Concomitant Targeting of Tumor Cells and Induction of T-cell Response Synergizes to Effectively Inhibit Trastuzumab-Resistant Breast Cancer

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Abstract

Trastuzumab is an iconic rationally designed targeted therapy for HER2-positive breast cancers. However, the low response rate and development of resistance call for novel approaches for the treatment of patients. Here, we report that concurrent targeting of tumor cells and activation of T cells in the tumor microenvironment results in a synergistic inhibitory effect on tumor growth and overcomes resistance in two distinct PTEN loss–mediated trastuzumab-resistant mammary tumor mouse models. In vivo combination treatment with HER2/Neu antibody and Akt inhibitor triciribine effectively inhibited tumor growth in both models via inhibiting PI3K/AKT and mitogen-activated protein kinase signaling accompanied by increased T-cell infiltration in the tumor microenvironment. We showed that both CD8+ and CD4+ T cells were essential to the optimal antitumor effect of this combination treatment in an IFN-γ–dependent manner. Importantly, the antitumor activities of HER2/Neu antibody and triciribine combination treatment were further improved when coinfibitory receptor cytotoxic T-lymphocyte–associated antigen 4 was blocked to enhance the T-cell response. Our data indicate that multitargeted combinatorial therapies targeting tumor cells and concomitantly enhancing T-cell response in the tumor microenvironment could cooperate to exert maximal therapeutic activity, suggesting a promising clinical strategy for treating trastuzumab-resistant breast cancers and other advanced malignancies. Cancer Res; 72(17); 1–12. ©2012 AACR.

Introduction

Rationally designed targeted therapies are sorely needed in the new era of personalized cancer medicine (1, 2). HER2/ErbB2 or neu is overexpressed in 20% to 30% of breast cancers and is associated with aggressive disease and poor clinical outcomes. HER2 is a receptor tyrosine kinase that promotes cell survival and proliferation by activating multiple pathways, including the phosphoinositide 3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinase (MAPK) pathway. Trastuzumab (Herceptin), a humanized monoclonal antibody (mAb) targeting the extracellular domain of HER2, has shown remarkable clinical efficacy in HER2-positive breast cancer (3–8). In addition to inhibition of HER2 signaling, the therapeutic effect of trastuzumab also depends on immune-mediated mechanisms. Several studies have shown that antibody-dependent cellular cytotoxicity mediated by Fc receptor–expressing innate immune cells such as natural killer (NK) cells and monocytes are essential to trastuzumab’s antitumor activity (3–8). A recent study showed that HER2/Neu antibody treatment also requires adaptive immune response to achieve maximal therapeutic effects (7).

Despite the reported efficacy of trastuzumab-containing regimens in treatment of early- and advanced-stage breast cancer, a significant number of patients fail to respond to initial trastuzumab treatment (de novo resistance) and many trastuzumab-responsive tumors develop resistance after continuous treatment (acquired resistance; refs. 9, 10). Hyperactivation of the PI3K/AKT pathway is a major trastuzumab resistance mechanism (11, 12). We previously first reported that loss of PTEN, a negative regulator of PI3K/AKT pathway, conferred trastuzumab resistance through enhanced PI3K/AKT signaling in HER2-overexpressing breast cancers (13). Studies in 2 other different patient cohorts further validated that activation of the PI3K/AKT axis, defined as PTEN loss or PI3K catalytic subunit (PIK3CA) gain-of-function mutations, correlated with worse response to trastuzumab (14, 15). These findings suggest that targeting PI3K/AKT may overcome trastuzumab resistance.

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We previously found that the combination of trastuzumab with a small-molecule Akt inhibitor triciribine could restore trastuzumab sensitivity in PTEN-deficient tumor cells in vitro and in a xenograft model in severe combined immunodeficiency mice (16). However, over the past years, it has increasingly been recognized that most cancer drugs developed on the basis of cell culture and xenograft studies have not translated well into the clinic. One potential possibility is that cell culture and xenograft models lack the appropriate tumor microenvironment and host immune system, which compromises their ability to fully recapitulate the behavior of the human malignant tumors. It is recognized that immune cells in the tumor microenvironment play critical roles in tumor development and in determining the therapeutic response to anticancer treatment as well (17–20). Hence, genetically engineered mouse (GEM) models that develop tumors in an immunocompetent setting and better mimic the initiation and progression of human cancer could circumvent the shortcomings of traditional models and may be more suitable for preclinical investigations, especially in regards to immune functions (21, 22).

In the present study, we tested whether immune response is functionally essential in overcoming trastuzumab resistance using GEM models. We report that HER2/Neu antibody and Akt inhibitor triciribine combination treatment effectively inhibits tumor growth in 2 PTEN loss−mediated HER2/Neu antibody−resistant breast cancer models. In addition to inhibiting PI3K/AKT and MAPK signaling, the combination treatment increases T-cell infiltration, including both CD8+ and CD4+ T cells into the tumor microenvironment, which contribute to the optimal antitumor effect of this combination treatment. Enhancement of T-cell response by blockade of cytotoxic T-lymphocyte−associated antigen 4 (CTLA-4, also known as CD152), a coinhibitory receptor that decreases T-cell function, increases T-cell infiltration, and further improves the antitumor activity of HER2/Neu antibody and triciribine combination treatment. Our data imply that multitargeted combinatorial therapies inhibiting tumor cells and enhancing immune cell response in the tumor microenvironment cooperates to promote maximal therapeutic effect.

Materials and Methods

Cell lines

3T3 and 3T3/Neu B7.1 cells were provided by Dr. E.M. Jaffee (Sidney Kimmel Cancer Center at Johns Hopkins and the Viragh Pancreatic Cancer Center, Baltimore, MD) 3 weeks before the assay; Neu expression was confirmed by flow cytometry.

Animals

MMTV-NIC (Neu-IRES-Cre) mice (23) were interbred with Flx−/−PTEN mice to generate HER2/Neu overexpression−PTEN heterozygous loss (PTEN+/−/NIC) and homozygous loss (PTEN−/−/NIC) mice (24). ErbB2K1 mice (25), MMTV-Cre mice (25), and Flx-PTEN mice were interbred to generate PTEN−/−/ErbB2K1 mice (26). All animal studies were approved by the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

In vivo treatment

Anti-HER2/neu mAb 7.16.4 was produced in house using hybridoma obtained from Dr. Mark Greene (University of Pennsylvania, Philadelphia, PA). PTEN+/−/NIC, PTEN−/−/NIC, and PTEN−/−/NIC mice were randomized to indicated treatments when first palpable tumor was 3 to 5 mm in diameter. 7.16.4 mAb and Akt inhibitor triciribine (Berry & Associates) were administered intraperitoneally (i.p.) at 2 mg/kg body weight every 3 days and 1 mg/kg in PBS daily, respectively. Mice were sacrificed after treatment for 3 weeks and tumors were harvested and weighted. PTEN+/−/ErbB2K1 mice were randomized to indicated treatment when tumors reached the size of 150 to 300 mm3. 7.16.4 mAb (1 mg/kg, every 3 days) and triciribine (1 mg/kg, daily) were administered, and the tumors were measured twice weekly using calipers and the volume was calculated by length × width2/2. CD8-depleting antibody (53.6.7) or CD4-depleting antibody (GK1.5) or both was injected (150 μg per mouse, i.p.) every 3 days, starting 1 day before Ab and triciribine treatment. For the IFN-γ neutralization, 150 μg antibody (R4-6A2) was injected at the same time as Ab treatment. Anti-CTLA-4 antibody (9H10) was injected (150 μg per mouse) every 3 days alone or together with Ab or/and triciribine treatment.

Results

PTEN loss leads to hyperactivation of Akt and confers resistance to anti-HER2/Neu antibody treatment in HER2/Neu overexpressing mammary tumors

Our laboratory has previously shown that PTEN activation contributes to the antitumor activity of trastuzumab and PTEN loss leads to trastuzumab resistance (13–15). To ascertain PTEN is critical for the antitumor activity of trastuzumab directly, we generated Neu-overexpressing mammary tumor mouse models with different PTEN levels by genetic modification and tested the tumor response to HER2/Neu mAb (7.16.4 mAb) that binds the mouse and rat Neu at the same site as trastuzumab binds to human HER2 (27). Consistent with prior findings, heterozygous or homozygous loss of PTEN accelerates Neu-induced mammary tumorigenesis (Supplementary Fig. S1). PTEN loss also leads to increased Akt phosphorylation in mammary tumors (Fig. 1A). We then treated mice with 7.16.4 mAb. Compared with the control (PBS) group, antibody treatment for 3 weeks significantly inhibited tumor growth in PTEN+/−/NIC mice (P = 0.0001) and PTEN−/−/NIC mice (P = 0.0008), but not in PTEN−/−/NIC mice (P = 0.2327; Fig. 1B). Furthermore, 7.16.4 treatment inhibited both Akt and ERK phosphorylation in mammary tumors of PTEN+/−/NIC mice, but did not inhibit Akt phosphorylation in PTEN−/−/NIC mice (Fig. 1C). These results show that PTEN loss leads to hyperactivation of Akt and confers resistance to HER2/Neu antibody treatment in mammary tumors of genetically engineered Neu-overexpressing PTEN loss PTEN−/−/NIC mice.

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HER2/Neu antibody and Akt inhibitor triciribine combination treatment effectively inhibits Neu overexpression–PTEN loss mammary tumors

On the basis of our previous findings (16), we next investigated whether addition of the Akt inhibitor triciribine could overcome 7.16.4 mAb resistance in the aggressive spontaneous mammary tumor model of PTEN\(^{-/-}\)/NIC mice. Combination treatment with 7.16.4 antibody and triciribine significantly decreased tumor burden \((P < 0.001;\) Fig. 2A) and multiplicity \((P < 0.01;\) Supplementary Fig. S2A) compared with control, antibody alone, or triciribine alone, whereas antibody or triciribine alone had no significant effect on tumor growth and multiplicity. Combination treatment also reduced lung metastasis (Supplementary Fig. S2B). We then examined the effects of antibody, triciribine, or combination treatment on PI3K/AKT and MAPK signaling (Fig. 2C). Single treatment with antibody decreased pErk but not pAkt; triciribine alone modestly decreased pAkt but produced no significant inhibition on pErk, whereas combination treatment reduced both pAkt and pErk. Histologic assessment showed large areas of cell loss or necrosis in tumors with combination treatment (Fig. 3A). Furthermore, immunohistochemical (IHC) analysis showed dramatically lower percentage of proliferating cells, more apoptotic cells, and fewer blood vessels in combination-treated tumors compared with antibody or triciribine alone–treated tumors (Fig. 3B–D). These data indicate that combination treatment with HER2/Neu antibody and the Akt inhibitor triciribine effectively inhibits tumor growth, angiogenesis, and induces tumor cell death in PTEN\(^{-/-}\)/NIC mice.

Considering that PTEN\(^{-/-}\)/NIC mouse models use a strong viral promoter to drive an activated Neu expression, they may not best simulate human breast cancer. Therefore, we extended our studies in the PTEN\(^{-/-}\)/ErbB2\(^{KI}\) model, which has physiologic levels of human HER2/ErbB2 expression and PTEN...
deficiency during mammary tumorigenesis (25, 26). Moreover, the PTEN$^{−/−}$/NIC model displays molecular characteristics of the luminal subtype of primary human breast cancer, whereas PTEN$^{−/−}$/ErbB2$^{KI}$ tumors display striking morphologic heterogeneity with features of both basal-like and HER2 human breast cancer (24, 26). Therefore, evaluation and/or comparison of the therapeutic efficacy in these 2 model systems could provide more insights for the development of potential treatments for human breast cancer.

Similar to the results seen in PTEN$^{−/−}$/NIC mice, combination treatment resulted in significant inhibition of tumor growth compared with antibody or triciribine monotherapy in PTEN$^{−/−}$/ErbB2$^{KI}$ mice ($P < 0.001$; Fig. 2B). Remarkably, mice with combination treatment developed only small tumors even after 7 weeks, whereas single treatment mice had to be sacrificed after 4 weeks because of excessive tumor burden. These data suggest that combination treatment induces rapid and sustained tumor suppression. Furthermore, treatment of PTEN$^{−/−}$/ErbB2$^{KI}$ mice led to significant inhibition of pAKT and pERK compared with individual treatments with antibody or triciribine or control (Fig. 2D). Consistently, histologic and IHC analysis of tumors derived from PTEN$^{−/−}$/ErbB2$^{KI}$ mice also revealed that combination treatment led to a significantly increased necrosis area, reduced proliferation, reduced blood vessels, and an enhanced level of apoptosis compared with individual treatments (Supplementary Fig. S3). Together, data from these 2 different spontaneous breast cancer models show that the combination of HER2/Neu antibody with Akt inhibitor triciribine could overcome PTEN loss–mediated HER2/Neu antibody (trastuzumab equivalent) resistance in mammary tumors.

Figure 2. Combination treatment with HER2/Neu antibody and Akt inhibitor triciribine effectively inhibits tumor growth in HER2/Neu overexpression-PTEN loss mammary tumors. A, top, tumor weight (mean ± SEM) of PTEN$^{−/−}$/NIC mice after Ctrl, Ab, triciribine, or combination (Ab + triciribine) treatment as described in Materials and Methods. Bottom, representative tumors from each treatment group. Scale bar, 1 cm. B, top, tumor volume (mean ± SEM) of PTEN$^{−/−}$/ErbB2$^{KI}$ mice that received Ctrl, Ab, triciribine, or Ab + triciribine treatment as described in Materials and Methods. Bottom, representative tumors after 4 weeks of indicated treatment. Scale bar, 1 cm. C and D, Western blots of pAkt and pErk using tumor lysates with the indicated treatments for 3 weeks in PTEN$^{−/−}$/NIC mice (C) and 4 weeks in PTEN$^{−/−}$/ErbB2$^{KI}$ mice (D). Data are representative of 3 experiments. Graph at the bottom illustrates the densitometry analysis of the pAkt/Akt ratio and pErk/Erk ratio (mean ± SEM). **, $P < 0.01$ versus Ctrl, Ab, or triciribine; $$$, $P < 0.01$ versus Ctrl or Ab; $$, $P < 0.01$ versus Ctrl or triciribine.
Combination treatment increases T-cell infiltration into tumor microenvironment

When conducting histologic examination, we observed, strikingly, that lymphocyte/leukocyte-like cells were highly infiltrated into tumors of both PTEN<sup>−/−</sup>/NIC and PTEN<sup>−/−</sup>/ErbB2<sup>K1</sup> mice after combination treatment (Supplementary Fig. S4). It is known that the antitumor effect of trastuzumab partly depends on immune-mediated mechanisms (3–8), and activation of the PI3K/Akt pathway is a mechanism for tumor immune evasion, which can be reversed by Akt inhibition (28). These observations led us to ask whether the immune response contributes to the potent antitumor effect of the combination treatment against PTEN loss–induced HER2/Neu antibody-resistant mammary tumors. Initially, we assessed what types of immune cells might be altered upon combination treatment. First, we quantified expression of markers for specific types of immune cells by quantitative reverse transcriptase PCR (qRT-PCR). Compared with Ctrl, Ab, and triciribine single treatment, combination treatment significantly increased (>3-fold, \(P<0.01\)) CD3ε and CD8α mRNAs in tumors from both PTEN<sup>−/−</sup>/NIC and PTEN<sup>−/−</sup>/ErbB2<sup>K1</sup> mice; combination treatment also increased the CD4 mRNA level in both models (\(P<0.05\); Fig. 4A and B). Expression of markers of other types of immune cells, such as B220 (B cells), F4/80 (macrophages), NK1.1 (NK cells), and CXCR1 (neutrophils), were either not significantly altered or not consistently altered in the 2 models (Supplementary Fig. S5). Therefore, we focused on CD3+ T cells as well as the CD8<sup>+</sup> and CD4<sup>+</sup> subtypes. IHC staining showed a marked increase of CD3<sup>+</sup> lymphocytes present in combination-treated tumors in both PTEN<sup>−/−</sup>/NIC (Fig. 4C) and PTEN<sup>−/−</sup>/ErbB2<sup>K1</sup> mice (Fig. 4D). IHC staining also revealed greater numbers of CD8<sup>+</sup> and CD4<sup>+</sup> T cells within
Figure 4. Combination treatment increases the infiltration of T cells into tumor. A and B, CD3, CD8α, and CD4 mRNAs were quantified by qRT-PCR in tumors of PTEN<sup>−/−</sup>/NIC mice (A) and PTEN<sup>−/−</sup>/ErbB2<sup>−/−</sup> mice (B) treated as described. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control and data were normalized to mRNA levels of Ctrl tumors. Data are presented as mean ± SEM (n = 3–5). C and D, CD3 immunohistochemical staining of tumor sections from each treatment group of PTEN<sup>−/−</sup>/NIC mice (C) and PTEN<sup>−/−</sup>/ErbB2<sup>−/−</sup> mice (D). E and F, CD8 (E) and CD4 (F) staining on frozen tumor sections of PTEN<sup>−/−</sup>/NIC mice. The CD3-positive (C and D), CD8-positive (E), or CD4-positive (F) cells per field (400×) were quantified (mean ± SEM) and representative images are shown. Scale bar, 50 μm in C–F. G, percent of CD8-positive or CD4-positive cells among all cells were determined by flow cytometry using fresh tumors from PTEN<sup>−/−</sup>/NIC mice (mean ± SEM). Data are derived from 2 independent experiments with at least 3 mice per group. *, P < 0.05; **, P < 0.01 by ANOVA.
the combination-treated tumors of PTEN\(^{-/-}\)/NIC mice (Fig. 4E and F). To better quantify these differences, freshly excised tumors from PTEN\(^{-/-}\)/NIC mice were analyzed by flow cytometry for CD4\(^+\) and CD8\(^+\) T cells. Higher CD4\(^+\) and CD8\(^+\) populations were detected in combination-treated tumors (Fig. 4G). Collectively, these results show that antibody and triciribine combination treatment increases intratumoral T-cell (both CD8\(^+\) and CD8\(^-\)) infiltration.

**Antibody and triciribine combination treatment enhances antitumor immunity**

CD8\(^+\) CTLs are crucial components of adaptive immunity that suppresses tumors growth (29). CD4\(^+\) T cells are divided into multiple subtypes that play distinct roles during tumorigenesis and progression, including Th1, Th2, Th17, T regulatory cells (Treg), and natural killer T (NKT) cells (29, 30). Th1 polarization promotes CTLs-mediated killing of cancer cells and tumor regression. In contrast, Th2-polarized immune responses promote tumor progression mainly through cytokine production (31). Therefore, we examined the expression profile of known Th1 and Th2 cytokines. Tumors from combination-treated mice had significantly elevated mRNA levels of IFN-\(\gamma\), interleukin (IL)-2, and IL-12 (Th1 cytokines; \(P < 0.01\)), but not IL-4, IL-10, and TGF-\(\beta\) (Th2 cytokines) when compared with those of control, antibody, or triciribine single–treatment tumors, in both PTEN\(^{-/-}\)/NIC (Fig. 5A) and PTEN\(^{-/-}\)/ErbB2\(^{KI}\) mice (Fig. 5B). In addition, combination treatment also significantly reduced VEGF\(\alpha\) mRNA expression (Fig. 5A and B), which promotes angiogenesis (32) and immune suppression (33). We also measured cytokine levels by ELISA using tumor lysates from PTEN\(^{-/-}\)/NIC mice with different treatments. Consistent with mRNA data, IFN-\(\gamma\) and IL-2 cytokine protein levels were significantly increased, whereas IL-4 and IL-10 were not significantly changed (Fig. 5C). These data indicate that combination treatment induced a dominant antitumor Th1 polarization in the tumor microenvironment. Moreover, splenocytes from combination–treated mice showed a significant increase in CTL activity against Neu-overexpressing 3T3 target cells compared with the other 3 treatment groups (\(P < 0.01\)), whereas splenocytes among 4 groups had no significant difference in lytic effect against 3T3 cells without Neu overexpression (Fig. 5D). These results indicate that combined treatment with HER2/Neu antibody and triciribine effectively induces Neu-specific CD8\(^+\) T-cell response. Taken together, antibody and triciribine combination treatment enhances antitumor immunity associated with Th1 polarization and Neu-specific CD8\(^+\) T-cell response.

**The optimal antitumor effect of the combination treatment requires CD8\(^+\), CD4\(^+\) T cells, and IFN-\(\gamma\)**

We have shown that combination treatment inhibited tumor growth with increased intratumor infiltration of CD8\(^+\) and CD4\(^+\) T lymphocytes. To determine whether CD8\(^+\) or/and CD4\(^+\) T cells are critical for the antitumor effect of combination treatment, PTEN\(^{-/-}\)/NIC mice were simultaneously treated with anti-CD8\(\alpha\) antibody or/and anti-CD4 antibody to deplete the CD8\(^+\) and CD4\(^+\) T lymphocytes (Supplementary Fig. S6A). Depletion of CD8\(\alpha\) or CD4\(^+\) or both T cells during combination treatment resulted in reduced tumor suppression. Compared with PBS-treated control group, concurrent CD8\(\alpha\) and CD4\(^+\) depletion resulted in highly significant (\(P < 0.01\)), and CD8\(\alpha\) or CD4 single depletion resulted in significant (\(P < 0.05\)) reductions of tumor suppression (Fig. 6A). These results indicate that both CD8\(^+\) and CD4\(^+\) T cells contribute to the optimal antitumor effect of HER2/Neu antibody and triciribine combination treatment. Notably, depletion of CD8\(^+\) or CD4\(^+\) T cells or both during combination treatment still inhibited tumors better than Ctrl, Ab, or triciribine single agent treatment (Supplementary Fig. S6B).

Given that IFN-\(\gamma\) level is markedly increased in tumors after combination treatment with antibody and triciribine in our models (Fig. 5A–C), we next investigated whether CD8\(^+\) or CD4\(^+\) T cells or both contribute to the increased IFN-\(\gamma\) levels. We found that depleting CD8\(^+\) or/and CD4\(^+\) T cells significantly reduced IFN-\(\gamma\) production, indicating both CD8\(^+\) and CD4\(^+\) T cells contribute to IFN-\(\gamma\) production (Fig. 6B). We next asked whether IFN-\(\gamma\) is a mediator of CD8\(^+\) and CD4\(^+\) T cells’ antitumor effect of combination treatment. Blocking IFN-\(\gamma\) function with neutralizing mAb (R4-6A2) significantly reduced the antitumor efficacy of combination treatment (\(P < 0.05\); Fig. 6C). These results indicate that IFN-\(\gamma\) from CD8\(^+\) and CD4\(^+\) T cells contributes to the therapeutic activity of combination treatment.

**Enhancing T-cell response by blocking CTLA-4 further improves the antitumor activity of HER2/Neu antibody and triciribine combination treatment**

Because increased infiltration of CD8\(^+\) and CD4\(^+\) T cells in the tumor microenvironment contributed to the optimal antitumor effect of combination treatment (Fig. 6), we wondered whether enhancing the activity of these T cells may further augment the antitumor effect. Given that costimulatory or coinhibitory signals provided by coreceptors of T-cell receptors are critical for T-cell activity (34), we examined the expression of 3 major coreceptors including costimulator CD28, inducible costimulator (ICOS), and inhibitory coreceptor cytotoxic CTLA-4 (or CD152). We found that along with increased expression of CD28 and ICOS, CTLA-4 expression was also increased markedly in tumors with combination treatment of both PTEN\(^{-/-}\)/NIC and PTEN\(^{-/-}\)/ErbB2\(^{KI}\) models (Fig. 7A and B). The highly significant and consistent increase of CTLA-4 expression suggests that the CTLA-4 inhibitory coreceptor may play an important role in the negative feedback regulation of T-cell response upon T-cell activation by combination treatment, and blockade of this negative signal during combination treatment might potentially improve the antitumor effect (35, 36). Remarkably, as shown in Fig. 7C, HER2/Neu antibody and triciribine combination with the addition of CTLA-4 blocking mAb significantly (\(P < 0.05\)) better inhibited tumors than without the anti-CTLA-4 mAb in PTEN\(^{-/-}\)/NIC mice. Anti-CTLA-4 mAb alone, anti-CTLA-4 mAb combined with HER2/Neu antibody only, or with triciribine only showed no significant enhancement of antitumor effect (Supplementary Fig. S7). Although the number of infiltrating T cells (including CD8\(^+\) and CD4\(^+\) T cells) was not significantly increased in the HER2/Neu antibody and
triciribine plus anti-CTLA-4 mAb triple combination–treated tumors compared with HER2/Neu antibody and triciribine without anti-CTLA-4 mAb treated tumors (Supplementary Fig. S8), the IFN-γ mRNA and protein levels were significantly increased (Fig. 7D and E), indicating enhanced activities of these T cells. Moreover, serum IFN-γ level was markedly higher in triple combination–treated mice compared with all other treatment groups (Supplementary Fig. S9), which correlated with tumor inhibition activity (Fig. 7). These results indicate that serum IFN-γ level is a potential biomarker for predicting or evaluating therapeutic response. Overall, our results suggest that enhancing T-cell response by blocking CTLA-4 further augments the therapeutic activities of HER2/Neu antibody plus triciribine treatment in HER2/Neu antibody–resistant mammary tumors.

Discussion
Trastuzumab resistance is a clinically devastating problem for HER2-positive breast cancers. In the present study, we used 2 distinct GEM models with HER2/Neu overexpression and PTEN loss as preclinical models closely mimicking trastuzumab-resistant human breast cancer with the aim of facilitating development of better strategies to overcome trastuzumab resistance in the clinic. Our results show that HER2/Neu...
antibody and the Akt inhibitor triciribine combination treatment not only suppressed Erk and Akt activities in tumor cells but also triggered T-cell response in the tumor microenvironment, which contributed to optimal antitumor effect. Therefore, both autonomous (inhibition of oncogenic signaling of tumor cells) and nonautonomous (immune response in tumor microenvironment) mechanisms were involved in the therapeutic activity of combination treatment. Importantly, modulation of immune response was shown to be essential in overcoming trastuzumab resistance.

Figure 6. The optimal antitumor effect of combination treatment requires CD8⁺ and CD4⁺ T cells in an IFN-γ-dependent manner. A, tumor weight of PTEN⁻/⁻/NIC mice upon depletion of CD8a⁺ or/and CD4⁺ T cells during combination treatment. B, IFN-γ level was determined by ELISA. C, tumor weight of PTEN⁻/⁻/NIC mice upon IFN-γ neutralization during combination treatment. Data are shown as mean ± SEM (n = 7 or 8 per group).

Figure 7. Enhancement of T-cell response by blockade of CTLA-4 augments antitumor activity of HER2/Neu antibody and triciribine combination treatment. A and B, expression of CD28, ICOS, and CTLA-4 mRNA was measured by qRT-PCR in tumors from PTEN⁻/⁻/NIC mice (A) and PTEN⁻/⁻/ErbB2KI mice (B). Data were normalized to mRNA level of glyceraldehyde-3-phosphate dehydrogenase. Data are presented as mean ± SEM (n = 3–5). **, P < 0.01 by ANOVA. C, tumor weight of PTEN⁻/⁻/NIC mice upon addition of anti-CTLA-4 antibody during combination treatment with HER2/Neu antibody and triciribine (n = 6–7 per group). D, expression of IFN-γ and IL-2 mRNA was measured by qRT-PCR in tumors derived from C (n = 4). E, IFN-γ level was determined by ELISA using tumor lysates derived from C (n = 6–7 per group). Data are shown as mean ± SEM. **, P < 0.01; *, P < 0.05 by t test in C–E.
Emerging evidence suggests that necrotic death of cancer cells triggered by certain chemotherapeutic drugs can induce tumor-specific adaptive immune responses, which are essential to the antitumor activity (19, 37–40). On the other hand, therapeutic mAbs are effective in enhancing the presentation of apoptotic tumor cells or soluble tumor antigens to T cells by dendritic cells (29, 41). We observed large areas of necrosis, more apoptotic cells, and significant infiltration of CD3+ T cells in combination-treated tumors but not in control, antibody, or triciribine single–treated tumors (Fig. 3A). Because HER2/Neu antibody and triciribine combination treatment enhanced T-cell response (Fig. 5), possibly through presentation of apoptotic cells and tumor debris by antigen-presenting cells (APC), we postulate that tumor cell death induced by combination treatment is necessary and as an initiator, then feedback positively, to induce T-cell responses via APCs in the combination treatment group.

CD8+ cytotoxic T lymphocytes are a major component of adaptive immunity that attacks tumor cells (29). Several studies have reported that tumor-infiltrating CD8+ lymphocytes or stromal expression of CD8e are significantly associated with better clinical outcomes of breast cancer (42–44). Tumor-associated T lymphocytes are also an independent predictor of response to anthracycline/taxane-based neoadjuvant chemotherapy for breast cancer (20). Recently, CD8+ T-cell–dependent adaptive immune response has been shown to be required for maximal therapeutic effects of anti-HER2/Neu antibody (7). In our study, we observed marked increase of CD8+ T-cell infiltration upon combination treatment compared with anti-HER2/Neu antibody alone and depletion of CD8+ T cells decreased the tumor inhibition effect by combination treatment. The data support a more important role of CD8+ T-cell–dependent adaptive immune response in antitumor effect of combination treatment. Interestingly, depletion of CD4+ T cells also diminished the antitumor potency of combination treatment, suggesting that CD4+ T cells are also required for antitumor effect of combination treatment. CD4+ T-cell response has been previously observed in patients with breast cancer treated with trastuzumab (45). Notably, increasing evidence indicates that CD4+ cells can play critical roles in antitumor immunity (46–52). CD4+ T cells were considered to play a critical role in maintaining/facilitating CD8+ T cells’ cytotoxic function by providing the appropriate cytokine milieu (29). Recent studies showed that CD4+ T cells are able to eradicate tumor directly (51); and may be even more efficient than CD8+ cells at tumor rejection (50). Consistently, our combination treatment induced a dominant antitumor Th1 polarization in the tumor microenvironment characterized by increased expression of IFN-γ, IL-2, and IL-12, thus favoring tumor regression by CTLs-mediated killing of cancer cells (Fig. 4 and 5). Depletion of either CD8+ or CD4+ T cells decreased antitumor effect in our model (Fig. 6), indicating that the cooperation of CD8+ and CD4+ T cells is required for the antitumor effect of combination treatment. Our data, along with others’ findings, show that optimal tumoricidal activity of anticancer therapies is typically achieved with both CD8+ and CD4+ T-cell responses (49). Therefore, our novel findings showed that immune response is functionally essential in overcoming trastuzumab resistance.

Because anti-CD4 mAb depletes both Th and Treg CD4+ cells, we were unable to dissect the role of CD4+ Treg cells separately in this study. Nevertheless, the overall immunosuppressive effect of CD4+ Treg cells during combination treatment is likely very limited because depletion of CD4+ T cells resulted in decreased rather than increased antitumor activity of combination treatment (Fig. 6A). Further experiments with specific depletion of Th cells or Treg cells would reveal definitive role of CD4+ T cells in our model.

Although combination therapy with HER2/Neu antibody and triciribine induced an initial immune response against HER2/Neu overexpression–PTEN loss tumors, the therapeutic effect was still incomplete, partly due to the development of immunosuppressive mechanisms. CTLA-4 is inducibly expressed in activated CD4+ and CD8+ effector T cells, which provides negative feedback signal to suppress T-cell activity. Combination treatment increased the numbers of both CD8+ and CD4+ T cells required for the optimal antitumor effect. At the same time, negative feedback was augmented through CTLA-4 by combination treatment to suppress T-cell activity and inhibit antitumor effect. The seemingly opposing effects of combination treatment on T-cell activity provide us a unique opportunity to further boost the antitumor response of combination treatment by blocking CTLA-4 to favor T-cell response and tumor elimination. Indeed, blockade of CTLA-4 further improved the therapeutic activity of HER2/Neu antibody and triciribine combination in our model (Fig. 7). In addition, CTLA-4 is constitutively expressed on a subset of Tregs. In support of this notion, concomitant blockade of CTLA-4 on both effector T cells and Tregs was previously shown to contribute to the maximal therapeutic effects of anti-CTLA-4 mAb treatment (53). It is clear that augmentation of immune response by inhibiting immunosuppressing molecules could further improve the efficacy of tumor cell–based targeted therapies, which may be a particularly effective anticancer strategy.

The findings in the current study can potentially be translated to the clinic for patients’ benefit. First, trastuzumab is part of the standard treatment for HER2-positive breast cancers. Second, triciribine is a tricyclic nucleoside inhibiting phosphorylation of Akt1, Akt2, and Akt3 (54), and Phase I and II clinical trials showed that triciribine’s safety and side effects were dose dependent (55–57). Although treatment with triciribine alone was not efficacious against advanced breast, colon, and lung cancer even at very high doses (55, 56), recent preclinical studies showed that triciribine combined with other anticancer agents was therapeutically effective against T-cell acute lymphoblastic leukemia (58), breast cancer (16, 59), and prostate cancer (60). In addition, other clinically applicable PI3K/Akt inhibitors may be used as alternatives of triciribine in combination treatment. Third, 2 CTLA-4 blocking antibodies, ipilimumab (Bristol-Myers Squibb) and tremelimumab (Pfizer) are currently under intense clinical investigation. CTLA-4 blockade has shown promise in the treatment of metastatic melanoma (61), and ipilimumab has been approved for first-
second-line treatment of advanced melanoma by the U.S. Food and Drug Administration and second-line treatment by the European Medicines Agency (62). Therefore, our findings open new avenues for preclinical and clinical studies to determine the safety and efficacy of the trastuzumab, tricrine (or other PI3K/Akt inhibitors), and infliumumab (or trekelinumumab) combinations in personalized treatment of trastuzumab-resistant breast cancer, especially mediated by PTEN deficiency and PI3K pathway activation.

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

Authors' Contributions
Conception and design: Q. Wang, S.W. Brady, D. Yu
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Wang, O. Sahin, E.M. Jaffee, G.N. Hortobagyi, D. Yu
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References

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