Priority Brief

Cancer Immunology Research

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

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Abstract

Vaccination strategies incorporating the immunodominant HLA-A2–restricted HER2/*neu*-derived peptide 369–377 ($HER2_{369–377}$) are increasingly utilized in HER2/*neu*-expressing cancer patients. The failure of postvaccination $HER2_{369–377}$-specific CD8$^+$ T cells to recognize HLA-A2^{pos}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 $HER2_{369–377}$ is directly recognized by high functional-avidity $HER2_{369–377}$-specific CD8$^+$ T cells—either genetically modified to express a novel $HER2_{369–377}$ TCR or sensitized using $HER2_{369–377}$-pulsed type 1–polarized dendritic cells (DC1)—on class I–abundant HER2^{low}, but not class I–deficient HER2^{high}, 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 HER2^{high} cancers, thereby facilitating recognition and lysis of these cells by $HER2_{369–377}$-specific CD8$^+$ T cells. Concomitant induction of PD-L1 on HER2/*neu*-expressing cells by IFNγ/TNF and trastuzumab, however, has minimal impact on DC1-sensitized $HER2_{369–377}$-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 $HER2_{369–377}$-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 (HER2^{pos}) 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 HER2^{pos} 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 ($HER2_{369–377}$; 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 $HER2_{369–377}$ (with adjuvant) to vaccinate patients with HER2^{pos} tumors generated postimmunization $HER2_{369–377}$-reactive CD8$^+$ T cells that failed to recognize HLA-A2^{pos} tumor cells expressing HER2 (9). In addition, $HER2_{369–377}$ peptide vaccination in GM-CSF (E75) induced immune responses and improved clinical outcomes in patients with low HER2-expressing (1+)—but not in classically HER2^{pos} (3+ or 2+/FISH-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 $HER2_{369–377}$ is endogenously presented by HER2-expressing cancer cells, and naturally recognized by $HER2_{369–377}$-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 $HER2_{369–377}$-CD8$^+$ T-cell targeting of HER2-overexpressing cancers. Concomitant induction of PD-L1

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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 $HER2_{369–377}$-$CD8^+$ 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 $HER2_{369–377}$-$CD8^+$ 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 ($HER2^{high}$), MCF-7 ($HER2^{intermediate}$), MDA-MB-231 ($HER2^{low}$; American Type Culture Collection), and ovarian cancer cell line SK-OV-3 ($HER2^{intermediate}$) stably transfected with the HLA-A2 gene (SK-OV-3^{A2}; kind gift of Mary Disis, University of Washington) were immediately resuscitated and maintained in RPMI supplemented with 10% FCS (Cellgro). HLA-A2 status was 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 (MIH1; ref. 15) or IgG1 isotype control (eBiosciences). Specific cell treatments are detailed in Supplementary Methods.

Generation of $HER2_{369–377}$-specific $CD8^+$ TCR clones

$HER2_{369–377}$-specific $CD8^+$ TCR clones were generated as previously described (16). Briefly, high-avidity $HER2_{369–377}$-reactive

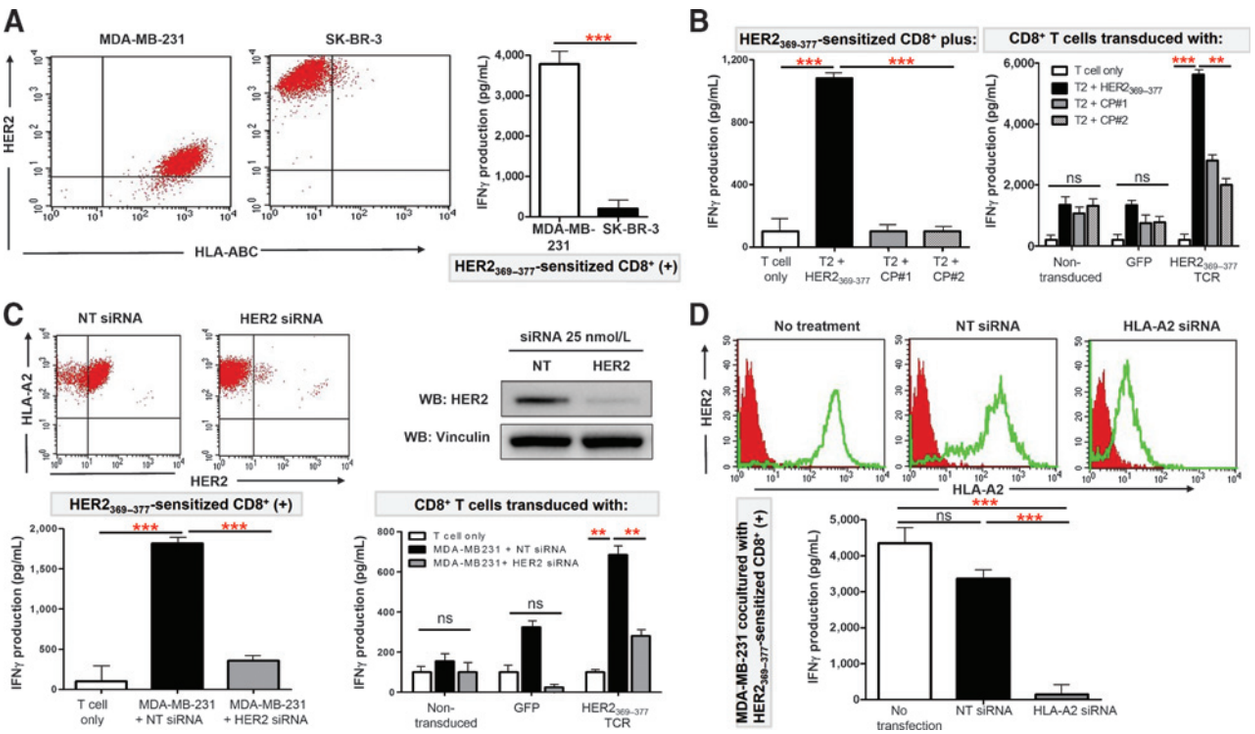

Figure 1.
HER2-expressing cancer cells are directly recognized by $HER2_{369–377}$-specific $CD8^+$ T cells in an MHC class I–dependent manner. A, by flow cytometric analysis, pan-class I surface expression on tumor cells correlated inversely with HER2 expression (MDA-MB-231: $HER2^{low}$, robust HLA-ABC; SK-BR-3: $HER2^{high}$, minimal HLA-ABC). Tumor cells were stained with anti-HER2 (PE: *y*-axis) and anti–HLA-ABC (APC: *x*-axis), with representative stainings shown. Tumor cells were challenged against postvaccination HLA-$A2^{pos}$ patient–derived $CD8^+$ T cells sensitized with $HER2_{369–377}$-pulsed DC1 (from 2 separate $HER2^{pos}$-DCIS patients), and antigen-specific recognition was evaluated by IFNγ release ELISA. B, recall responses of $HER2_{369–377}$-sensitized $CD8^+$ T cells from four separate donors (left) or $HER2_{369–377}$-TCR–transduced, GFP-transduced, or nontransduced $CD8^+$ T-cell clones (right) to $HER2_{369–377}$ or two control class I peptide-pulsed T2 hybridoma cells demonstrate markedly specific antigen-specific recognition of target cells in IFNγ release assays. 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-HER2:*x*-axis vs. FITC anti–HLA-A2:*y*-axis) and Western blot analysis (vinculin as loading control); representative images are shown. $HER2_{369–377}$-sensitized (left) and $HER2_{369–377}$-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. $HER2_{369–377}$-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* HER2$_{369–377}$ stimulation of CD8$^+$ T cells obtained from a patient with HLA-A2^{pos}HER2^{pos} ductal carcinoma *in situ* who was previously vaccinated with HER2$_{369–377}$-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 HER2$_{369–377}$ T cells, allowed for isolation of HER2$_{369–377}$-specific CD8$^+$ TCR. Primary human CD8$^+$ T cells genetically modified to express this HER2$_{369–377}$ TCR specifically bound HER2$_{369–377}$-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-A2^{pos} HER2^{pos}-DCIS donors, who had undergone HER2$_{369–377}$-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 HER2$_{369–377}$ 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 HER$_{2369–377}$-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 HER2$_{369–377}$ or control class I peptides (1 μg/mL), and tested against MDA-MB-231 with or without HER2/HLA-A2 siRNA transfection, SK-OV-3^{A2}, MCF-7, and SK-BR-3 cell lines as indicated. HER2$_{369–377}$-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, 3×10^5 MDA-MB-231 cells were transfected with HER2, HLA-A2, or nontargeting siRNA sequences (25 nmol/L; ON-TARGETplus; Dharmacon) using RNAi Max Lipofectamine (Life Technologies) in serum-free medium. After 1 hour, medium was supplemented with 10% FBS; 20 hours later, cells were serum-starved for 48 hours, followed by coculture with HER2$_{369–377}$-sensitized or HER2$_{369–377}$-TCR–transduced CD8$^+$ T cells. Before coculture, aliquots from individual treatment groups were obtained to confirm HER2/HLA-A2 silencing via flow cytometry or Western blot (Supplementary Methods), or both.

Flow cytometry

Cell suspensions were prepared in FACS buffer (PBS + 1% FCS + 0.01% azide); 7-AAD (viability stain), and FITC/APC/phycoerythrin (PE)–conjugated mouse anti-human CD11c, CD8, HLA-ABC, HLA-A2, HER2, IFNγRα/β, TNFαR1, PD-L1, PD-1, or subclass-matched controls (BD Bioscience) were utilized as indicated. HER2$_{369–377}$/HLA-A*0201 tetramers (MCL) were used to identify HER2$_{369–377}$-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 HER2$_{369–377}$-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. $P \leq 0.05$ was considered statistically significant. Analysis was performed using Prism 5.0 (GraphPad Inc.).

Results and Discussion

HER2$_{369–377}$ is recognized on HER2-expressing cancer cells by HER2$_{369–377}$-specific CD8$^+$ T cells

The HER2$_{369–377}$ peptide is widely regarded as the immunodominant epitope recognized by lymphocytes from HLA-A2^{pos} patients with breast/ovarian cancer (8). HER2$_{369–377}$ was used to immunize mice transgenic for both HLA-A2.1 and human CD8; postimmunization splenocytes recognized HLA-A2^{pos}HER2^{pos} human tumor cells (18). In spite of supportive preclinical evidence, the failure of CTLs from HER2$_{369–377}$-immunized patients to recognize HER2^{pos} 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 HER2^{low} 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 HER2$_{369–377}$-pulsed autologous DC1s recognized MDA-MB-231, but not SK-BR-3, cells (Fig. 1A). While refractory to HER2$_{369–377}$-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 HER2$_{369–377}$-CD8$^+$ T cells to recognize

antigen-loaded target cells in IFNγ release assays. By IFNγ ELISA,DC1-sensitized $HER2_{369–377}$-$CD8^+$ T cells showed highly specific recognition of $HER2_{369–377}$-loaded T2 cells, compared with control peptide–loaded T2 cells. A similarly specific recognition of $HER2_{369–377}$-loaded T2 cells, compared with control peptide–loaded T2 cells, was observed when cocultured with $HER2_{369–377}$-TCR–transduced $CD8^+$ T cells, but not with nontransduced or GFP-transduced $CD8^+$ T-cell controls (Fig. 1B).

Importantly, in order to determine if $HER2_{369–377}$-$CD8^+$ T cells could recognize HER2-expressing cancer cells, DC1-sensitized $HER2_{369–377}$-$CD8^+$ T cells were cocultured with HLA-$A2^{pos}$ $HER2^{low}$-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 $HER2_{369–377}$-$CD8^+$ 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 $HER2_{369–377}$-TCR–transduced $CD8^+$ T cells, compared with control nontransduced or GFP-transduced $CD8^+$ T cells. This $HER2_{369–377}$-specific tumor recognition, however, was eliminated by silencing HER2 expression in MDA-MB-231 cells (Fig. 1C, bottom right).

In an effort to explore if $HER2_{369–377}$-$CD8^+$ recognition of HER2-expressing cancer cells was contingent on HLA-A2, DC1-sensitized $HER2_{369–377}$-$CD8^+$ T cells were cocultured with nontransfected, NT siRNA, and HLA-A2 siRNA-transfected MDA-MB-231 cells. $HER2_{369–377}$-$CD8^+$ T cells only recognized nontransfected or NT siRNA-transfected, but not HLA-A2–silenced (via HLA-A2 siRNA), MDA-MB-231 cells (Fig. 1D). Together, these data indicate that $HER2_{369–377}$ is endogenously presented by HER2-expressing cancers; importantly, high-avidity $HER2_{369–377}$-specific $CD8^+$ T cells can naturally recognize the $HER2_{369–377}$ epitope on $HER2^{low}$ cancer cells maintaining abundant class I/HLA-A2 expression, but not on surface class I/HLA-A2–deficient $HER2^{high}$ cells.

Combination of $CD4^+$ Th1 cytokines IFN γ 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 $CD8^+$-mediated recognition and lysis. Although $CD4^+$ Th1 cytokine IFNγ upregulates surface class I expression in HER2-overexpressing murine models *in vitro* (11)—restoring $CD8^+$-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 pathways (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 trastuzumab 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^{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^{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^{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 $HER2^{high}$ [BT-474 ($P = 0.006$); SK-BR-3 ($P = 0.03$)], but not in $HER2^{intermediate}$ (MCF-7 or SK-OV-3^{A2}) or $HER2^{low}$ (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 TNFαR1 (data not shown) expression was qualitatively similar across all cell lines tested. In $HER2^{intermediate}$/$HER2^{high}$ 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 TNFα/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 $HER2^{intermediate}$ MCF-7/SK-OV-3^{A2}—but not $HER2^{high}$ BT-474/SK-BR-3—cells to class I restoration. These data raise the intriguing possibility that trastuzumab—in concert with even moderate levels of Th1 cytokines in the tumor microenvironment—may aid $CD8^+$ recognition of tumors without classical HER2 overexpression.

Consistent with findings in $HER2^{pos}$-gastric/esophageal cancer cells (22), treatment of $HER2^{high}$ SK-BR-3/BT-474 cells with lapatinib alone did not restore class I expression appreciably. Moreover, compared with both IFNγ/TNFα treatment and trastuzumab/IFNγ/TNFα treatment, an attenuated upregulation in class I expression was observed when lapatinib was combined with IFNγ/TNFα (Supplementary Fig. S3). Mechanisms underlying 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 $HER2^{pos}$ breast cancer patients (23).

Synergism between IFNγ, TNFα, and trastuzumab enhances $HER2_{369–377}$-$CD8^+$ T-cell recognition and lysis of HER2-overexpressing cells

We next sought to determine the impact of class I restoration on $HER2_{369–377}$-$CD8^+$ T-cell recognition and lysis in $HER2^{intermediate/high}$ cancers. Compared with untreated cells, treatment with TNFα, IFNγ (data not shown), or trastuzumab alone (Fig. 2B) did not augment $HER2_{369–377}$-$CD8^+$ T-cell recognition or lysis of $HER2^{intermediate}$ MCF-7/SK-OV-3^{A2}, and $HER2^{high}$ SK-BR-3 cells. Dual treatment with IFNγ and TNFα, however, significantly enhanced $HER2_{369–377}$-$CD8^+$-mediated

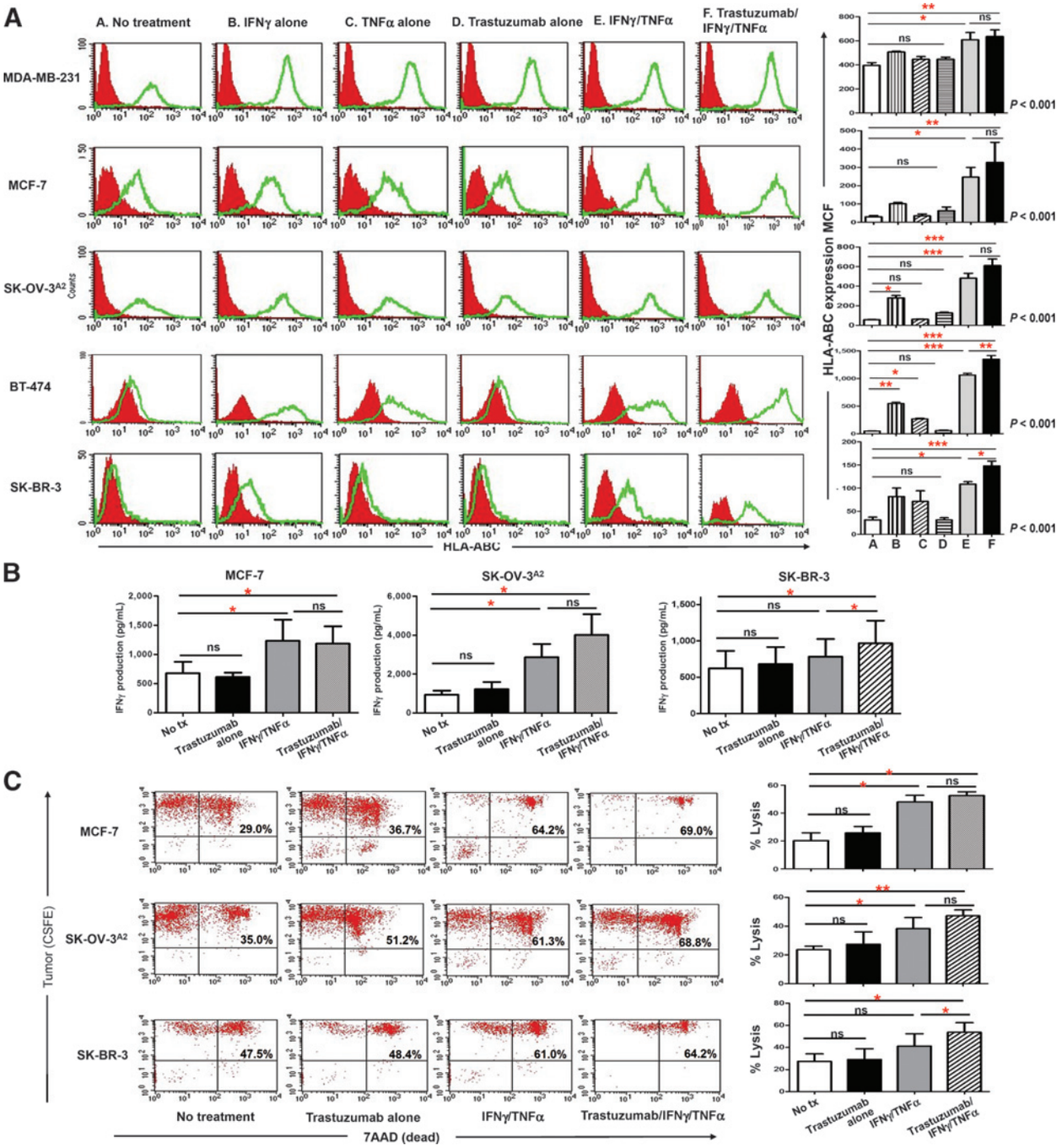

Figure 2.

Effect of CD4$^+$ Th1 cytokines and HER2-targeted antibodies on MHC class I restoration and HER2$_{369–377}$-CD8$^+$ T-cell targeting of HER2-expressing cancer cells. HER2^{low} MDA-MB-231, HER2^{intermediate} MCF-7 and SK-OV-3^{A2}, and HER2^{high} 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 HER2$_{369–377}$-sensitized CD8$^+$ T cells was assessed by IFNγ ELISA. HER2^{intermediate} MCF-7, SK-OV-3^{A2}, and HER2^{high} SK-BR-3 cells—pretreated with trastuzumab alone, IFNγ + TNFα, or trastuzumab + IFNγ + TNFα—were cocultured 1:1 with HER2$_{369–377}$-sensitized CD8$^+$ T cells; coculture supernatant was harvested and subjected to IFNγ ELISA. Results, representative of three experiments using cells from different HER2^{pos}-DCIS donors, are expressed as mean IFNγ (pg/mL) ± SEM. C, cytotoxicity of HER2-expressing cells induced by HER2$_{369–377}$-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. *, $P \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with post hoc Tukey testing.

recognition and lysis of HER2^{intermediate} MCF-7 and SK-OV-3^{A2}, but not HER2^{high} SK-BR-3, cells. The addition of trastuzumab to IFNγ/TNFα rendered HER2^{high} SK-BR-3 cells susceptible to both recognition and lysis by HER2$_{369–377}$-CD8$^+$ T cells; the addition of trastuzumab to IFNγ/TNFα did not incrementally improve recognition or lysis of HER2^{intermediate} MCF-7/SK-OV-3^{A2} cells (Fig. 2B and C).

PD-L1 induction on HER2-expressing cells by IFNγ/TNFα/trastuzumab has minimal impact on DC1-sensitized HER2$_{369–377}$-CD8$^+$ T-cell–mediated cytotoxicity

Because IFNγ 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 combinations 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^{A2}, MCF-7, SK-BR-3), despite negligible endogenous levels, PD-L1 expression was inducible with IFNγ but not with TNFα or trastuzumab treatment alone. Dual IFNγ/TNFα treatment further enhanced PD-L1 expression in all cells; however, addition of trastuzumab to IFNγ/TNFα did not incrementally improve PD-L1 expression (Fig. 3A).

Next, we evaluated the competing effects of cytokine/trastuzumab-mediated class I and PD-L1 upregulation on HER2$_{369–377}$-CD8$^+$ T-cell recognition of these tumors. Using HER2$_{369–377}$/HLA-A*0201 tetramers, scant PD-1 expression on DC1-sensitized HER2$_{369–377}$-reactive CD8$^+$ T cells was observed (mean, 1.21 ± 0.2% of CD8$^+$ T cells; Fig. 3B). Not surprisingly, treatment with PD-1–neutralizing, compared with isotype-control, antibody did not significantly affect HER2$_{369–377}$-CD8$^+$ lysis of HER2^{intermediate} MCF-7/SK-OV-3^{A2} or HER2^{high} SK-BR-3 cells (Fig. 3C). Although these data may explain the minimal impact of inhibitory PD-1/PD-L1 interactions on HER2$_{369–377}$-CD8$^+$ 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.

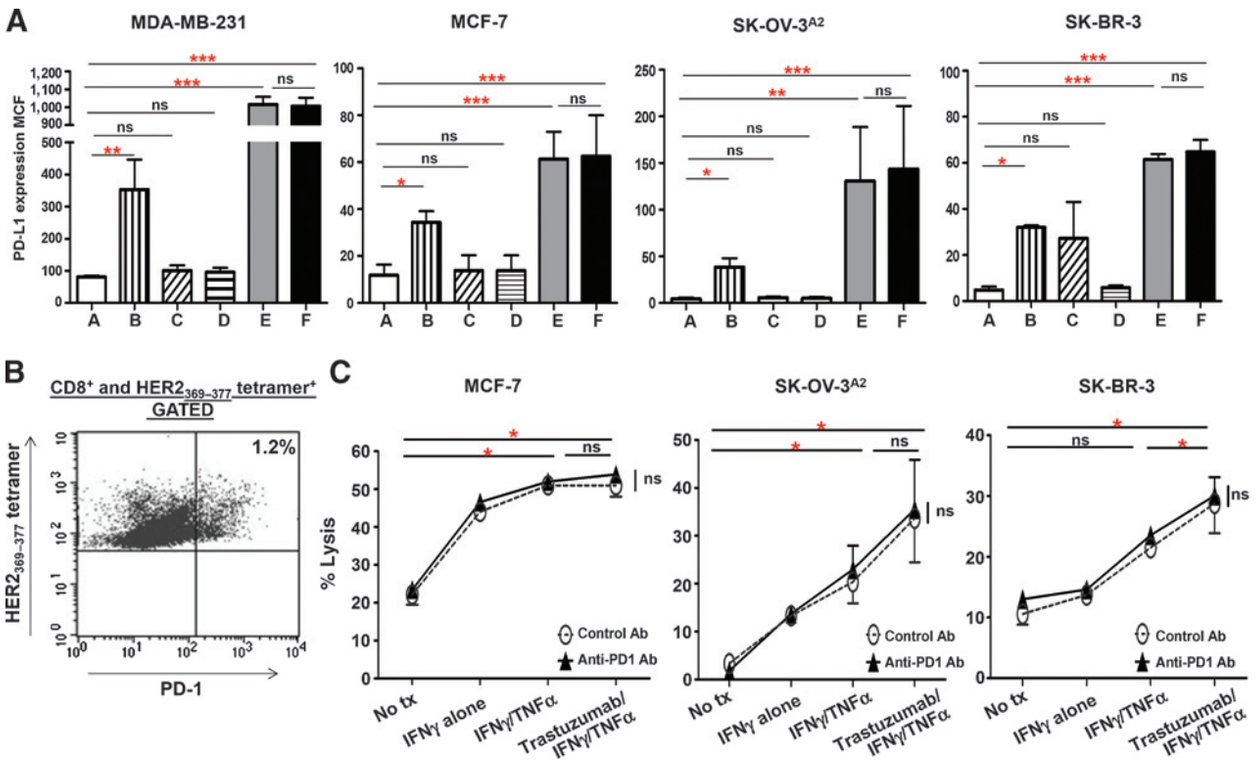

Figure 3.
PD-L1 induction by IFNγ/TNFα and trastuzumab has minimal impact on DC1-sensitized HER2$_{369–377}$-CD8$^+$ T-cell targeting of HER2-expressing cancer cells. A, HER2^{low} MDA-MB-231, HER2^{intermediate} MCF-7 and SK-OV-3^{A2}, and HER2^{high} SK-BR-3 cells were treated with the following: no treatment (A), rhIFNγ alone (B), rhTNFα alone (C), trastuzumab alone (D), IFNγ + TNFα (E), or trastuzumab + IFNγ + TNFα (F), and PD-L1 expression was examined by flow cytometry. Results are representative of three experiments and quantified as average PD-L1 mean channel fluorescence (MCF) ± SEM. B, DC1-sensitized HER2$_{369–377}$-CD8$^+$ T cells were stained with anti-CD8, anti–PD-1, and HER2$_{369–377}$/HLA-A*0201 tetramers. A representative dot-plot panel with gating on CD8$^+$ and HER2$_{369–377}$-tetramer$^+$ cells is shown; percentage of HER2$_{369–377}$-tetramer$^+$PD-1$^+$ cells is indicated (top right quadrant). C, following indicated cell treatments (*x*-axis), CFSE-labeled MCF-7, SK-OV-3^{A2}, and SK-BR-3 cells were cocultured 1:1 with HER2$_{369–377}$-CD8$^+$ T cells, and treated with neutralizing anti–PD-1 (▲) or isotype control (○) antibodies. Harvested cells were stained with 7-AAD and FITC:anti-CD8, and CSFE$^+$ 7-AAD$^+$CD8$^-$ cells (apoptotic) were assessed by flow cytometry. Results are representative of three experiments and expressed as percentage of apoptotic tumor cells ± SEM (% lysis) in coculture minus background. *, $P \leq \leq 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with post hoc Tukey testing.

Inhibition of EGFR and HER3 receptors rescues EGF and Heregulin-induced resistance to class I restoration and CD8$^+$ T-cell–mediated cytotoxicity by IFNγ/TNFα/trastuzumab

The HER2 RTK 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 has been implicated as an important mechanism of resistance to trastuzumab (28). Therefore, we investigated the impact of EGFR and HER3 signaling on class I expression *in vitro*. HER2^{high} 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 EGFR/HER3 together and HER3 alone, but not EGFR alone, rendered HER2^{high} 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 HER2$_{369–377}$-CD8$^+$ T-cell lysis (Fig. 4C), of HER2^{high} cells.

We have previously shown that HLA-A2^{pos} donor-derived CD8$^+$ T cells, sensitized with HER2$_{369–377}$-pulsed DC1, directly recognize HER2-expressing cancer cells via an IL12-dependent mechanism (17). In the current study, we extend these observations by demonstrating that the HER2$_{369–377}$ epitope is not only endogenously presented on HER2-expressing cancers, but also naturally recognized on class I–abundant HER2^{low}, but not on class I–deficient HER2^{high}, cancer cells by HER2$_{369–377}$-specific CD8$^+$ T cells. Our findings, therefore, may explain the clinical benefit paradoxically observed in patients with HER2^{low} (1+), but not classically HER2^{high} (3+), tumors following HER2$_{369–377}$ vaccinations in GM-CSF (10).

Because HER2-driven class I downregulation impedes CD8$^+$ T-cell recognition of even immunodominant epitopes, such as HER2$_{369–377}$ 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, thereby rendering them susceptible to HER2$_{369–377}$-specific CD8$^+$ T-cell–mediated targeting. Intriguingly, although synergism between Th1 cytokines IFNγ and TNFα appears sufficient for class I restoration and HER2$_{369–377}$-CD8$^+$ T-cell targeting of HER2$^{low/intermediate}$

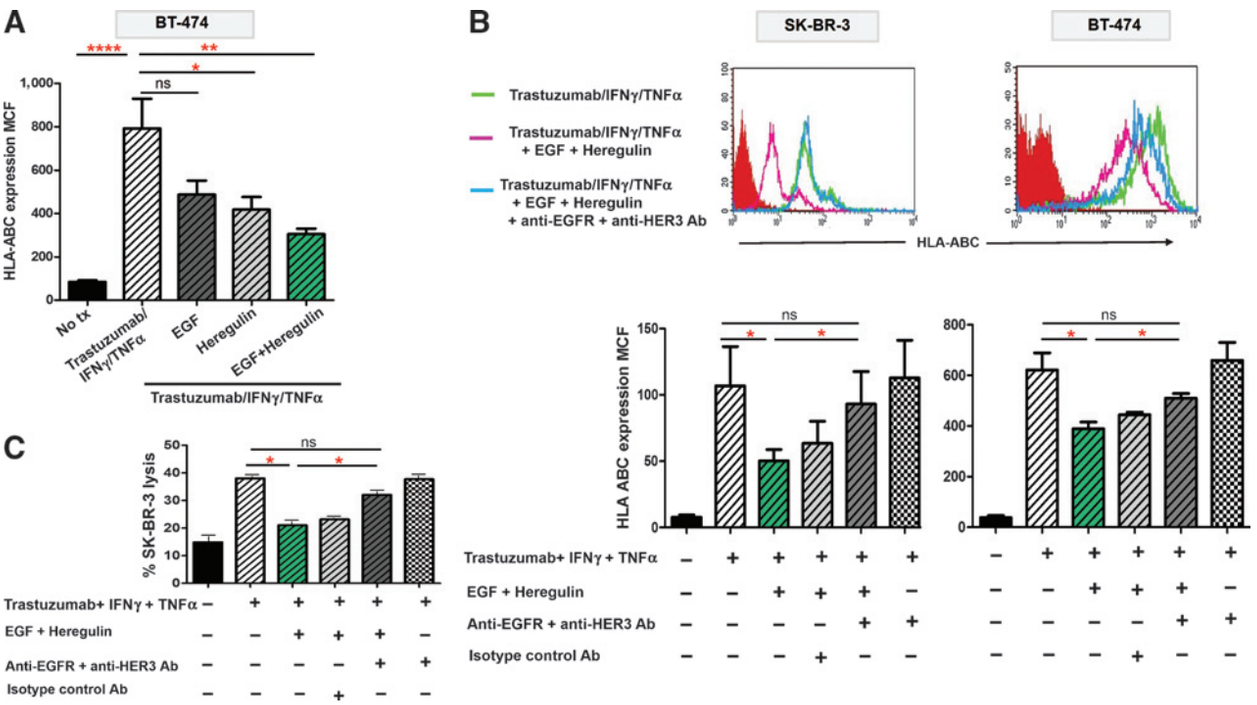

Figure 4.

Resistance to Th1 cytokine/trastuzumab-mediated class I restoration and HER2$_{369–377}$-CD8$^+$ T-cell targeting of HER2-expressing cancers by EGF/Heregulin is rescued with inhibition of EGFR and HER3 signaling. A, effect of EGFR- and HER3-mediated signaling on class I restoration by trastuzumab and Th1 cytokines. Trastuzumab-treated HER2^{high} BT-474 cells were serum starved and activated with EGF, Heregulin, or both, followed by IFNγ and TNFα treatment. Harvested cells were assessed for HLA-ABC expression by flow cytometry. Results are representative of three experiments, and expressed as mean HLA-ABC mean channel fluorescence (MCF) ± SEM. B, trastuzumab/IFNγ/TNFα-treated HER2^{high} SK-BR-3 (left) and BT-474 (right) cells with or without EGF + Heregulin activation were subsequently treated with anti-EGFR + anti–HER3-neutralizing or IgG1 isotype control antibodies. Harvested cells were assessed for HLA-ABC expression by flow cytometry. In representative panels, filled traces represent isotype-matched control staining, and open traces represent HLA-ABC staining. Color-coded cell treatments are indicated to the left of the graphs. Adjoining results in histograms are representative of three experiments, and expressed as mean HLA-ABC MCF ± SEM; cell treatments are indicated below the histograms. C, following treatments indicated in B above, CFSE-labeled HER2^{high} SK-BR-3 cells were cocultured 1:1 with HER2$_{369–377}$-sensitized CD8$^+$ T cells. Tumor cells were harvested, stained with 7-AAD and FITC:anti-CD8, and CSFE$^+$7-AAD$^+$CD8$^-$ cells (apoptotic) were assessed by flow cytometry. Results are representative of three experiments, and expressed as a percentage of apoptotic tumor cells ± SEM (% lysis) in coculture minus background. *, $P \leq 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by one-way ANOVA with post hoc Tukey testing. ns, not statistically significant.

cancers, class I restoration and ensuing targeting of HER2-overexpressing ($HER2^{high}$) cells is critically dependent on the cooperation between IFNγ/TNFα 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 IFNγ/TNFα potentiate class I upregulation in $HER2^{high}$ 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 $CD4^+$ helper epitopes may potentiate anti-HER2 $CD8^+$ T-cell–directed immunotherapies and improve clinical outcomes in patients with HER2-overexpressing tumors. Indeed, we have recently initiated a phase I trial in $HER2^{pos}$ 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 $CD8^+$ T-cell–mediated cytotoxicity in trastuzumab-treated $HER2^{pos}$ 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.

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