

## Trastuzumab Increases HER2 Uptake and Cross-Presentation by Dendritic Cells

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### Abstract

Early-phase clinical trials evaluating CD8<sup>+</sup> T cell-eliciting, HER2-derived peptide vaccines administered to HER2<sup>+</sup> breast cancer patients in the adjuvant setting suggest synergy between the vaccines and trastuzumab, the mAb targeting the HER2 protein. Among 60 patients enrolled in clinical trials evaluating the E75 + GM-CSF and GP2 + GM-CSF vaccines, there have been no recurrences in patients vaccinated after receiving trastuzumab as part of standard therapy in the per treatment analyses conducted after a median follow-up of greater than 34 months. Here, we describe a mechanism by which this synergy may occur. Flow cytometry showed that trastuzumab facilitated uptake of HER2 by dendritic cells (DC), which was mediated by the Fc receptor and was specific to trastuzumab. *In vitro*, increased HER2 uptake by DC increased cross-presentation

of E75, the immunodominant epitope derived from the HER2 protein, an observation confirmed in two *in vivo* mouse models. This increased E75 cross-presentation, mediated by trastuzumab treatment, enabled more efficient expansion of E75-specific cytotoxic T cells (E75-CTL). These results demonstrate a mechanism by which trastuzumab links innate and adaptive immunity by facilitating activation of antigen-specific T cells. On the basis of these data, we conclude that HER2-positive breast cancer patients that have been treated with trastuzumab may experience a more robust antitumor immune response by restimulation of T cells with the E75 peptide vaccine, thereby accounting for the improved disease-free survival observed with combination therapy. *Cancer Res*; 77(19); 5374–83. ©2017 AACR.

### Introduction

Despite advances in breast cancer treatment, approximately 20% of patients recur after standard-of-care therapy and ultimately succumb to their disease. To address this, our group has been investigating strategies to augment antitumor immune responses using HER2-derived peptide vaccines administered to patients in the adjuvant setting. One of these vaccines incorporates E75, the immunodominant epitope derived from the HER2 protein's extracellular domain, combined with the immunoadjuvant gran-

ulocyte macrophage colony-stimulating factor (GM-CSF). E75 is an MHC I peptide that, when administered with GM-CSF, stimulates an antigen-specific CD8<sup>+</sup> T-cell response. In a phase I/II clinical trial enrolling 187 assessable patients, the 5-year disease-free survival (DFS) rate was 90% in vaccinated patients versus 80% in unvaccinated control patients ( $P = 0.08$ ; ref. 1). A second CD8<sup>+</sup> T cell-eliciting vaccine that we have been investigating is GP2, an MHC class I peptide derived from the HER2 protein's intracellular domain. In a phase II trial evaluating the GP2 + GM-CSF vaccine, the Kaplan–Meier estimated 5-year DFS rate was 94% in vaccinated patients versus 85% in patients receiving GM-CSF alone in the per treatment analysis ( $P = 0.17$ ; ref. 2). Although both of these trials failed to achieve statistical significance with respect to their primary endpoint of DFS, they were informative in that they confirmed the vaccines to be safe and capable of stimulating an antigen-specific immune response. They also identified a potential strategy for further investigation, specifically combination immunotherapy using a CD8<sup>+</sup> T cell-eliciting vaccine in combination with trastuzumab, the mAb that targets the HER2 protein.

The trials described above enrolled breast cancer patients with any degree of HER2 expression (IHC 1+, 2+, or 3+). Between the two trials, in the per treatment analyses, there were 60 patients with HER2<sup>+</sup> breast cancer that were vaccinated after receiving trastuzumab as part of their standard-of-care therapy. After a median follow-up of over 34 months, there were no recurrences (100% DFS) in any of these patients. In contrast, in the GP2 trial, the DFS rate in HER2<sup>+</sup> patients that received trastuzumab but were randomized to the control arm of the study was 89% (2).

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This observation suggests synergy between trastuzumab and these CD8<sup>+</sup> T cell-eliciting vaccines.

Trastuzumab is a humanized IgG1k mAb with two antigen-specific sites that bind to the extracellular domain of HER2 and a conserved Fc portion (3). Multiple mechanisms of action have been proposed for trastuzumab, including the inhibition of downstream HER2 signaling, the inhibition of cell proliferation, as well as the activation of both innate and adaptive cellular immunity (4). *In vivo* models have confirmed antibody-dependent cell-mediated cytotoxicity as one of trastuzumab's mechanisms of action with clinical efficacy correlating with both the presence of natural killer cells and the expression of FcγR polymorphisms by natural killer cells (5). Adaptive immunity has also been shown to play a role in mediating breast cancer cell lysis by trastuzumab in murine models, with CD8<sup>+</sup> and CD4<sup>+</sup> T-cell depletion resulting in a blunted response to treatment with trastuzumab (6, 7). In patients with metastatic HER2<sup>+</sup> breast cancer, trastuzumab has been shown to induce anti-HER2 CD4<sup>+</sup> T-cell responses as well as anti-HER2 antibody responses with anti-HER2 antibody responses being associated with improved progression-free and overall survival (8, 9). In addition, trastuzumab-treated breast cancer cells are more susceptible to killing by antigen-specific cytotoxic T lymphocytes (CTL) due to increased internalization of HER2 and presentation of HER2-derived peptides on MHC class I molecules (10).

Tumor cell lysis using a variety of therapeutic modalities exposes tumor antigens for uptake by antigen-presenting cells (APC), and subsequent processing and presentation of antigen-derived peptides on HLA, hence potentiating the priming of an immune response. Dendritic cells (DC) highly express receptors that are involved in antigen uptake and cross-presentation, including Fc receptors (FcR), MHC class I and II molecules, as well as costimulatory molecules, making them the most efficient APCs in the immune system and essential in stimulating a CTL response (11). A number of studies have shown that tumor lysis by chemotherapy and radiotherapy facilitates antigen release by tumors and subsequent uptake and processing by APCs (12, 13). Given the fact that DCs express FcR and present antigen to T cells, we hypothesized that trastuzumab, like other therapeutic antibodies that share a conserved Fc domain (14), could facilitate HER2 uptake by DCs for subsequent cross-priming of an anti-HER2 immune response. The immune priming that is mediated by trastuzumab would then facilitate a more potent immune response following vaccination with E75 peptide.

In this study, we evaluated the effect of trastuzumab on the uptake and cross-presentation of HER2-derived peptides by DCs. We show that soluble HER2 is taken up more efficiently by DCs than cell-associated HER2 and that HER2 uptake is enhanced by treatment with trastuzumab. We also show, in *in vitro* and *in vivo* models, that HER2 uptake results in cross-presentation of E75 peptide by DCs. Finally, we demonstrate that cross-presentation of E75 by DCs leads to priming of an antitumor immune response with increased antigen-specific CTL generation.

## Materials and Methods

### Cell lines

The breast cancer cell lines SKBR3, BT474, and MDA-MB-231 as well as the ovarian cancer cell line SKOV3 were obtained from ATCC in 2013. After purchase, cell lines were expanded and frozen after one to three passages. Cell lines were validated in our

institutional sequencing facility using short tandem repeat DNA fingerprinting. Cells were cultured in DMEM: Nutrient Mixture F-12 (Sigma-Aldrich) supplemented with 10% FBS (Corning) and 1% penicillin-streptomycin solution (HyClone). TUBO cells, which were originally derived from mammary gland tumors of BALB-neuT transgenic mice that overexpress rat *neu* under the MMTV promoter (15, 16), were obtained from Haifa Shen at the Methodist Research Institute (Houston, TX). All cells were grown in a humidified incubator set at 37°C and 5% CO<sub>2</sub>. Mycoplasma testing was performed quarterly using the MycoAlert Kit (Lonza). After thawing, cells were used for experiments within 5 passages.

### Mice

Balb/c mice were obtained from The Jackson Laboratory. FVB/N-Tg(MMTV-*neu*) breeding pairs were obtained from Dr. Dihua Yu at the University of Texas MD Anderson Cancer Center (Houston, TX). These mice overexpress the activated form of the mouse HER2/*neu* protein and have a 50% tumor incidence rate of HER2/*neu*-overexpressing mammary tumors in females by 6 to 12 months of age (17, 18). Male FVB/N-Tg(MMTV-*neu*) mice were crossbred with female BALB/c mice to create a generation of mice heterozygous for the activated MMTV-*neu* transgene as well as the H-2Kd MHC class I haplotype, off which our tetramer assay is based. Inheritance of the activated MMTV-*neu* transgene was confirmed in these crossbred mice using PCR analysis. All animal studies were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

### Antibodies and other reagents

Trastuzumab and rituximab were obtained from the MD Anderson pharmacy. The E75 antibody fragment (Fab) was kindly provided by Dr. Edward Collins at the University of North Carolina (Chapel Hill, NC; ref. 19). Purified human FcR-binding inhibitor was purchased from eBioscience. The mouse anti-HER2/*neu* mAb 7.16.4 was produced *in vitro* by the mAb core facility at MD Anderson using a hybridoma purchased from ATCC. The 7.16.4 mAb is an IgG2a antibody that is closely related to trastuzumab and competes with 4D5, the precursor to trastuzumab, for binding cell surface HER2/*neu* and inhibition of tumor proliferation (20). Purified mouse IgG2a isotype control antibody was purchased from BioLegend (clone MG2a-53).

### Flow cytometry

Anti-human flow cytometry antibodies used include PE-conjugated anti-HER2/*neu* (clone Neu 24.7; BD Biosciences), PE-conjugated anti-HLA-A2 (clone BB7.2; BD Biosciences), FITC-conjugated anti-CD11c (clone B-ly6; BD Biosciences), PE-conjugated as well as allophycocyanin (APC)-Cy7-conjugated anti-HLA-DR (clone L243; BioLegend), FITC-conjugated anti-CD3 (clone OKT3; BioLegend), APC-conjugated anti-CD8a (clone OKT8; Tonbo Biosciences), Pacific Blue (PB)-conjugated anti-CD14 (clone M5E2; BioLegend), PB-conjugated anti-CD56 (clone MEM-188; BioLegend), PB-conjugated anti-CD19 (clone HIB19; BioLegend), PB-conjugated anti-CD16 (clone 3G8; BioLegend), and PE-conjugated E75/HLA-A2 dextramer (Immunodex).

Anti-mouse flow cytometry antibodies include FITC-conjugated anti-CD3 (clone 145-2C11; BioLegend), peridinin chlorophyll (PerCP)-Cy5.5-conjugated anti-CD8 (clone 53-6.7; BioLegend), and PE-conjugated HER2/*neu*-peptide (TYLPANASL)-loaded mouse H-2Kd tetramer produced by the Baylor College of



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Medicine MHC Tetramer Core (Houston, TX; ref. 21). TYLPANASL (HER2/neu aa: 63-71) is a known immunogenic HER2/neu-derived antigen with binding affinity for the H-2Kd MHC class I haplotype found in BALB/c mice (22).

Ghost Violet 510 viability dye (Tonbo Biosciences) was used in all flow panels to determine cell viability. Flow cytometry data acquisition and analysis was performed on a LSRFortessa X-20 cell analyzer (BD Biosciences) and FlowJo data analysis software (Tree Star, Inc.) or Amnis ImageStreamX (EMD Millipore).

#### Detection of HER2 surface expression and shedding of HER2 into the culture media by tumor cells

SKBR3, BT474, MDA-MB-231, and SKOV3 cells were evaluated for HER2 surface expression by direct staining of  $1 \times 10^6$  cells in 100  $\mu$ L of PBS (Lonza) with PE-conjugated anti-HER2/neu for 30 minutes at 4°C. Cells were then washed and analyzed for mean fluorescence intensity (MFI) by flow cytometry. These same cell lines were then assayed for HER2 shedding from the cell surface by incubating  $2 \times 10^6$  cells in 10 mL of serum-free RPMI (Hyclone) for 48 hours. The supernatant was then removed and assayed for soluble HER2 by following the manufacturer's protocol for a commercially available HER2 ELISA Kit (R&D Systems, Inc., DHER20). To determine the effects of chemotherapy on HER2 shedding,  $5 \times 10^5$  cells in 2.5 mL of serum-free RPMI were treated with doxorubicin or paclitaxel for 24 hours. The supernatant was removed and assayed for soluble HER2 using the HER2 ELISA Kit. To determine the effects of radiation on HER2 shedding,  $5 \times 10^5$  cells in 2.5 mL of serum-free RPMI were irradiated for 25 minutes. Supernatant was removed after 24 hours, and soluble HER2 levels were assayed using the HER2 ELISA Kit.

#### Generation of mature DCs

Mature DCs were generated from monocytes isolated through a previously described adherence culturing methodology (23). Briefly, Buffy coats from healthy human donors were obtained from the MD Anderson Cancer Center Blood Bank. These buffy coats were screened for HLA-A2 by staining an aliquot of blood with PE-conjugated anti-HLA-A2 and analyzing with flow cytometry. Peripheral blood mononuclear cells (PBMC) were then isolated from HLA-A2<sup>+</sup> buffy coats by separation with Histopaque-1077 (Sigma Aldrich). A total of  $1 \times 10^7$  PBMCs were seeded in each well of a 6-well plate suspended in 3 mL of Macrophage-Serum Free Media (M-SFM; Gibco) and allowed to incubate and adhere for 1.5 hours. Nonadherent cells were then removed and utilized for CTL generation, the remaining adherent cells, which include monocytes, were cultured in M-SFM supplemented with 100 ng/mL of GM-CSF (Sanofi-Aventis) and 100 ng/mL of IL4 (BioLegend). After 48 hours of incubation at 37°C, 10 ng/mL of TNF $\alpha$  (BioLegend) was added to allow for DC maturation. Mature DCs were ready for use 48 hours later.

#### Effect of trastuzumab on the uptake of HER2 by APCs

SKBR3, BT474, MDA-MB-231, or SKOV3 cells ( $3 \times 10^5$ ) were added to each well of a 6-well plate containing mature DCs in M-SFM supplemented with GM-CSF, IL4, and TNF $\alpha$ . Trastuzumab was then added to the media at various concentrations (0–80  $\mu$ g/mL), and cells were cultured for up to 24 hours. Rituximab was used as an isotype control, and purified human Fc $\gamma$ R-binding inhibitor (eBioscience), which inhibits Fc $\gamma$ R-mediated binding, was used to evaluate the importance of the FcR in trastuzumab-

mediated uptake. After 24 hours, cells were removed from the plates with vigorous pipetting and were stained for DC surface markers with FITC-conjugated anti-CD11c and APC-Cy7-conjugated anti-HLA-DR. Cells were then fixed in 2% paraformaldehyde (Thermo Fisher Scientific) for 15 minutes, permeabilized with Perm/Wash buffer (BD Biosciences) for 20 minutes, and stained in 100  $\mu$ L of Perm/Wash buffer with PE-conjugated anti-HER2/neu. Flow cytometry was used to identify the MFI of HER2-PE in DCs (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>), an indicator of HER2 uptake.

#### Evaluating antigen cross-presentation

Mature DCs were cocultured with  $3 \times 10^5$  SKBR3 cells and 10  $\mu$ g/mL of trastuzumab for 24 hours. Cells were then removed with vigorous pipetting and the cell surface was stained for DC markers, with FITC-conjugated anti-CD11c and PE-conjugated anti-HLA-DR, as well as E75 using an anti-E75 Fab fragment conjugated to Alexa Fluor 647 (19). Flow cytometry was used to determine the MFI of E75-Alexa Fluor 647 within the DC population (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>), a direct measure of E75 cross-presentation.

#### Effect of trastuzumab on T-cell stimulation by APCs

*In vitro*. The nonadherent HLA-A2<sup>+</sup> lymphocytic cells from our DC preparation were maintained in RPMI supplemented with 10% FBS, 1% penicillin-streptomycin, and 10 ng/mL of IL7 (BioLegend). These cells were then divided evenly and added to mature DCs that had been pulsed for 24 hours with SKBR3 cells or trastuzumab-treated (10  $\mu$ g/mL) SKBR3 cells. Lymphocytes and DCs were cocultured on 6-well plates containing 4 mL per well of complete RPMI supplemented with IL7 at 37°C. After 48 hours, 50 IU/mL of IL2 (R&D Systems) was added to stimulate CTL expansion. After T-cell activation and expansion with IL7 and IL2, cells were stained with FITC-conjugated anti-CD3 and APC-conjugated anti-CD8 $\alpha$  to select for CD8<sup>+</sup> T cells; PB-conjugated anti-CD14, anti-CD56, anti-CD19, and anti-CD16 to select against other immune cells; and PE-conjugated E75/HLA-A2 dextramer to enumerate the percentage of E75-specific, CD8<sup>+</sup>, T cells present in our samples. Analysis was completed using flow cytometry.

*In vivo*. Female mice heterozygous for the activated MMTV-neu gene (inherited from FVB/N-Tg(MMTV-neu) males) and the H-2Kd MHC class I haplotype (inherited from BALB/c females) were used to evaluate the change in immune response associated with mAb treatment. As these mice began to grow spontaneous mammary tumors >200 mm<sup>3</sup> in size, they were assigned to receive either 4 mg/kg of 7.16.4 mAb or 4 mg/kg of purified mouse IgG2a isotype control antibody twice a week via intraperitoneal injection. For each mouse, tail vein blood was sampled before the first dose of mAb (pretreatment sample) and after 7 days (2 doses) of mAb treatment (posttreatment sample). Each blood sample was stained with FITC-conjugated anti-CD3 and PerCP Cy5.5-conjugated anti-CD8 to select for CD8<sup>+</sup> T cells, as well as PE-conjugated TYLPANASL/H-2Kd tetramer to enumerate the percentage of TYLPANASL-specific, CD8<sup>+</sup> T cells present in our pretreatment and posttreatment samples. Analysis was completed using flow cytometry. The change in number of antigen-specific CTLs was determined for each mouse after 1 week of mAb treatment and the results between treatment with 7.16.4 mAb and isotype control were compared. Experiments were repeated using a transplantable model. Briefly, TUBO cells ( $1 \times 10^6$  cells/mouse) were suspended

in Matrigel/PBS (1:1) and then implanted into the mammary gland fat pads of Balb/c mice. Once the tumors were palpable, the mice were randomly assigned to receive either 4 mg/kg of 7.16.4 mAb or 4 mg/kg of purified mouse IgG2a isotype control antibody twice a week via intraperitoneal injection. Blood draws occurred and analyses for TYLPANASL-specific CD8<sup>+</sup> T cells were performed at the previously described time points.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01. Data were compared by means of one-way ANOVA utilizing Tukey multiple comparisons test as well as two-way ANOVA with Sidak multiple comparisons test. Probabilities less than 0.05 were considered significant.

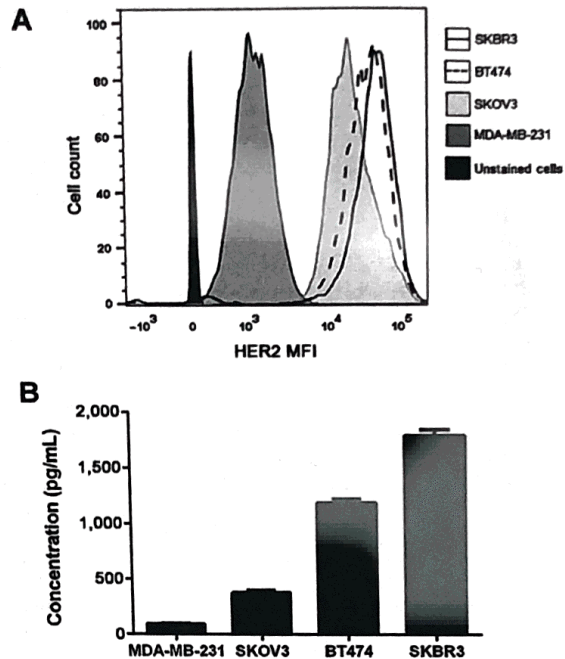
## Results

### HER2 surface expression and shedding by multiple cancer cell lines

To determine the ideal cell line to use in our studies, SKBR3, BT474, SKOV3, and MDA-MB-231 cells were stained with PE-conjugated anti-HER2 mAb to determine levels of cell surface HER2 expression. We confirmed high HER2 expression by SKBR3, BT474, and SKOV3 cells, and lower HER2 expression by MDA-MB-231 cells (Fig. 1A; refs. 24, 25). We next determined the level of HER2 shedding from the cell surface in these cell lines. Shed HER2 was quantified in the culture media by ELISA. As shown in Fig. 1B, there were significantly higher levels of HER2 shedding from SKBR3 and BT474 cells when compared with SKOV3 and MDA-MB-231 cells ( $P < 0.0001$ ). Taken together, our data show that SKBR3 and BT474 cells express and shed high levels of HER2, SKOV3 cells express a high level of HER2 but shed a lower amount of soluble HER2 in comparison with SKBR3 and BT474 cells, while MDA-MB-231 cells express and shed low levels of HER2. Furthermore, irradiated SKBR3 cells or SKBR3 cells treated with doxorubicin or paclitaxel chemotherapy showed increased HER2 shedding compared with untreated SKBR3 cells (Supplementary Fig. S1), suggesting that tumors treated with either of those modalities would result in soluble HER2.

### Trastuzumab enhances HER2 uptake by DCs

Using cell lines with various levels of HER2 expression and shedding, we were able to evaluate the efficiency of HER2 uptake by DCs and the contribution of trastuzumab to the mechanism of uptake. We first cocultured mature DCs with SKBR3 cells for 24 hours, after which, cells were stained for DC markers and intracellular HER2 expression. Analysis by Amnis imaging flow cytometry demonstrated HER2 uptake by DCs (Fig. 2A). We next determined whether HER2 uptake by DCs correlates with the amount of shed HER2. To test this, we cocultured mature DCs with SKBR3, BT474, SKOV3, or MDA-MB-231 cells for 24 hours and then tested DCs for HER2 uptake using flow cytometry. As shown in Fig. 2B, significantly higher levels of HER2 were taken up by DCs after coculture with SKBR3 and BT474 cells (high HER2 expressing/shedding;  $P < 0.0001$ ). Coculture of DCs with SKOV3 cells (high HER2 expressing/low shedding) or MDA-MB-231 cells (low HER2 expressing/shedding) showed no significant increase in HER2 uptake when compared with the DC alone group. These data suggest that DCs take up soluble HER2 more efficiently given that coculture with SKOV3 cells, despite having high HER2



**Figure 1.**

Surface expression and shedding of HER2 by cancer cell lines. **A**, Surface expression of HER2 measured by flow cytometry in multiple breast cancer cell lines. Results indicate high HER2 surface expression by the breast cancer cell lines SKBR3 and BT474, and the SKOV3 ovarian cancer cell line. MDA-MB-231 cells express low levels of HER2. **B**, Levels of soluble HER2 shedding by multiple cancer cell lines as determined by ELISA. SKBR3, BT474, SKOV3, and MDA-MB-231 cells were cultured for 48 hours in serum-free media, after which, the supernatant was assayed for HER2 concentration by ELISA. SKBR3 and BT474 cells shed greater amounts of HER2 into the culture media compared with SKOV3 and MDA-MB-231 cells.

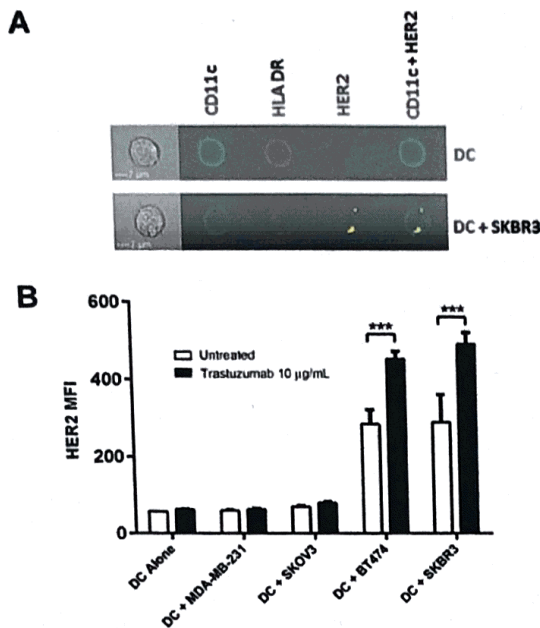
expression, did not result in significant HER2 uptake by DCs when cocultured.

To determine the effect of trastuzumab treatment on HER2 uptake, each cell line was cocultured with either DCs alone or with DCs + trastuzumab (10  $\mu$ g/mL) for 24 hours. Significant increases in HER2 uptake by DCs were observed when the DCs were cultured with SKBR3 or BT474 cells + trastuzumab compared with the cells alone ( $P < 0.0001$ ; Fig. 2B). These data indicate that trastuzumab facilitates the uptake of HER2 by DCs when cocultured with cancer cell lines that demonstrate high HER2 expression and shedding.

We next investigated the effects of treatment duration and dose on the uptake of HER2 by DCs. SKBR3 was chosen as our primary source of HER2 given its high levels of both HER2 expression and shedding. SKBR3 cells and mature DCs were cocultured with and without trastuzumab (10  $\mu$ g/mL) for 1, 4, 8, and 24 hours. Increasing amounts of HER2 uptake was observed over time, with the enhancing effect of trastuzumab on HER2 uptake reaching significance at 24 hours of incubation ( $P < 0.0001$ ; Fig. 3A). SKBR3 cells and mature DCs were also cocultured with trastuzumab at various doses (10, 20, 40, and 80  $\mu$ g/mL) to determine whether trastuzumab's effect on HER2



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**Figure 2.** Trastuzumab enhances HER2 uptake by DCs. **A**, Mature DCs were cocultured with SKBR3 breast cancer cells. Cells were then stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed using Amnis imaging flow cytometry, which demonstrated uptake of HER2 by DCs. **B**, Mature DCs were cocultured with SKBR3, SKOV3, BT474, or MDA-MB-231 cells for 24 hours ± trastuzumab (10 µg/mL). Cells were then stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed by flow cytometry. Results are expressed as average HER2 MFI for DCs cultured with each cancer cell line ± trastuzumab. Our results show increased HER2 uptake when DCs were in culture with SKBR3 and BT474 cells (high HER2 shedding) when compared with SKOV3 and MDA-MB-231 cells (low HER2 shedding). \*\*\*, *P* < 0.0001.

uptake was dose dependent. With the lowest trastuzumab dose (10 µg/mL), there was a 50% increase in HER2 uptake after 24 hours of coculture when compared with DCs cultured with SKBR3 cells alone (*P* < 0.01). This effect was observed with all trastuzumab doses tested without significant differences between low (10 µg/mL) and high dose (80 µg/mL) treatment (Fig. 3B).

**Facilitated uptake of HER2 by DCs is FcR mediated and specific for trastuzumab**

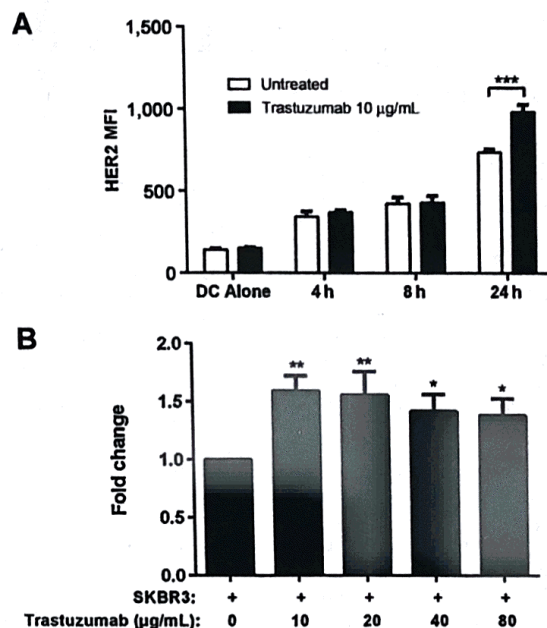
We hypothesized that trastuzumab facilitates HER2 uptake via an FcR-mediated mechanism. To test this, we cultured mature DCs with SKBR3 cells + trastuzumab, in addition to purified human FcR-binding inhibitor. The inhibition of FcR-mediated uptake abrogated the enhancing effect of trastuzumab on HER2 uptake (*P* < 0.0001; Fig. 4A). These data implicate the FcR in trastuzumab-mediated uptake of HER2 by DCs.

We next sought to confirm that the effect of trastuzumab on HER2 uptake by DCs was specific to trastuzumab. To evaluate this, rituximab, which binds CD20 but shares the same Fc region (IgG1) with trastuzumab, was used as a control mAb. Mature DCs were cocultured with SKBR3 cells in the presence of either trastuzumab (10 µg/mL) or rituximab (10 µg/mL). After 24 hours,

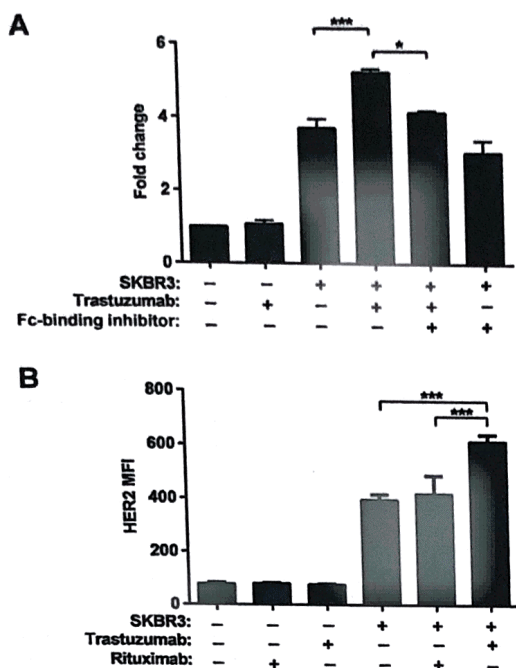
HER2 uptake was analyzed by flow cytometry. Treatment of SKBR3 cells with rituximab did not enhance HER2 uptake by DCs when compared with DCs cultured with SKBR3 cells alone (Fig. 4B), confirming that the effect of trastuzumab on HER2 uptake by DCs is specific.

**Trastuzumab increases cross-presentation of E75 and subsequent priming of T cells**

Having shown increased HER2 uptake by DCs in the presence of trastuzumab, we next investigated the functional consequences of the increased uptake. Because DCs are the principal cell type that presents antigens to T cells and leads to T-cell expansion (11), we investigated whether the increased uptake of HER2 by DCs results in increased cross-presentation of E75, an HER2-derived peptide that has strong binding affinity for HLA-A2 (18.95 nmol/L; refs. 26, 27) and has been targeted therapeutically by a number of investigators, including our own group, which has completed clinical trials evaluating E75 mixed with the immunoadjuvant GM-CSF as a vaccine strategy in breast cancer patients (1, 28, 29). To study this, we employed



**Figure 3.** Effect of treatment time and dose of trastuzumab on HER2 uptake by DCs. **A**, Mature DCs were cocultured with SKBR3 cells ± trastuzumab at increasing durations up to 24 hours. Cells were then surface-stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed using flow cytometry. HER2 uptake by DCs is expressed using average HER2 MFI. Increasing HER2 uptake was observed over time, with trastuzumab contributing significantly to uptake at 24 hours of treatment. **B**, Mature DCs were cocultured with SKBR3 cells at various doses of trastuzumab. Cells were stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed by flow cytometry. HER2 uptake by DCs is expressed using fold change in average HER2 MFI. All comparisons were made to DCs cocultured with SKBR3 cells alone. A 50% increase in HER2 uptake was noted with trastuzumab treatment at all doses tested, with little difference between low (10 µg/mL) and high (80 µg/mL) dose treatment. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.0001.



**Figure 4.**

Trastuzumab's enhancing effect on HER2 uptake by DCs is specific to trastuzumab and is mediated by the Fc receptor. **A**, Mature DCs were cocultured with SKBR3 cells and trastuzumab (10  $\mu$ g/mL)  $\pm$  human FcR-binding inhibitor. Cells were stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed with flow cytometry. HER2 uptake by DCs is expressed as fold change in average HER2 MFI when compared with DCs alone. Data indicate that FcR inhibition abrogates the effect of trastuzumab on HER2 uptake by DCs. **B**, Mature DCs were cocultured with SKBR3 cells  $\pm$  trastuzumab or rituximab for 24 hours. Rituximab (10  $\mu$ g/mL) was used as an antibody isotype control. Cells were stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) and intracellular HER2 and analyzed with flow cytometry. Results are represented by average HER2 MFI. No significant increase in HER2 uptake was noted in DCs cultured with SKBR3 cells + rituximab compared with SKBR3 cells alone. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0001$ .

a T-cell receptor mimic antibody-binding fragment (Fab) that binds the conformational epitope formed by E75/HLA-A2 (19). Mature DCs were cultured with SKBR3 cells + trastuzumab (10  $\mu$ g/mL) for 24 hours and then stained with the anti-E75 Fab fragment conjugated to Alexa Fluor 647. Increased E75 was shown on the surface of DCs that were cultured with SKBR3 cells and trastuzumab in contrast with DCs that were cultured with SKBR3 cells alone ( $P < 0.05$ ; Fig. 5A). These data directly demonstrate increased cross-presentation of the HER2 peptide E75 by DCs following the addition of trastuzumab.

In addition to using the TCR-like Fab, we also investigated the ability of DCs to activate and expand E75-specific cytotoxic T lymphocytes (E75-CTLs) when cultured with SKBR3 cells + trastuzumab. This was accomplished by investigating the ability of mature DCs that were cultured with SKBR3 cells  $\pm$  trastuzumab to expand E75-CTLs from healthy donor PBMCs. Using PE-conjugated E75/HLA-A2 dextramer, which stains E75-CTLs, we showed a higher frequency of E75-CTLs (i.e., CD3<sup>+</sup>/CD8<sup>+</sup>/

E75-HLA-A2 dextramer<sup>+</sup>/CD16<sup>-</sup>/CD19<sup>-</sup>/CD56<sup>-</sup>/CD14<sup>-</sup>/live) after coculture of PBMCs with DCs that were cultured with SKBR3 cells + trastuzumab, when compared with DCs that were cultured with SKBR3 cells alone ( $P < 0.05$ ; Fig. 5B and C). These data provide further evidence that the improved generation of E75-CTLs seen with trastuzumab treatment is a result of increased E75 cross-presentation.

To further reinforce these findings, we used two mouse models to evaluate the effect of mAb treatment on antigen-specific CTL expansion *in vivo*. In the first model, a spontaneous tumor model, female mice heterozygous for the activated MMTV-neu gene and the H-2Kd MHC class I haplotype were treated with either 7.16.4 mAb (4 mg/kg) or mouse IgG2a isotype control antibody (4 mg/kg) after developing mammary tumors. Expansion of CTLs specific for TYLPANASL, an immunogenic HER2/neu-derived peptide with strong binding affinity for H-2Kd (39 nmol/L; refs. 26, 27), was evaluated after 1 week (2 doses) of treatment. Using PE-conjugated TYLPANASL/H-2Kd tetramer, which stains TYLPANASL-specific CTLs, we evaluated the frequency of TYLPANASL-CTLs (i.e., CD3<sup>+</sup>/CD8<sup>+</sup>/TYLPANASL-H-2Kd tetramer<sup>+</sup>/live) after 1 week of treatment with 7.16.4 mAb. Figure 6A shows the gating strategy used. As shown in Fig. 6B, there was a higher frequency of TYLPANASL-CTL after 7.16.4 mAb treatment when compared with pretreatment levels ( $P < 0.05$ ). This increase was not seen in mice that received isotype control antibody. Aggregate representation of TYLPANASL-CTL expansion in multiple mice is shown in Fig. 6C. Experiments were repeated in the TUBO Balb/c implantable tumor model, which confirmed the finding of increased expansion of TYLPANASL-specific CTL in mice treated with 7.16.4 (Fig. 6B and C). These data support our *in vitro* findings of trastuzumab treatment facilitating HER2 uptake and cross-presentation.

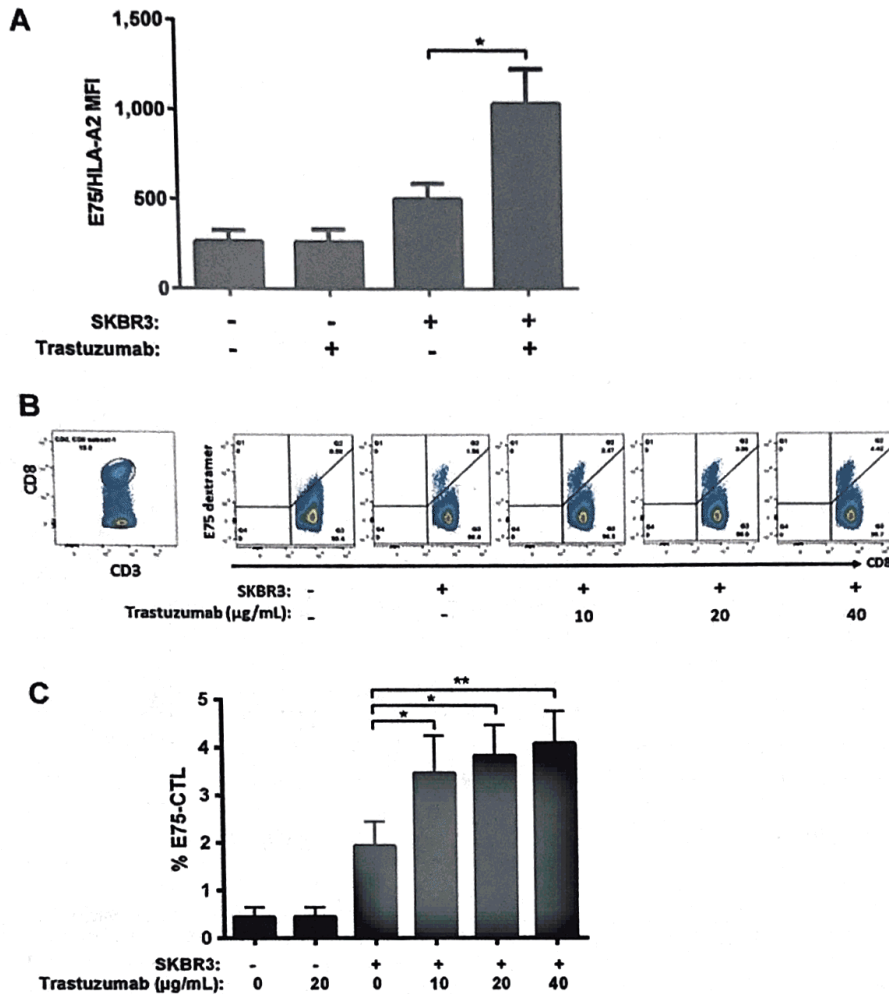
## Discussion

In this study, we demonstrate a mechanism by which trastuzumab links innate and adaptive immunity. Using flow cytometry, we have shown that trastuzumab treatment facilitates increased uptake of soluble HER2 by DCs *in vitro*. This effect is specific to trastuzumab and is FcR mediated. Increased antigen uptake led to increased antigen-specific CTL generation *in vitro* and *in vivo* secondary to increased cross-presentation by DCs. This is the first report to describe a mechanism by which trastuzumab could facilitate the cross-presentation of E75 by human DCs from soluble HER2. As DCs are known to be the most potent APCs and the most efficient activators of naive T cells, these findings further characterize the effects of trastuzumab on both innate and adaptive immunity (11).

We first observed that uptake of HER2 by DCs occurs more efficiently with cells that shed higher amounts of soluble HER2. This would suggest that, with regards to tumor antigens, monocyte-derived DCs are more specialized for internalization of soluble antigen than cell-associated antigen. This finding is consistent with published data that distinguishes different subsets of DCs. Multiple studies have found that in both humans and mice, there exist populations of DCs that are more highly specialized in the internalization of soluble antigens and that the uptake mechanism in this process is dependent on the presence and function of the FcR (30–32). In addition, we have had prior experience with DCs taking up soluble tumor-associated antigens and have previously published data showing that DCs from healthy donors are



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**Figure 5.**

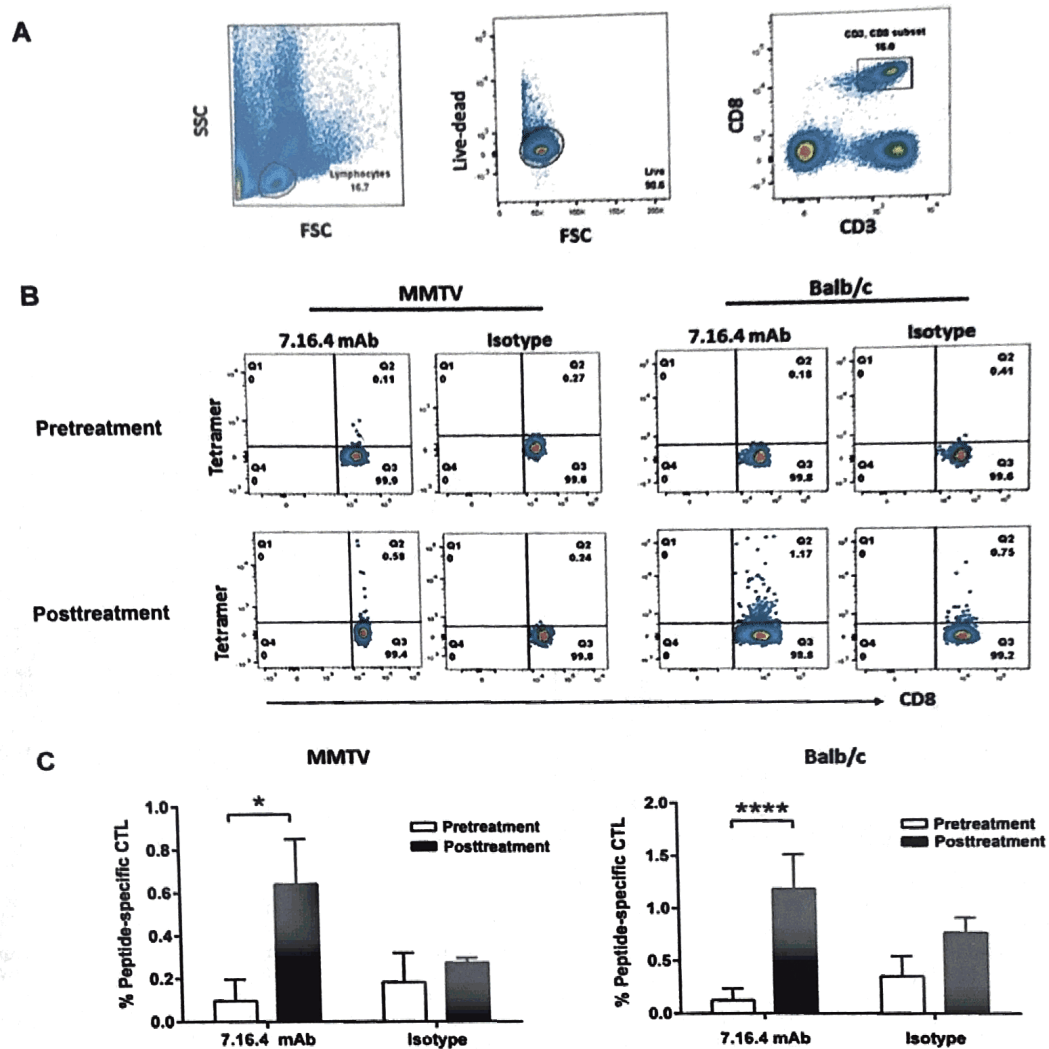
Trastuzumab increases cross-presentation of E75 by DCs. **A**, Mature DCs were cultured with SKBR3 cells  $\pm$  trastuzumab (10  $\mu\text{g/mL}$ ) for 24 hours, after which, the cell surface was stained for DC markers (CD11c<sup>+</sup>, HLA-DR<sup>+</sup>) as well as an Fab-targeting E75 peptide/HLA-A2. Cells were analyzed with flow cytometry, and results are expressed as average E75/HLA-A2 Fab MFI. DCs cultured with SKBR3 cells + trastuzumab resulted in increased E75 cross-presentation compared with DCs cultured with SKBR3 cells alone. **B** and **C**, Mature DCs were cultured with SKBR3 cells  $\pm$  trastuzumab (10–40  $\mu\text{g/mL}$ ) to activate and expand E75-CTLs from HLA-A2<sup>+</sup> PBMCs. After 1 week of coculture, enumeration of E75-CTLs was performed using an E75/HLA-A2 dextramer. Results are expressed as percent CD8<sup>+</sup>/E75<sup>+</sup> cells from a parent gating of live/CD16<sup>-</sup>/CD19<sup>-</sup>/CD56<sup>-</sup>/CD14<sup>-</sup>/CD3<sup>+</sup>/CD8<sup>+</sup> cells. DCs that had been cultured with SKBR3 cells + trastuzumab resulted in more efficient expansion of E75-CTLs from donor PBMCs. **B**, Representative scatter plots. **C**, Aggregate results of three experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

capable of taking up exogenous soluble neutrophil elastase and proteinase 3, which are parent proteins for the HLA-A\*0201-restricted leukemia antigen PR1 (33).

We also showed that trastuzumab facilitates the uptake of soluble HER2 by an FcR-mediated mechanism and results in increased cross-presentation of the E75 antigen. *In vitro* work has shown that immature DCs have higher amounts of macropinocytosis, resulting in more nonspecific uptake of soluble antigen, whereas mature DCs have significant downregulation of macropinocytosis and rely on receptor-mediated internalization of antigen/antibody complexes (34). This finding supports the observation that trastuzumab enhances FcR-mediated uptake of HER2 by mature DCs that are more dependent on receptor-mediated uptake for antigen processing. Enhancement of FcR-mediated tumor antigen uptake has also been reported in a murine model that showed increased uptake of a GM-CSF-secreting whole-cell tumor vaccine when administered with an mAb directed against mouse HER2/neu. In this model, increased tumor cell uptake led to a subsequent increase in antigen-specific CTL generation. This effect was lost when mAb lacking an Fc domain was used (35). Other experimental mouse models have

also demonstrated the benefit of administering preformed antibody-antigen complexes to facilitate antigen uptake and cross-presentation (36–39). In all of these studies, the FcR is central to the uptake mechanism with limited uptake noted in FcR-knock-out mice.

Whereas cross-presentation can lead to immune tolerance in addition to immune priming, our data demonstrate that cross-presentation of HER2 facilitated by trastuzumab leads to immune priming of naïve CD8<sup>+</sup> T cells by mature DCs and increased generation of E75-specific CTLs. The balance between immune tolerance and priming in the setting of cross-presentation has been studied in the past, with previous data suggesting that presentation by immature DCs results in a tolerogenic T-cell phenotype, while presentation by mature DCs results in an antigen-specific response (40, 41). It has also been shown that mature DCs, in the presence of antigen/antibody complexes, are better able to initiate a T-cell response when compared with mature DCs cultured with soluble antigen alone (34, 42, 43). This is consistent with our experimental findings in mature DCs and supports increased T-cell priming with trastuzumab treatment. In the case of neutrophil elastase and proteinase 3 uptake by



**Figure 6.** Treatment with 7.16.4 mAb increases expansion of antigen-specific CTLs *in vivo*. MMTV-  $neu^+$ /H-2Kd<sup>+</sup> female mice or Balb/c mice implanted with TUBO tumor cells were treated with 7.16.4 mAb (4 mg/kg) or mouse IgG2a isotype control (4 mg/kg) twice a week for one week, during which, pretreatment tail vein blood and tail vein blood after 1 week of treatment were sampled and stained for TYLPANASL-specific CTLs utilizing TYLPANASL/H-2Kd tetramer. **A**, A representative scatter plot from one mouse is shown to demonstrate gating strategy. FSC, forward scatter; SSC, side scatter. **B**, Representative scatter plots demonstrating an increase in TYLPANASL-specific CTL after treatment with 7.16.4 mAb. Isotype antibody was used as a treatment control. **C**, Aggregate representation of the expansion of TYLPANASL-CTL from mice treated with 7.16.4 mAb ( $n = 3$  for each tumor model). Isotype antibody was used as a treatment control ( $n = 2$  for each experiment). \*,  $P < 0.05$ .

DCs, we have observed that DCs have the ability to prime naïve CD8<sup>+</sup> T cells to varying degrees depending on the primary source of antigen (33). This was also noted in the case of myeloma, where loading DCs with mAb-treated myeloma cells led to superior elicitation of tumor-specific CTLs when compared with DCs loaded with apoptotic cells or peptide alone (14). We did not

directly investigate whether HER2-loaded DCs promote HER2-specific CTL reactivation/restimulation. However, published data suggest that this could be an important process in eliciting antitumor immunity. Although the majority of studies show an important role for DCs in priming T cells against tumors, which is the foundation for the implementation of DC-based vaccines,



there are reports that suggest expansion of mature T cells by DCs. For example, studies have shown that the restimulation of tissue-resident antigen-specific T cells was dependent upon the composition of the proinflammatory microenvironment, to include the expression of distinct molecules by the DCs (44, 45). In another study, Clarkson and colleagues demonstrated a role for DCs in expanding antigen-specific T cells in the central nervous system; however, this was dependent on the expression of the chemokine receptor CCR2 by the DCs (46). Nevertheless, these data should be cautiously interpreted as a number of DC-associated factors can shape the outcome of the immune response, to include not only immune activation, but also tolerance (40, 41).

As trastuzumab can affect uptake by DCs via the FcR, it is worth noting that DCs express both activating (e.g. FcγRIIIa) and inhibitory (e.g. FcγRIIb) FcRs. Trastuzumab, along with other mAbs, has been found to interact with both FcR types, and experiments using FcγRIIb knockout mice show an increased antitumor response to mAb treatment secondary to the absence of inhibitory FcR (47, 48). Although inhibitory FcRs modulate the effect of mAbs, the relatively lower affinity of IgG1 for FcγRIIb may still result in a net activating effect, which was the case with trastuzumab in our experiments (49). This does, however, raise the question of whether an mAb that selectively binds to activating FcRs would have a more profound effect on DC cross-presentation.

The current study describes a mechanism by which trastuzumab could synergize with peptide vaccines to produce a stronger antitumor immune response. To evaluate this clinically, we are currently conducting two clinical trials in breast cancer patients investigating this combination therapy. In one trial (NCT02297698), patients with high-risk HER2<sup>+</sup> breast cancer that are receiving trastuzumab as part of their standard of care therapy are being randomized to vaccine (E75 + GM-CSF) or GM-CSF alone. The second trial (NCT01570036) is enrolling patients with low HER2-expressing tumors (1+ or 2+ by IHC) who would not be receiving trastuzumab as part of their standard therapy. These patients are being randomized to receive trastuzumab + vaccine (E75 + GM-CSF) or trastuzumab + GM-CSF alone. Both trials are enrolling patients in the adjuvant setting with the primary endpoint being DFS.

In conclusion, our study demonstrates an additional mechanism by which trastuzumab can affect both innate and adaptive

immunity. By enhancing DC uptake and cross-presentation of HER2-derived peptides through an FcR-mediated mechanism, trastuzumab can enhance the generation of peptide-specific CTLs. After this initial immune response, we believe that restimulation with vaccination results in a more robust antitumor response, thereby resulting in the synergistic effect that has been observed in our clinical trials.

#### Disclosure of Potential Conflicts of Interest

E.A. Mittendorf is a consultant/advisory board member for Amgen, Astra-Zeneca, OBI Pharma, Peregrine Pharmaceuticals, and Roche. No potential conflicts of interest were disclosed by the other authors.

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## Trastuzumab Increases HER2 Uptake and Cross-Presentation by Dendritic Cells

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