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 (TCN) effectively inhibited tumor growth in both models via inhibiting PI3K/AKT and MAPK signaling accompanied by increased T cell infiltration in the tumor microenvironment. We demonstrated that both CD8+ and CD4+ T cells were essential to the optimal anti-tumor effect of this combination treatment in an IFN-γ-dependent manner. Importantly, the anti-tumor activities of HER2/Neu antibody and TCN combination treatment were further improved when co-inhibitory receptor cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) was blocked to enhance the T cell response. Our data indicate that multi-targeted 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.

Precis: Breast cancers resistant to anti-HER2 therapy can be destroyed by blocking the AKT pathway and degrading immune tolerance, thereby effectively restoring T cell-mediated anti-tumor immunity.
Introduction

Rationally designed targeted therapies are sorely needed in the new era of personalized cancer medicine (1-2). Human epidermal growth factor receptor-2 (HER2/ErbB2 or neu) is overexpressed in 20-30% of breast cancers and is associated with aggressive disease and poor clinical outcomes. HER2 is a receptor tyrosine kinase (RTK) that promotes cell survival and proliferation by activating multiple pathways, including the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and the mitogen-activated protein kinase (MAPK) pathway. Trastuzumab (Herceptin), a humanized monoclonal antibody 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 (ADCC) mediated by Fc receptor-expressing innate immune cells such as natural killer (NK) cells and monocytes are essential to trastuzumab’s anti-tumor activity (3-8). A recent study demonstrated 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) (9-10). Hyperactivation of the PI3K/AKT pathway is a major trastuzumab resistance mechanism (11-12). We previously first reported that loss of the phosphatase and tensin homolog (PTEN), a negative regulator of PI3K/AKT pathway, conferred trastuzumab resistance through enhanced PI3K/AKT
signaling in HER2-overexpressing breast cancers (13). Studies in two 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.

We previously found that the combination of trastuzumab with a small-molecule Akt inhibitor triciribine (TCN) 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 cells. It is recognized that immune cells in the tumor microenvironment play critical roles in tumor development and in determining the therapeutic response to anti-cancer 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 (TCN) combination treatment effectively inhibits tumor growth
in two 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 anti-tumor 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 co-inhibitory receptor that decreases T-cell activation, further improves the anti-tumor activity of HER2/Neu antibody and TCN combination treatment. Our data imply that multi-targeted 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. Jaffee (Sidney Kimmel Cancer Center at Johns Hopkins and the Viragh Pancreatic Cancer Center) 3 weeks before the assay, Neu expression was confirmed by flow cytometry.

**Animals.** MMTV-NIC (Neu-IRES-Cre) mice (23) were interbred with Flox-PTEN mice to generate HER2/Neu overexpression-PTEN heterozygous loss (PTEN+/−/NIC) and homozygous loss (PTEN−/−/NIC) mice (24). ErbB2KI mice (25), MMTV-Cre mice (25) and Flox-PTEN mice were interbred to generate PTEN−/−/ErbB2KI 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 monoclonal antibody 7.16.4 was produced in house using hybridoma obtained from Dr. Mark Greene (University of Pennsylvania). PTEN+/+/-NIC, PTEN+/+/-NIC and PTEN+/-/-NIC mice were randomized to indicated treatments when first palpable tumor was 3-5 mm in diameter. 7.16.4 mAb and Akt inhibitor triciribine (TCN) (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+/-/ErbB2KI mice were randomized to indicated treatment when tumors reached the size of 150-300 mm³. 7.16.4 mAb (1 mg/kg, every 3 days) and TCN (1 mg/kg, daily) were administered, and the tumors were measured twice weekly using calipers and the volume was calculated by length x 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 one day before Ab and TCN 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 TCN 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 anti-tumor
activity of trastuzumab and PTEN-loss leads to trastuzumab resistance (13-15). To ascertain PTEN is critical for the anti-tumor 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 monoclonal antibody (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 (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 to 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 demonstrate 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.

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

Based on our previous findings (16), we next investigated whether addition of the Akt inhibitor TCN 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 TCN significantly decreased tumor burden ($P < 0.001$) (Fig. 2A) and multiplicity ($P < 0.01$) (Fig. S2A) compared to control, antibody alone, or TCN alone, whereas antibody or TCN alone had no significant effect on tumor growth and multiplicity. Combination treatment also reduced lung metastasis (Fig. S2B). We then examined the effects of antibody, TCN or combination treatment on PI3K/AKT and MAPK signaling (Fig. 2C). Single treatment with antibody decreased pErk but not pAkt; TCN alone modestly decreased pAkt but produced no significant inhibition on pErk; whereas combination treatment reduced both pAkt and pErk. Histological 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 TCN alone treated tumors (Fig. 3B-D). These data indicate that combination treatment with HER2/Neu antibody and the Akt inhibitor TCN effectively inhibits tumor growth, angiogenesis and induces tumor cell death in PTEN$^{-}$/NIC mice.

Considering that PTEN$^{-}$/NIC mouse models utilize 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 physiological 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, while 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 two 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 TCN monotherapy in PTEN−/−/ErbB2KI mice (P<0.001) (Fig. 2B). Remarkably, mice with combination treatment developed only small tumors even after 7 weeks, while single treatment mice had to be sacrificed after 4 weeks due to excessive tumor burden. These data suggest that combination treatment induces rapid and sustained tumor suppression. Furthermore, treatment of PTEN−/−/ErbB2KI mice led to significant inhibition of pAKT and pERK compared to individual treatments with antibody or TCN or control (Fig. 2D). Consistently, histological and IHC analysis of tumors derived from PTEN−/−/ErbB2KI 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 to individual treatments (Fig. S3). Together, data from these two different spontaneous breast cancer models demonstrate that the combination of HER2/Neu antibody with Akt inhibitor TCN could overcome PTEN loss-mediated HER2/Neu antibody (trastuzumab equivalent) resistance in mammary tumors.

**Combination treatment increases T cells infiltration into tumor microenvironment**

When performing histological examination, we observed, strikingly, that lymphocyte/leukocyte-like cells were highly infiltrated into tumors of both PTEN−/−/NIC and PTEN−/−/ErbB2KI mice after combination treatment (Fig. S4). It is known that the anti-tumor 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 anti-tumor 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 qRT-PCR. Compared with Ctrl, Ab and TCN single treatment, combination treatment significantly increased (>3 fold, \( P < 0.01 \)) CD3\( \varepsilon \) and CD8\( \alpha \) mRNAs in tumors from both PTEN\(^{-/-}\)/NIC and PTEN\(^{-/-}\)/ErbB2\(^{KI}\) 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 two models (Fig. S5). Therefore, we focused on CD3\(^{+}\) T cells as well as the CD8\(^{+}\) and CD4\(^{+}\) subtypes. IHC staining showed a marked increase of CD3\(^{+}\) lymphocytes presence in combination treated tumors in both PTEN\(^{-/-}\)/NIC (Fig. 4C) and PTEN\(^{-/-}\)/ErbB2\(^{KI}\) mice (Fig. 4D). IHC staining also revealed greater numbers of CD8\(^{+}\) and CD4\(^{+}\) T cells within 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 demonstrate that antibody and TCN combination treatment increases intra-tumoral T cell (both CD4\(^{+}\) and CD8\(^{+}\)) infiltration.
Antibody and TCN combination treatment enhances anti-tumor immunity

CD8+ cytotoxic T lymphocytes (CTLs) are crucial components of adaptive immunity that suppresses tumor 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-γ, IL-2 and IL-12 (Th1 cytokines) ($P < 0.01$), but not IL-4, IL-10 and TGF-β (Th2 cytokines) when compared with those of control, antibody or TCN single treated tumors, in both PTEN<sup>-/-</sup>/NIC (Fig. 5A) and PTEN<sup>-/-</sup>/ErbB2<sup>Ki</sup> mice (Fig. 5B). In addition, combination treatment also significantly reduced vascular endothelial growth factor α (VEGFα) 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<sup>-/-</sup>/NIC mice with different treatment. Consistent with mRNA data, IFN-γ and IL-2 cytokine protein levels were significantly increased while IL-4 and IL-10 were not significantly changed (Fig. 5C). These data indicate that combination treatment induced a dominant anti-tumor 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 to the other 3 treatment groups ($P < 0.01$), while 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 TCN effectively induces Neu-specific CD8+ T cell response. Taken together, antibody and TCN combination treatment enhances anti-tumor immunity associated with Th1 polarization and Neu-specific CD8+ T cell response.

**The optimal anti-tumor effect of the combination treatment requires CD8+, CD4+ T cells and IFN-γ**

We have shown that combination treatment inhibited tumor growth with increased intra-tumor infiltration of CD8+ and CD4+ T lymphocytes. To determine whether CD8+ or/and CD4+ T cells are critical for the anti-tumor effect of combination treatment, PTEN−/−/NIC mice were simultaneously treated with anti-CD8α antibody or/and anti-CD4 antibody to deplete the CD8+ and CD4+ T lymphocytes (Fig. S6A). Depletion of CD8α+ or CD4+ or both T cells during combination treatment resulted in reduced tumor suppression. Compared with PBS-treated control group, concurrent CD8α and CD4 depletion resulted in highly significant \( P<0.01 \), and CD8α 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 anti-tumor effect of HER2/Neu antibody and TCN combination treatment. Notably, depletion of CD8+ or CD4+ T cells or both during combination treatment still inhibited tumors better than Ctrl, Ab or TCN single agent treatment (Fig. S6B).

Given that IFN-γ level is markedly increased in tumors after combination treatment with antibody and TCN in our models (Fig. 5A-C), we next investigated whether CD8+ or CD4+
T cells or both contribute to the increased IFN-γ levels. We found that depleting CD8+ or/and CD4+ T cells significantly reduced IFN-γ production, indicating both CD8+ and CD4+ T cells contribute to IFN-γ production (Fig. 6B). We next asked whether IFN-γ is a mediator of CD8+ and CD4+ T cells’ anti-tumor effect of combination treatment. Blocking IFN-γ function with neutralizing mAb (R4-6A2) significantly reduced the anti-tumor efficacy of combination treatment ($P < 0.05$) (Fig. 6C). These results indicate that IFN-γ 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 anti-tumor activity of HER2/Neu antibody and TCN combination treatment**

Since increased infiltration of CD8+ and CD4+ T cells in the tumor microenvironment contributed to the optimal anti-tumor effect of combination treatment (Fig. 6), we wondered whether enhancing the activity of these T cells may further augment the anti-tumor effect. Given that co-stimulatory or co-inhibitory signals provided by co-receptors of T cell receptors are critical for T cell activity (34), we examined the expression of three major co-receptors including co-stimulator CD28, inducible co-stimulator (ICOS) and inhibitory co-receptor cytotoxic T-lymphocyte-associated antigen 4 (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 co-receptor 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 anti-tumor effect (35-36). Remarkably, as shown in Fig. 7C, HER2/Neu antibody and TCN 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 TCN only showed no significant enhancement of anti-tumor effect (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 TCN plus anti-CTLA-4 mAb triple combination treated tumors compared to HER2/Neu antibody and TCN without anti-CTLA-4 mAb treated tumors (Fig. S8), the IFN-$\gamma$ mRNA and protein levels were significantly increased (Fig. 7D and E), indicating enhanced activities of these T cells. Moreover, serum IFN-$\gamma$ level was markedly higher in triple combination treated mice compared with all other treatment groups (Fig. S9), which correlated with tumor inhibition activity (Fig. 7). These results indicate that serum IFN-$\gamma$ 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 TCN 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 utilized two distinct genetically engineered mouse 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 demonstrate that HER2/Neu antibody and the Akt inhibitor TCN 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 anti-tumor effect. Therefore, both autonomous (inhibition of oncogenic signaling of tumor cells) and non-autonomous (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.

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 anti-tumor activity (19, 37-40). On the other hand, therapeutic monoclonal antibodies 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 TCN single treated tumors (Fig. 3A). Since HER2/Neu antibody and TCN combination treatment enhanced T cell response (Fig. 5), possibly through presentation of apoptotic cells and tumor debris by antigen presenting cells (APCs), 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 CD8α 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 to 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 anti-tumor effect of combination treatment. Interestingly, depletion of CD4+ T cells also diminished the anti-tumor potency of combination treatment, suggesting that CD4+ T cells are also required for anti-tumor effect of combination treatment. CD4+ T-cell response has been previously observed in breast cancer patients treated with trastuzumab (45). Notably, increasing evidence indicates that CD4+ cells can play critical roles in anti-tumor 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 anti-tumor 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 anti-tumor effect in our model (Fig. 6), indicating that the cooperation of CD8+ and CD4+ T cells is required for the anti-tumor effect of combination treatment. Our data, along with others’ findings, demonstrate that optimal tumoricidal activity of anticancer therapies is typically achieved with both CD8+ and CD4+ T cells’ responses (49). Therefore, our novel findings demonstrated that immune response is functionally essential in overcoming trastuzumab resistance.

Since 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 anti-tumor 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 TCN induced an initial immune response against HER2/Neu overexpression-PTEN loss tumors, the therapeutic effect was still incomplete, partly due to the development of immune-suppressive 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 anti-tumor effect. At the same time, negative feedback was augmented through CTLA-4 by combination
treatment to suppress T-cell activity and inhibit anti-tumor effect. The seemingly opposing effects of combination treatment on T-cell activity provide us a unique opportunity to further boost the anti-tumor 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 TCN 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 immune-suppressing 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, TCN is a tricyclic nucleoside inhibiting phosphorylation of Akt1, Akt2 and Akt3 (54), and Phase I and II clinical trials showed that TCN’s safety and side effects were dose-dependent (55-57). Although treatment with TCN alone was not efficacious against advanced breast, colon, lung cancer even at very high doses (55-56), recent preclinical studies showed that TCN combined with other anti-cancer agents was therapeutically effective against T-cell acute lymphoblastic leukemia (58), breast cancer (16, 59), and prostate cancer (60). Additionally, other clinically applicable PI3K/Akt inhibitors may be used as alternatives of TCN in combination treatment. Third, two 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- and second-line treatment of advanced melanoma by the U.S. Food and Drug Administration (FDA) 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, TCN (or other PI3K/Akt inhibitors) and ipilimumab (or tremelimumab) combinations in personalized treatment of trastuzumab-resistant breast cancer, especially mediated by PTEN deficiency and PI3K pathway activation.
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References


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Figure legends

Figure 1. PTEN loss leads to increased Akt phosphorylation and confers resistance to anti-HER2/Neu antibody treatment in HER2/Neu-overexpression mammary tumors. (A) Western blotting for Pten, pAkt S473, Akt, and Neu using tumor lysates from PTEN+/−/NIC, PTEN+/−/NIC and PTEN−/−/NIC. β-actin is shown as a loading control. Data represent three experiments. Graph on the right illustrates the densitometry analysis of the pAkt/Akt ratio.
(mean ± s.e.m). **, \( P < 0.01 \) versus PTEN\(^{+/+}\)/NIC or PTEN\(^{+/+}\)/NIC; *, \( P < 0.05 \) versus PTEN\(^{+/+}\)/NIC. (B) Tumor weight (mean ± s.e.m) of indicated mice treated with PBS (Ctrl) or 7.16.4 mAb (Ab) as described in Materials and Methods. \( P \)-values were determined by t-test. (C) Western blot analysis of indicated proteins in tumor lysates from PTEN\(^{+/+}\)/NIC and PTEN\(^{+/+}\)/NIC mice with Ctrl or Ab treatment for 3 weeks. Graph on the right illustrates the densitometry analysis of the pAkt/Akt ratio and pErk/Erk ratio (mean ± s.e.m).

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 ± s.e.m) of PTEN\(^{-/-}\)/NIC mice after Ctrl, Ab, triciribine (TCN) or combination (Ab+TCN) treatment as described in Materials and Methods. Bottom, representative tumors of each treatment group. Scale bar, 1 cm. (B) Top, tumor volume (mean ± s.e.m) of PTEN\(^{-/-}\)/ErbB2\(^{KI}\) mice received Ctrl, Ab, TCN or Ab+TCN 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 three experiments. Graph at the bottom illustrates the densitometry analysis of the pAkt/Akt ratio and pErk/Erk ratio (mean ± s.e.m). **, \( P < 0.01 \) versus Ctrl, Ab or TCN; ###, \( P < 0.01 \) versus Ctrl or Ab; $$, \( P < 0.01 \) versus Ctrl or TCN.

Figure 3. Histological and immunohistochemical analysis of tumors derived from PTEN\(^{-/-}\)/NIC mice. (A-D) Quantification of the following parameters in tumors with the
indicated treatment (mean ± s.e.m): hypocellular regions using H&E staining of tumors (scale bar, 200 μm) (A); proliferation by Ki-67 staining (scale bar, 50 μm) (B); apoptosis by TUNEL (scale bar, 50 μm) (C); and blood vessel density by CD34 staining (scale bar, 100 μm) (D). Representative images for each treatment group are shown on the right. **, P < 0.01, by ANOVA.

**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^{-/-}/NIC mice (A) and PTEN^{-/-}/ErbB2^KI mice (B) treated as described. GAPDH was used as an internal control and data were normalized to mRNA levels of Ctrl tumors. Data are presented as mean ± s.e.m (n=3-5). (C and D) CD3 immunohistochemical staining of tumor sections from each treatment group of PTEN^{-/-}/NIC mice (C) and PTEN^{-/-}/ErbB2^KI mice (D). (E and F) CD8 (E) and CD4 (F) staining on frozen tumor sections of PTEN^{-/-}/NIC mice. The CD3-positive (C, D), CD8-positive (E) or CD4-positive (F) cells per field (400 ×) were quantified (mean ± s.e.m) and representative images were 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^{-/-}/NIC mice (mean ± s.e.m). Data are derived from two independent experiments with at least three mice per group. *, P < 0.05; **, P < 0.01 by ANOVA.

**Figure 5. Combination treatment with HER2/Neu antibody and TCN enhances anti-tumor immune response.** (A and B) mRNA expression of the indicated cytokine was quantified by qRT-PCR in tumors from PTEN^{-/-}/NIC mice (A) and PTEN^{-/-}/ErbB2^KI mice (B).
Data are presented as in Fig. 4A (n=3-5). (C) IFN-γ, IL-2, IL-4 and IL-10 cytokines were measured by ELISA. Data are shown as mean ± s.e.m (n=6-8 per group). (D) Neu-specific lytic effect of splenocytes from PTEN<sup>−/−</sup>/NIC mice with indicated treatments. Percent of specific lysis are shown as mean ± s.e.m (n=3 per group). *, P < 0.05; **, P < 0.01 by ANOVA.

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

Figure 7. Enhancement of T cell response by blockade of CTLA-4 augments anti-tumor activity of HER2/Neu antibody and TCN combination treatment. (A and B) Expression of CD28, ICOS and CTLA 4 mRNA was measured by qRT-PCR in tumors from PTEN<sup>−/−</sup>/NIC mice (A) and PTEN<sup>−/−</sup>/ErbB2<sup>KI</sup> mice (B). Data were normalized to mRNA level of GAPDH. Data are presented as mean ± s.e.m (n=3-5). **, P < 0.01 by ANOVA. (C) Tumor weight of PTEN<sup>−/−</sup>/NIC mice upon addition of anti-CTLA4 antibody during combination treatment with HER2/Neu antibody and TCN (n=6-7 per group). (D) Expression of IFN-γ and IL2 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 ± s.e.m. *, P < 0.05; **, P < 0.01 by t-test in C-E.
Figure 1

A

PTEN+/-/NIC  PTEN+/-/NIC  PTEN-/-/NIC

Pten  pAkt S473  Akt  Neu  β-actin

B

Tumor weight (g)

Ctrl  Ab  Ctrl  Ab  Ctrl  Ab  Ctrl  Ab

P=0.2327

P=0.0001

P=0.0008

PTEN+/+/NIC  PTEN+/-/NIC  PTEN-/-/NIC

PTEN+/-/NIC  PTEN-/-/NIC

Tumor weight (g)

Ctrl  Ab  Ctrl  Ab  Ctrl  Ab  Ctrl  Ab

P=0.2327

P=0.0001

P=0.0008

PTEN+/+/NIC  PTEN+/-/NIC  PTEN-/-/NIC

C

PTEN+/+/NIC  PTEN+/-/NIC

Pten  pAkt S473  Akt  pErk  Erk  Neu  β-actin

PTEN+/+/NIC  PTEN+/-/NIC  PTEN-/-/NIC

P=0.0001

P=0.0001

P=0.0001

pAkt/Akt ratio (Relative intensity)

pErk/Erk ratio (Relative intensity)
Figure 2

A

Tumor weight (g)

P < 0.001

Ctrl Ab TCN Ab+TCN

B

Tumor volume (mm³)

P < 0.001

Time after treatment (Weeks)

C

PTEN⁺/-/NIC mice

Ctrl Ab TCN Ab+TCN

pAkt 473

Akt

pErk

Erk

β-actin

D

PTEN⁺/-/ErbB2KI mice

Ctrl Ab TCN Ab+TCN

pAkt 473

Akt

pErk

Erk

β-actin

Relative intensity

pAkt/Akt pErk/Erk

Relative intensity

pAkt/Akt pErk/Erk

Ctrl Ab TCN Ab+TCN

Ctrl Ab TCN Ab+TCN

**

##

#
Figure 3

(A) Hypocellularity (%)

(B) Ki-67 + cells (%)

(C) TUNEL + cells (%)

(D) Blood vessels/Field

Control (Ctrl) vs. Ab vs. TCN vs. Ab+TCN
Figure 5

A: PTEN−/−/NIC mice

B: PTEN−/−/Erbb2KI mice

C

D

**% of specific lysis**

Effector : Target=40:1  Effector : Target=10:1
Figure 6

A

Tumor weight (g)

PBS Anti-CD8 Anti-CD4 Anti-CD8 Anti-CD4

Ab+TCN

P < 0.05

P < 0.05

B

IFN-γ (pg/mg protein)

PBS Anti-CD4 Anti-CD8 Anti-CD4

Ab+TCN

P < 0.01

C

Tumor weight (g)

PBS Anti-IFN-γ

Ab+TCN

P < 0.05

P < 0.05

P < 0.01

P < 0.01
Figure 7

A  
PTEN<sup>−/−</sup>/NIC mice

CD28 mRNA level (X10<sup>-3</sup>)

B  
PTEN<sup>−/−</sup>/ErbB2<sup>KI</sup> mice

CD28, ICOS, CTLA 4 mRNA levels

C  
Tumor weight (g)

D  
Relative mRNA level

E  
IFN-γ, IL2 production
Concomitant targeting of tumor cells and induction of T cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer

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