, including breast, ovarian, gastric, and pancreatic cancers (1). This HER2 receptor tyrosine kinase (RTK) is critically involved in early uncontrolled growth, enhanced invasiveness, and metastatic spread (2, 3). Although the combination of HER2-targeted monoclonal antibodies (trastuzumab) with chemotherapy has dramatically improved outcomes in patients with HER2-overexpressing tumors, HER2/neu-targeted breast cancer (4, 5), significant resistance to therapy occurs, leading to recurrence (6).

There are emerging data that both CD4⁺ and CD8⁺ T-cell antitumor responses are critical in these aggressive tumors. Not surprisingly, enhanced infiltration of these immune cell subsets is associated with favorable clinical outcomes in HER2low tumors (7). The tumoricidal activity of antigen-specific CD8⁺ CTLs is also widely appreciated; indeed, CD8⁺ T cells recognizing the HLA-A2–restricted peptide 369–377 (HER2369–377; KIFGSLAFL) have been identified in tumors from breast and ovarian cancer patients (8). Controversy exists, however, whether this epitope is actually processed and presented by HER2-expressing cancers. Utilizing HER2369–377 (with adjuvant) to vaccinate patients with HER2pos tumors generated postimmunization HER2369–377-reactive CD8⁺ T cells that failed to recognize HLA-A2low tumour cells expressing HER2 (9). In addition, HER2369–377 peptide vaccination in GM-CSF (E75) induced immune responses and improved clinical outcomes in patients with low HER2-expressing (1+) —but not in classically HER2pos (3+ or 2+/ISH-positive)—breast cancer patients (10). The failure of HER2-specific CD8⁺ T-cell recognition may be explained by evidence that HER2 overexpression downregulates MHC class I expression by inducing defects in the antigen-processing machinery (APM; refs. 11–14), thereby mediating escape from immune surveillance.

In the current study, we attempted to reconcile this controversy by demonstrating that HER2369–377 is endogenously presented by HER2-expressing cancer cells, and naturally recognized by HER2369–377-specific CD8⁺ T cells in a class I–dependent manner. Furthermore, we demonstrate a critical cooperation between CD4⁺ T-helper type-1 (Th1) cytokines IFNγ/TNFα and HER2-targeted antibody trastuzumab in mediating restoration of class I expression and facilitating HER2369–377 CD8⁺ T-cell targeting of HER2-overexpressing cancers. Concomitant induction of PD-L1

¹Department of Surgery, University of Pennsylvania Perelman School of Medicine, Philadelphia. ²Department of Pathology and Laboratory Medicine; University of Pennsylvania Perelman School of Medicine, Philadelphia. ³Rena Rowen Breast Center, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania.

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*J. Datta and S. Xu contributed equally to this article.

Corresponding Author: Brian J. Czerniecki, University of Pennsylvania, 3400 Civic Center Drive, 2 Perelman Center, Philadelphia, PA 19104. Phone: 215-615-1696; Fax: 215-615-0555; E-mail: brian.czerniecki@uphs.upenn.edu
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on HER2/neu-expressing cells by IFNγ/TNF and trastuzumab, however, has minimal impact on type 1–polarized dendritic cell (DC1)–sensitized HER2369–CD8+ T-cell–mediated cytotoxicity. Although activation of EGFR and HER3 signaling significantly abrogates IFNγ/TNFα/trastuzumab-induced class I restoration, EGFR and HER3 receptor blockade rescues class I expression as well as HER2369–CD8+ cytotoxicity of HER2/neu-expressing cells. As such, our novel findings have important implications for vaccine design and T-cell–directed therapies in patients with HER2-expressing cancers.

**Materials and Methods**

**Cell lines**

HER2-expressing breast cancer cell lines SK-BR-3 and BT-474 (HER2high), MCF-7 (HER2intermediate), MDA-MB-231 (HER2low, American Type Culture Collection), and ovarian cancer cell line SK-OV-3 (HER2 intermediate) stably transfected with the HLA-A2 gene (SK-OV-3A2; kind gift of Mary Disis, University of Washington) were immediately resuscitated and maintained in RPMI supplemented with 10% FCS (Cellgro). HLA-A2 status and HER2 status of cell lines were verified (LABType SSO) by the Clinical Immunology laboratory at the Hospital of the University of Pennsylvania. HLA-A2/HER2 status of cell lines was verified by flow cytometry (Fig. 1A, data not shown).

**Treatment with cytokines, ligands, and targeted antibodies**

HER2-expressing cells were treated with the following, either alone or in designated combinations: rhTNFα, rhIFNγ (BD Biosciences), trastuzumab (Genentech), lapatinib (Santa Cruz Biotechnology); rh-EGF (BD Biosciences), rh-Heregulin (Sigma-Aldrich); neutralizing anti-EGFR (LA1) and/or anti-HER3 (H3.105.5) antibodies, or IgG1 isotype control antibody (all Millipore); and neutralizing anti–PD-1 (mH11; ref. 15) or IgG1 isotype control (eBiosciences). Specific cell treatments are detailed in Supplementary Methods.

**Generation of HER2369–specific CD8+ TCR clones**

HER2369–specific CD8+ TCR clones were generated as previously described (16). Briefly, high-avidity HER2369–reactive T cells in an MHC class I–dependent manner. A, by flow cytometric analysis, pan-class I surface expression on tumor cells correlated inversely with HER2 expression (MDA-MB-231: HER2low, robust HLA-ABC; SK-BR-3: HER2high, minimal HLA-ABC). Tumor cells were stained with anti-HER2 (PE: anti-HER2 x C, surface HER2 and HLA-A2 expression in MDA-MB-231 cells following treatment with nontargeting (NT) or HER2 siRNA evaluated by flow cytometry (PE anti-HER2x-axis vs. FITC anti–HLA-A2y-axis) and Western blot analysis (vinculin as loading control); representative images are shown. HER2369–sensitized (left) and HER2369–TCR-transduced (right) CD8+ T-cell recognition of MDA-MB-231 cells was abrogated by HER2 silencing. D, HLA-A2 silencing with HLA-A2, but not NT, siRNA confirmed by flow cytometry. In representative panels, filled traces represent isotype-matched control staining, and open traces represent FITC anti–HLA-A2 staining. HER2369–sensitized CD8+ T-cell recognition of MDA-MB-231 was abrogated by silencing HLA-A2. All results are representative of three experiments and are expressed as mean ± SEM. **P < 0.01; ***P < 0.001.
CD8+ T cells were isolated after in vitro HER2369-377 stimulation of CD8+ T cells obtained from a patient with HLA-A2pos/HER2pos ductal carcinoma in situ who was previously vaccinated with HER2369-377-pulsed autologous DC1. The vaccination protocol is summarized in Supplementary Methods. CD8+ T-cell stimulation with HLA-A2–matched or unmatched HER2-expressing cell lines, with subsequent sorting for tumor-activated HER2369-377 T cells, allowed for isolation of HER2369-377-specific CD8+ TCR. Primary human CD8+ T cells genetically modified to express this HER2369-377 TCR specifically bound HER2369-377-containing HLA-A2+ tetramers (16). GFP-transduced and nontransduced clones served as controls.

In vitro sensitization of CD8+ T cells
Monocyte-derived DCs from four HLA-A2pos/HER2pos-DCIS donors, who had undergone HER2369-377-pulsed DC1 vaccinations (Supplementary Table S1), were matured to a DC1 phenotype (high IL12-secreting) via IFNγ (1,000 U/mL) and LPS (10 ng/mL; ref. 17), and pulsed with HER2369-377 peptide (Genscript; 50 μg/mL) 2 hours before harvest. DC1s were sensitized with postvaccination CD8+ T cells, isolated by immunomagnetic separation (EasySep; Stem Cell Technologies), by coculturing at a 20:1 (T cell:DC1) ratio in RPMI 1640 + 5% human serum. IL2 (30 IU/mL) was added on day 2. As previously described (17), this technique generates high functional-avidity HER2369-377-specific CD8+ T cells. After 6 to 7 days, sensitized CD8+ T cells were harvested and tested 1:1 with HLA-A2 transporter (TAP)–deficient T2 hybridoma cells pulsed with HER2369-377 or control class I peptides (1 μg/mL), and tested against MDA-MB-231 with or without HER2/HLA-A2 siRNA transfection, SK-OV-3A2, MCF-7, and SK-BR-3 cell lines as indicated. HER2369-377-TCR-transduced, GFP-transduced, and nontransduced CD8+ T-cell clones were also tested against T2 and HER2-expressing cells. Supernatants and tumor cells were harvested after 24 hours, and CD8+ T-cell recognition (by IFNγ ELISA) and cytotoxicity (by flow cytometry) were assessed.

RNA interference
For HER2 or HLA-A2 silencing, 3 × 106 MDA-MB-231 cells were transfected with HER2, HLA-A2, or nontargeting siRNA sequences (25 nmol/L; ON-TARGETplus; Dharmacon) using RNAiMax Lipofectamine (Life Technologies) in serum-free medium. After 1 hour, medium was supplemented with 10% FBS; 20 μg/mL RNAi Max Lipofectamine (Life Technologies) in serum-free medium, allowed for isolation of HER2369-377-specific CD8+ T-cells. After 6 to 7 days, sensitized CD8+ T cells were harvested and tested 1:1 with HLA-A2 transporter (TAP)–deficient T2 hybridoma cells pulsed with HER2369-377 or control class I peptides (1 μg/mL), and tested against MDA-MB-231 with or without HER2/HLA-A2 siRNA transfection, SK-OV-3A2, MCF-7, and SK-BR-3 cell lines as indicated. HER2369-377-TCR-transduced, GFP-transduced, and nontransduced CD8+ T-cell clones were also tested against T2 and HER2-expressing cells. Supernatants and tumor cells were harvested after 24 hours, and CD8+ T-cell recognition (by IFNγ ELISA) and cytotoxicity (by flow cytometry) were assessed.

Flow cytometry
Cell suspensions were prepared in FACS buffer (PBS + 1% FCS + 0.01% azide); 7-AAD (viability stain), and FITC/APC/phycocerythrin (PE)–conjugated mouse anti-human CD11c, CD8, HLA-ABC, HLA-A2, HER2, IFNγR1, TNFαR1, PD-1, PD-L1, or subclass-matched controls (BD Bioscience) were utilized as indicated. HER2369-377/HLA-A*0201 tetramers (MCL) were used to identify HER2369-377–specific CD8+ T cells. Flow cytometry was performed using BD FACSCalibur; datasets were analyzed using CellQuest Pro software.

Cytotoxicity assays
Following cell treatments as indicated, CFSE-labeled tumor cells were cocultured 1:1 with HER2369-377–CD8+ T cells for 24 hours. Cells were harvested, stained with 7-AAD and FITC-anti-CD8, and subjected to flow cytometry. CFSE-positive, but not CD8-positive, cells were gated and analyzed; the percentage of apoptotic cells was calculated as 7-AAD+/(7-AAD+ + 7-AAD−) × 100%. Cytotoxicity was calculated as percentage of apoptotic tumor cells in CD8+ coculture minus background (i.e., apoptotic cells in tumor culture alone).

Antibody-dependent cell-mediated cytotoxicity assays
Refer to Supplementary Methods.

ELISA
Capture and biotinylated detection antibodies and standards for IFNγ (BD Pharmingen) were used according to the manufacturer’s protocols.

Statistical analysis
One-way ANOVA with post hoc Tukey paired testing was used for all ≥3-group comparisons. Student t test (parametric) or Mann–Whitney tests (nonparametric) were used for two-group comparisons. P ≤ 0.05 was considered statistically significant. Analysis was performed using Prism 5.0 (GraphPad Inc.).

Results and Discussion
HER2369-377 is recognized on HER2-expressing cancer cells by HER2369-377–specific CD8+ T cells
The HER2369-377 peptide is widely regarded as the immunodominant epitope recognized by lymphocytes from HLA-A2pos patients with breast/ovarian cancer (8). HER2369-377 was used to immunize mice transgenic for both HLA-A2.1 and human CD8; postimmunization splenocytes recognized HLA-A2pos/HER2pos human tumor cells (18). In spite of supportive preclinical evidence, the failure of CTLs from HER2369-377–immunized patients to recognize HER2pos tumor cells (9) has generated skepticism regarding the utility of this peptide for HER2-directed immunotherapy. More recently, several groups have attempted to explain this phenomenon by demonstrating that overexpression of a signal-competent HER2 RTK dramatically impairs MHC class I expression and APM components, thereby impairing CD8+ recognition of HER2-expressing cancers (12–14).

In the present study, although HER2low MDA-MB-231 maintained robust surface class I expression, class I expression was severely diminished on HER2-overexpressing SK-BR-3 cells; postvaccination patient-derived CD8+ T cells sensitized with HER2369-377-pulsed autologous DC1s recognized MDA-MB-231, but not SK-BR-3, cells (Fig. 1A). While refractory to HER2369-377–sensitized CD8+–mediated lysis, SK-BR-3 cells were significantly vulnerable, however, to natural killer (NK)–mediated antibody-dependent cell-mediated cytotoxicity (ADCC; Supplementary Fig. S1). These data reinforce evidence that HER2 overexpression, and the associated downregulation of class I expression, reduces susceptibility of tumor cells to class I–dependent CD8+–mediated, but not to class I–independent NK-mediated, lysis.

Next, we evaluated the ability of postvaccination patient-derived high-avidity HER2369-377–CD8+ T cells to recognize...

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antigen-loaded target cells in IFNγ release assays. By IFNγ ELISA, DC1-sensitized HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cells showed high-
ly specific recognition of HER2\textsubscript{369-377}-loaded T2 cells, com-
pared with control peptide–loaded T2 cells. A similarly specific
recognition of HER2\textsubscript{369-377}-loaded T2 cells, compared with
control peptide–loaded T2 cells, was observed when cocultured with
HER2\textsubscript{369-377}-TCR–transduced CD8\textsuperscript{+} T cells, but not with
nontransduced or GFP-transduced CD8\textsuperscript{+} T-cell controls (Fig. 1B).

Importantly, in order to determine if HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cells could recognize HER2-expressing cancer cells, DC1-sensitized
HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cells were cocultured with HLA-A2\textsuperscript{+} HER2\textsuperscript{low}-expressing MDA-MB-231 cells with or without HER2 siRNA transfection. Compared with nontargeting (NT) control
siRNA, HER2 siRNA transfection resulted in depletion of HER2 protein expression by Western blot, as well as loss of HER2 surface expression by flow cytometry. Notably, HLA-A2 expression on MDA-MB-231 cells remained unaffected by HER2 interference (Fig. 1C, top). While NT siRNA-transfected MDA-
MB-231 cells were specifically recognized by DC1-sensitized HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cells, this recognition was abrogated by
silencing HER2 in HER2 siRNA-transfected MDA-MB-231 cells (Fig. 1C, bottom left). In order to corroborate these observations, a similarly specific recognition of NT siRNA-transfected MDA-
MB-231 cells was demonstrated by HER2\textsubscript{369-377}-TCR–transduced CD8\textsuperscript{+} T-cells, compared with control nontransduced or GFP-transduced CD8\textsuperscript{+} T cells. This HER2\textsubscript{369-377}-specific tumor recog-
nition, however, was eliminated by silencing HER2 expression in MDA-
MB-231 cells (Fig. 1C, bottom right).

In an effort to explore if HER2\textsubscript{369-377}-CD8\textsuperscript{+} recognition of HER2-expressing cancer cells was contingent on HLA-A2, DC1-sensitized HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cells were cocultured with nontransfected, NT siRNA, and HLA-A2 siRNA-transfected MDA-
MB-231 cells. HER2\textsubscript{369-377}-CD8\textsuperscript{+} T cells only recognized non-
transfected or NT siRNA-transfected, but not HLA-A2–silenced (via HLA-A2 siRNA), MDA-MB-231 cells (Fig. 1D). Together, these data indicate that HER2\textsubscript{369-377} is endogenously pre-
dented by HER2-expressing cancers; importantly, high-avidity HER2\textsubscript{369-377}–specific CD8\textsuperscript{+} T-cells can naturally recognize the
HER2\textsubscript{369-377} epitope on HER2\textsuperscript{low} cancer cells maintaining abun-
dant class I/HLA-A2 expression, but not on surface class I/HLA-
A2–deficient HER2\textsuperscript{high} cells.

Combination of CD4\textsuperscript{+} Th1 cytokines IFNγ and TNFα with trastuzumab restores class I expression on HER2-expressing cancer cells

The immune escape provoked by HER2 overexpression on cancer cells warrants a search for strategies that restore surface MHC class I expression and improve sensitivity to CD8\textsuperscript{+}–mediated recognition and lysis. Although CD4\textsuperscript{+} Th1 cytokine IFNγ upregulates surface class I expression in HER2-overexpressing murine models in vivo (11)—restoring CD8\textsuperscript{+}–mediated lysis and/or tumor cell rejection in vivo (19)—it is comparatively less effective in reverting class I suppression in human HER2-driven tumors (13, 20). HER2 signaling is also increasingly recognized in activating the MAPK- and PI3K/AKT signal transduction path-
ways (21), suggesting that targeting these pathways may influence
class I expression (14, 22). In view of this evidence, we evaluated the effect of HER2-targeted tyrosine kinase inhibitors trastuzu-
mab and lapatinib, as well as Th1 cytokines IFNγ and TNFα, on class I expression in HER2-expressing cancers.

A spectrum of HER2-expressing cell lines (MDA-MB-231, MCF-
7, SK-OV-3\textsuperscript{A2}, BT-474, and SK-BR-3) was treated with IFNγ, TNFα, or trastuzumab alone, or in designated combinations. Compared with untreated tumor cells, treatment with TNFα or IFNγ alone increased class I expression in select (TNFα: BT-474; IFNγ: SK-OV-
3\textsuperscript{A2}, BT-474), but not all, HER2-expressing cells. Dual IFNγ and
TNFα treatment, however, significantly restored class I expression
on all HER2-expressing cell lines evaluated (P < 0.05). Treatment
with trastuzumab alone had little impact on class I expression
compared with that in untreated cells; however, the combination of trastuzumab, IFNγ, and TNFα dramatically enhanced class I expression on all cells [MDA-MB-231 (P = 0.015), MCF-7 (0.05), SK-OV-3\textsuperscript{A2} (P < 0.001), BT-474 (P < 0.0001), and SK-
BR-3 (P < 0.001)]. Interestingly, class I expression was restored
more effectively following triple therapy with trastuzumab/ IFNγ/TNFα than with dual IFNγ/TNFα treatment in HER2\textsuperscript{high}/
[BT-474 (P = 0.006), SK-BR-3 (P = 0.03)], but not in HER2intermediate (MCF-7 or SK-OV-3\textsuperscript{A2}) or HER2low (MDA-MB-
231), cells (P > 0.05; Fig. 2A).

Next, we determined if variability in MHC class I expression
was related to IFNγ/TNFα receptor expression or treatment
dose. By flow cytometry, IFNγRα/β (Supplementary Fig. S2A)
and TNFRα1 (data not shown) expression was qualitatively
similar across all cell lines tested. In HER2intermediate/HER2high
cells, a dose–response relationship for class I expression
was observed with increasing rhIFNγ doses (250–2,000 U/mL). A
dose-saturation effect beyond the 1,000 U/mL rhIFNγ dose
was observed following combination treatment with TNFα or
TNFRα1/trastuzumab (Supplementary Fig. S2B); consequently,
this standard dose was utilized for further experiments. The
addition of trastuzumab to lower IFNγ concentrations (i.e.,
250 or 500 U/mL) improved sensitivity of HER2intermediate
MCF-7/SK-OV-3\textsuperscript{A2}—but not HER2high/ BT-474/SK-BR-3—cells
to class I restoration. These data raise the intriguing possi-
Bility that trastuzumab—in concert with even moderate
levels of Th1 cytokines in the tumor microenvironment—may
aid CD8\textsuperscript{+} recognition of tumors without classical HER2
overexpression.

Consistent with findings in HER2\textsuperscript{neo}–gastric/esophageal
cancer cells (22), treatment of HER2\textsuperscript{high} SK-BR-3/BT-474 cells with
lапatinib alone did not restore class I expression appreciably. Moreover, compared with both IFNγ/TNFα treatment and trastu-
zumab/IFNγ/TNFα treatment, an attenuated upregulation in
class I expression was observed when lapatinib was combined
with IFNγ/TNFα (Supplementary Fig. S3). Mechanisms underly-
ing this observation warrant investigation; importantly, these
findings have clinical implications and may explain the superior
head-to-head clinical efficacy of trastuzumab versus lapatinib
observed in HER2\textsuperscript{neo} breast cancer patients (23).

Synergism between IFNγ, TNFα, and trastuzumab enhances HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cell recognition and lysis of HER2-
overexpressing cells

We next sought to determine the impact of class I restoration
on HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cell recognition and lysis in
HER2intermediate/high cancers. Compared with untreated
cells, treatment with TNFα, IFNγ (data not shown), or trastuzumab
alone (Fig. 2B) did not augment HER2\textsubscript{369-377}-CD8\textsuperscript{+} T-cell recognition or lysis of HER2intermediate MCF-7/SK-OV-3\textsuperscript{A2}, and
HER2\textsuperscript{high} SK-BR-3 cells. Dual treatment with IFNγ and
TNFα, however, significantly enhanced HER2\textsubscript{369-377}-CD8\textsuperscript{+}–mediated
Figure 2. 
Effect of CD4+ Th1 cytokines and HER2-targeted antibodies on MHC class I restoration and HER2369–377-CD8+ T-cell targeting of HER2-expressing cancer cells. MDA-MB-231, MCF-7, SK-OV-3, BT-474, and SK-BR-3 cells were treated with the following: no treatment (A), rhIFNγ alone (B), rhTNFα alone (C), trastuzumab alone (D), IFNγ + TNFα (E), or trastuzumab + IFNγ + TNFα (F). For each cell line, representative panels show flow cytometric analysis of APC anti-HLA-ABC expression; filled traces represent isotype-matched control staining, and open traces represent specific Ab staining. Results in adjoining histograms are representative of three experiments, and quantified as average HLA-ABC mean channel fluorescence (MCF) ± SEM. B, direct tumor recognition of HER2-expressing cells by HER2369–377-sensitized CD8+ T cells was assessed by IFNγ ELISA. HER2intermediate MCF-7, SK-OV-3, and SK-BR-3 cells—pretreated with trastuzumab alone, IFNγ + TNFα, or trastuzumab + IFNγ + TNFα—were cocultured 1:1 with HER2369–377-sensitized CD8+ T cells; coculture supernatant was harvested and subjected to IFNγ ELISA. Results, representative of three experiments using cells from different HER2pos-DCIS donors, are expressed as mean IFNγ (pg/mL) ± SEM. C, cytotoxicity of HER2-expressing cells induced by HER2369–377-sensitized CD8+ T cells was assessed by flow cytometry. CFSE-labeled tumor cells from cocultures in B were harvested and stained with 7-AAD and FITC:anti-CD8. As shown in representative dot-plot panels for each cell line, CSFE+ (y-axis), but not CD8+, cells were gated and proportion of 7-AAD+ (x-axis) cells was assessed. Results shown in adjoining histograms are representative of three experiments, and expressed as a percentage of apoptotic tumor cells (±SEM) in coculture minus background. ns, not statistically significant; tx, treatment. *, P ≤ 0.05; **, P < 0.01; ***, P < 0.001 by one-way ANOVA with post hoc Tukey testing.
recognition and lysis of HER2\textsuperscript{intermediate} MCF-7 and SK-OV-3\textsuperscript{A2}, but not HER2\textsuperscript{high} SK-BR-3, cells. The addition of trastuzumab to IFN\textgamma}/TNF\alpha rendered HER2\textsuperscript{high} SK-BR-3 cells susceptible to both recognition and lysis by HER2\textsuperscript{high,377-CD8\textsuperscript{+}} T cells; the addition of trastuzumab to IFN\textgamma}/TNF\alpha did not incrementally improve recognition or lysis of HER2\textsuperscript{intermediate} MCF-7/SK-OV-3\textsuperscript{A2} cells (Fig. 2A).

PD-L1 induction on HER2-expressing cells by IFN\textgamma}/TNF\alpha/ trastuzumab has minimal impact on DC1-sensitized HER2\textsuperscript{369-377-CD8\textsuperscript{+}} T-cell-mediated cytotoxicity

Because IFN\textgamma} is known to induce immunosuppressive programmed death (PD)-ligand-1 (PD-L1) in cancer cells (24, 25), we examined the effect of Th1 cytokine/trastuzumab combination on PD-L1 expression in HER2-expressing cell lines. Consistent with previous findings (25), constitutive PD-L1 expression was observed only in basal breast cancer subtype MDA-MB-231 cells. In all other HER2-expressing cells (SK-OV-3\textsuperscript{A2}, MCF-7, SK-BR-3), despite negligible endogenous levels, PD-L1 expression was inducible with IFN\textgamma} but not with TNF\alpha or trastuzumab treatment alone. Dual IFN\textgamma}/TNF\alpha treatment further enhanced PD-L1 expression in all cells; however, addition of trastuzumab to IFN\textgamma}/TNF\alpha did not incrementally improve PD-L1 expression (Fig. 3A).

Next, we evaluated the competing effects of cytokine/trastuzumab-mediated class 1 and PD-L1 upregulation on HER2\textsuperscript{369-377-CD8\textsuperscript{+}} T-cell recognition of these tumors. Using HER2\textsuperscript{369-377}/HLA-A\textsuperscript{A-0201} tetramers, scant PD-1 expression on DC1-sensitized HER2\textsuperscript{369-377-CD8\textsuperscript{+}} T cells was observed (mean, 1.21 ± 0.2% of CD8\textsuperscript{+} T cells; Fig. 3B). Not surprisingly, treatment with PD-1–neutralizing, compared with isotype-control, antibody did not significantly affect HER2\textsuperscript{369-377-CD8\textsuperscript{+}} lysis of HER2\textsuperscript{intermediate} MCF-7/SK-OV-3\textsuperscript{A2} or HER2\textsuperscript{high} SK-BR-3 cells (Fig. 3C). Although these data may explain the minimal impact of inhibitory PD-1/PD-L1 interactions on HER2\textsuperscript{369-377-CD8\textsuperscript{+}} cytotoxicity following a single in vitro DC1 sensitization, the upregulation of PD-L1 following Th1 cytokine/trastuzumab treatment justifies exploration of combination therapy with HER2-targeted antibodies, HER2-Th1 immune interventions, and PD-1/PD-L1 axis inhibition in HER2-expressing cancers.
Inhibition of EGFR and HER3 receptors rescues EGF and Herregulin-induced resistance to class I restoration and CD8+ T-cell–mediated cytotoxicity by IFNγ/TNFα/trastuzumab

The HER2 BTK-signaling domain is activated upon heterodimerization with other HER family members (EGFR, HER3, HER4) or upon homodimerization (26). Given the inability of trastuzumab to inhibit EGFR/HER2 and HER2/HER3 heterodimers (27), escape signaling via EGF and/or HER3 receptors trastuzumab to inhibit EGFR/HER2 and HER2/HER3 hetero-HER4) or upon homodimerization (26). Given the inability of dimerization with other HER family members (EGFR, HER3, HER4) the inability of trastuzumab to inhibit EGFR/HER2 and HER2/HER3 hetero-dimers (27), escape signaling via EGF and HER3 receptors.

Therefore, we investigated the impact of EGFR and HER3 signaling on class I expression in vitro. HER2high BT-474 and SK-BR-3 cells were pretreated with EGF (EGFR ligand) or Heregulin (HER3 ligand) and subjected to treatment with trastuzumab and IFNγ/TNFα. Activation of signaling via EGF/HER2 together and HER3 alone, but not EGF alone, rendered HER2high BT-474 (Fig. 4A) and SK-BR-3 (data not shown) cells significantly resistant to the class I–restoring effect of trastuzumab/IFNγ/TNFα. Importantly, inhibition of EGFR and HER3-driven signaling with anti-EGFR and anti-HER3 antibodies rescued EGF and Heregulin-mediated resistance to class I restoration (Fig. 4B), and consequently HER2369-377-CD8+ T-cell lysis (Fig. 4C), of HER2high cells.

We have previously shown that HLA-A2pos donor-derived CD8+ T cells, sensitized with HER2369-377-pulsed DC1, directly recognize HER2-expressing cancer cells via an II.12-dependent mechanism (17). In the current study, we extend these observations by demonstrating that the HER2369-377 epitope is not only endogenously presented on HER2-expressing cancers, but also naturally recognized on class I–abundant HER2low, but not on class I–deficient HER2high, cancer cells by HER2369-377–specific CD8+ T cells. Our findings, therefore, may explain the clinical benefit paradoxically observed in patients with HER2low (1+), but not classically HER2high (3+), tumors following HER2369-377 vaccinations in GM-CSF (10).

Because HER2–driven class I downregulation impedes CD8+ T-cell recognition of even immunodominant epitopes, such as HER2369-377 in HER2-overexpressing cancers (13), we uncover a critical collaboration between cellular (IFNγ and TNFα) and humoral (trastuzumab) immunity in restoring class I expression in HER2-overexpressing cells, whereby rendering them susceptible to HER2369-377–specific CD8+ T-cell–mediated targeting. Intriguingly, although synergism between Th1 cytokines IFNγ and TNFα appears sufficient for class I restoration and HER2369-377-CD8+ T-cell targeting of HER2low/intermediate...
cancers, class I restoration and ensuing targeting of HER2-overexpressing (HER2\textsuperscript{high}) cells is critically dependent on the cooperation between IFN\textgamma/TNF\alpha and trastuzumab-mediated HER2 blockade. Recent evidence suggests that MAPK signaling inhibition predominantly regulates MHC class I induction in HER2-overexpressing cancer cells, whereas preserved PI3K/AKT signaling further enhances class I expression compared with MAPK inhibition alone (13, 22). In the setting of trastuzumab-mediated inhibition of presumably both MAPK and PI3K/AKT signaling, it is possible that IFN\textgamma/TNF\alpha potentiate class I upregulation in HER2\textsuperscript{high} cells by (i) more complete abrogation of MAPK signaling and/or (ii) preferential rescue of PI3K/AKT signaling. Future studies should investigate signaling cascades underlying such effects.

The translational relevance of these novel observations bears emphasis. Combinations of HER2-targeted antibodies and immune interventions incorporating CD4\textsuperscript{+} helper epitopes may potentiate anti-HER2 CD8\textsuperscript{+} T-cell–directed immunotherapies and improve clinical outcomes in patients with HER2-overexpressing tumors. Indeed, we have recently initiated a phase I trial in HER2\textsuperscript{+} breast cancer patients investigating such combinations in the neoadjuvant setting. Ultimately, given the impact of EGFR/HER3-mediated escape signaling on class I expression as well as CD8\textsuperscript{+} T-cell–mediated cytotoxicity in trastuzumab-treated HER2\textsuperscript{+} cancers, a multivalent strategy targeting other HER family members—in addition to HER2—may prove most effective.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Datta, S. Xu, B. J. Czerniecki
Development of methodology: J. Datta, S. Xu, C. Rosemblit, J. B. Smith, D. J. Powell Jr, B. J. Czerniecki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Datta, S. Xu, C. Rosemblit, J. B. Smith, J. A. Cintolo, D. J. Powell Jr, B. J. Czerniecki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Datta, S. Xu, C. Rosemblit, J. A. Cintolo, B. J. Czerniecki
Writing, review, and/or revision of the manuscript: J. Datta, S. Xu, C. Rosemblit, J. A. Cintolo, D. J. Powell Jr, B. J. Czerniecki
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Datta, S. Xu, J. B. Smith, B. J. Czerniecki
Study supervision: D. J. Powell Jr, B. J. Czerniecki

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References

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Jashodeep Datta, Shuwen Xu, Cinthia Rosemblit, et al.


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