Supplementary Experimental Procedures

**Immunoblotting.** Lysates from flash frozen tumor tissue were prepared using the following lysis buffer: 1% Triton X-100, 50mM Hepes PH 7.4, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, 10mM Na Pyruvate, 1mM Na3VO4, 10% glycerol, with protease inhibitors (Sigma) and phosphatase inhibitors (Roche). The protein concentration was determined by BCA assay (Thermo Scientific), and 40 μg total protein was loaded for immunoblotting. Each lane represents tumor lysate from an individual mouse. Pten, pAkt S473, Akt, pErk and Erk antibodies were obtained from Cell Signaling Technology. Neu and β-actin antibodies were obtained from Santa Cruz Biotechnology and Sigma, respectively. The blots were incubated with HRP-conjugated secondary antibodies and visualized by ECL (Amersham). Densitometry analysis was carried out to determine the intensity of bands by Adobe Photoshop software.

**Histology and IHC.** Tumor tissues and lungs were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Tumors from PTEN^+/NIC mice were also embedded in OCT and frozen at -80°C. H&E staining of paraffin-embedded tumor sections were used to quantify the hypocellular regions. Paraffin-embedded sections (4 μm) were subjected to antigen retrieval in a pressure cooker with sodium citrate buffer (PH=6.0) and incubated with antibodies specific for CD3 (Epitomics, 1:300), Ki-67 (DAKO, 1:200), CD34 (eBioscience, 1:50) overnight at 4°C. Frozen sections (10μm) were used for CD4 (eBioscience, 1:50) and CD8 (eBioscience, 1:50) IHC staining. Biotin-conjugated secondary
antibodies were used. Remaining steps were performed using Vectastain ABC kits (Vector Laboratories). Paraffin-embedded sections were also subjected to TUNEL staining (Roche) according to the manufacturer’s instructions. Slides were counterstained with hematoxylin. To quantify the positive staining cells, the numbers of infiltrating CD3+, CD8+, CD4+ cells and blood vessels were counted per field of view after examination of at least 10 fields of each section (T cells: 400X, blood vessels: 200X), and the percentages of positive cells for Ki-67 and TUNEL staining were evaluated by two pathologists (Hai Wang and Yan Xiong). Images were acquired using a Zeiss microscope with Axiovision software (Carl Zesis, Inc.).

**Quantitative real-time reverse transcription-PCR.** Total RNA was extracted from frozen (-80°C) tumor samples using TRIzol® Reagent (Invitrogen) according to the manufacturer’s instruction. c-DNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen) by inputting 2 μg of total RNA. Quantitative PCR was performed by using iTaq SYBR Green Supermix and CFX96 Touch Real-Time PCR Detection System (Bio-Rad) as recommended by the manufacturer. Each sample was done in triplicate and transcript levels were normalized to GAPDH. Primer sequences are listed in Supplemental Table.

**Flow cytometry.** Fresh tumors were excised and minced with sterile scissors into approximately 1- to 2- mm³ pieces, then digested in DMEM/F12 medium containing 10% fetal calf serum, 2 mg/ml collagenase (Sigma), 0.02 mg/ml hyaluronidase (Sigma), and 0.01 mg/ml DNase I (Sigma) for 3 hours at 37°C with gentle agitation. The supernatant was filtered through 40-µm nylon mesh (BD Biosciences) to remove clumps. Red blood cells
were removed by treatment with ACK buffer and washed with cold DMEM/F12. To confirm the depletion of CD8\(^+\) cells, CD4\(^+\) cells or both during HER2/Neu antibody and triciribine combination treatment, the spleen was excised and gently homogenized on a 40 µm cell strainer followed by red blood cell lysis using ACK buffer. Cells were suspended in PBS with 0.5% BSA and stained with FITC-conjugated anti-mouse CD8 (eBioscience) and PE-conjugated anti-mouse CD4 (eBioscience) antibodies for 30 minutes on ice. The samples were run on a FACSCanto flow cytometer (BD Biosciences) and the data were analyzed using FlowJo software.

**Cytokine measurements.** Lysates from flash frozen tumor samples were prepared using the protocol described above for immunoblotting. 100 µl lysate was subjected to ELISA analysis according to the manufacturer’s instructions. The following mouse-specific kits were used: IFN\(\gamma\) (Cat # 88-8314-86; eBioscience), IL2 (Cat # 88-7024-86; eBioscience), IL4 (Cat# 88-7044-86; eBioscience), IL10 (Cat# 88-7104-86; eBioscience). Cytokine levels were normalized to the protein concentrations of lysates as determined by BCA assay.

**In vitro cytotoxicity assay.** Splenocytes were isolated from PTEN\(^{+/−}\)/NIC mice that received the indicated treatments by mechanical disaggregation through a 70-µm cell strainer. After treatment with ACK buffer to lyses red blood cells, splenocytes were cultured in DMEM/F12 containing 10% fetal bovine serum, 0.5% 50 mM L-Glutamine, 50 mM β-mercaptoethanol and 20 IU/ml of recombinant human IL-2 (Peprotech). To assess Neu-specific lysis, splenocytes obtained from three mice per group, were pooled and incubated with 3T3/Neu
B7.1 cells as targets in a 96-well U-bottomed plate in triplicate for 16 hours at 37°C. 3T3 cells without Neu antigen were used as negative controls. The level of lactate dehydrogenase (LDH), a cytosolic enzyme that is released upon cell lysis was measured by the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega) according to the manufacturer’s instruction. The percentage of specific cytotoxicity was calculated according to the following formula: % specific lysis = [(Experimental - Effector Spontaneous - Target Spontaneous) / (Target Maximum - Target Spontaneous)] ×100.

**Statistics.** Statistical differences were assessed with two-tailed Student’ *t* test, one-way ANOVA or the nonparametric alternatives as indicated. The GraphPad Prism 5 Program (GraphPad Software) was used to perform all statistical analyses. *P* values less than 0.05 were considered statistically significant.
Supplementary Figure Legends

Figure S1. Characterization of PTEN<sup>+/−</sup>/NIC mouse model. (A) Representative genotyping result for NEU, CRE and PTEN Loxp. In the PTEN Loxp genotyping: the single lower band represents both alleles are wild type, double bands represent heterozygous mutant (one allele with a loxp sequence insertion and the other is wild type), the single upper band represents homozygous mutant (both allele with loxp insertion). Lanes 1, 2 and 3 are genotyping of PTEN<sup>+/+</sup>/NIC, PTEN<sup>+/-</sup>/NIC and PTEN<sup>-/-</sup>/NIC, respectively; lane 4 is a wild type control. (B) The percentages of tumor free mice were plotted according to their age at onset of the first palpable mammary tumors (about 3-5 mm in diameter). T50 represents the age of median tumor onset. (C) Whole-mount staining of inguinal mammary glands from mice with indicated genotypes at 5 weeks (32 to 35 days) of age and the representative images were shown. Scale bar, 5 cm. The arrowhead indicates tumor mass.

Figure S2. HER2/Neu antibody 7.16.4 and Akt inhibitor TCN combination treatment reduced tumor multiplicity and lung metastasis in PTEN<sup>+/−</sup>/NIC mice. Mice received indicated treatment starting when first palpable tumor was 3-5 mm in diameter and sacrificed after treatment for 3 weeks. (A) Number of tumors was counted. (B) Percentage of mice with lung metastases with the indicated treatment. Ctrl (n=11), Ab(n=12), TCN(n=6), Ab+TCN(n=6). **, P < 0.01 compared with Ctrl, Ab or TCN alone treated group by Kruskal-Wallis analysis with Dunn's multiple comparison test.
Figure S3. Histological and immunohistochemical analysis of tumors derived from PTEN$^{-/-}$/ErbB2$^{Kl}$ mice receiving single or combination treatment. (A-D) Quantification of the following parameters in tumors with indicated treatment: hypocellular regions by H&E staining slides (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). Quantification was determined as described in Methods. Representative images for each treatment group are shown. Horizontal line, mean; error bars, s.e.m. **, $P < 0.01$, by ANOVA in A-D.

Figure S4. The observation of more lymphocytes/leukocytes infiltration after HER2/Neu antibody and Akt inhibitor TCN combination treatment. Representative images of H&E-stained mammary tumor sections with indicated treatments from PTEN$^{-/-}$/NIC mice (A) and PTEN$^{-/-}$/ErbB2$^{Kl}$ mice (B). Cells with tiny, dense nuclei indicated by arrowheads showing lymphocytes/leukocytes in tumors. Scale bar, 50 μm.

Figure S5. Evaluation of markers of other tumor-infiltrating immune cells. B220 (for B cells), F4/80 (for macrophages), NK1.1 (for natural killer cells) and CXCR.1 (for neutrophils) mRNA levels in tumors from PTEN$^{-/-}$/NIC mice (A) and PTEN$^{-/-}$/ErbB2$^{Kl}$ mice (B) with indicated treatment were quantified by qRT-PCR. 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). $P$-value was not significant determined by one-way ANOVA analysis.
Figure S6. Depletion of CD8+ cells, CD4+ cells or both during HER2/Neu antibody and triciribine combination treatment. (A) Depletion of CD8+ or CD4+ or both cells was confirmed by flow cytometry analysis using splenocytes from PTEN^-/-/NIC mice with indicated treatment. Single cell of splenocyte suspensions were stained with antibodies specific to CD4 and CD8 and analyzed by flow cytometry. Representative dot plots from two independent experiments with at least 3 mice per group are shown. (B) Depletion of CD8+ or/and CD4+ cells during HER2/Neu antibody and triciribine combination treatment still showed lower tumor burden compared with Ctrl, Ab or TCN single treatment. P-value determined by unpaired Student’s t test is shown.

Figure S7. Anti-CTLA-4 mAb alone, anti-CTLA-4 mAb combined with HER2/Neu antibody only, or with TCN only showed no significant enhancement of antitumor effect. PTEN^-/-/NIC mice were randomized to receive indicated treatment starting when their first palpable tumors were 3-5 mm in diameter. Ab and TCN was given i.p. 2 mg/kg body weight every 3 days and 1 mg/kg daily, respectively. Anti-CTLA-4 antibody (9H10, 150 μg/mouse) was administered together with HER2/Neu antibody. Mice were sacrificed after treatment for 3 weeks and tumors were harvested and weighed. Horizontal line, mean; error bars, s.e.m (n=6-8 per group). The P values were determined by unpaired Student’s t test.

Figure S8. No significant changes in the number of tumor infiltrating T cells (including CD8+ and CD4+ ) upon the addition of anti-CTLA-4 mAb to HER2/Neu antibody and triciribine combination treatment. PTEN^-/-/NIC mice received treatment as in Fig. S7. (A)
CD3ε, CD8α and CD4 mRNAs were measured by qRT-PCR. GAPDH was used as an internal control and data were normalized to mRNA levels of Ab+TCN treated tumors. Data are presented as mean ± s.e.m (n=3-5). (B) Immunohistochemical staining for CD3 was performed on paraffin-embedded tumor sections from indicated treatment group. Frequencies of CD3-positive cells per field (400 ×) were quantified and representative images were shown. (C and D) Staining of frozen tumor sections from mice in the indicated treatment groups for CD4 (C) and CD8 (D). The CD4-positive (C) and CD8-positive (D) cells per field (400 ×) were quantified and representative images were shown. Horizontal line, mean; error bars, s.e.m. Scale bar, 50 μm in B to D. (E) Fresh mammary tumors were digested with collagenase, and single cell suspensions were stained with antibodies specific to CD4 and CD8 and analyzed by flow cytometry. The percentages of positive cells among all cells were shown as mean ± s.e.m. Data are derived from two independent experiments with at least three mice per group. Representative dot plots are shown.

Figure S9. HER2/Neu antibody and TCN plus anti-CTLA-4 mAb triple combination treatment increased serum IFN-γ level. Mice received treatment as in Fig. S7. Blood was collected from the orbital sinuses of mice just prior to sacrifice and allowed to coagulate at room temperature for 1 hour. Serum was then collected by centrifugation at 13,000 rpm for 20 minutes at room temperature. IFN-γ level was determined by ELISA using 100 μl of serum. Data are shown as mean ± s.e.m (n=6-7 per group). **, P < 0.01 versus other group by 2-tailed Student’s t test.