Supporting Information

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SI Materials and Methods

Flow Cytometry. H2N100 tumor cells (5×10^5 cells) were injected into WT BALB/c mice and treated with 100 μg anti–ErbB-2 mAb (clone 7.16.4) or control Ig (clone MAC4) injected i.p. on days 12 and 16. On day 20, tumors were harvested, cut into pieces, and incubated in a solution of collagenase type IV (1 mg/mL) and DNase I (20 μg/mL) for 45 min at 37 °C. Cells were washed (PBS 1% FCS) and incubated with the FcR-blocking mAb 2.4G2 for 10 min, anti-mouse CD8-biotin mAb (BD Bioscience) for 15 min, followed by anti-biotin magnetic microbeads (Myltenyi) for 15 min. Cells were washed and subjected to AUTOMACS (Myltenyi) positive selection sorting. Sorted CD8+ cells were incubated with plate-bound anti-CD3 (1 μg/mL) in the presence of Golgi Plug (BD Bioscience) for 4 h at 37 °C. Cells were washed and incubated with allophycoerythrin-conjugated anti-CD8 mAb (BD Bioscience) and intracellular phycoerythrin-conjugated IFN-γ mAb according to the manufacturer's instructions. Flow cytometry was performed on a BD LSR II.

Gene Array Analysis. The breast cancer dataset was previously described in Esteva et al. (1), in which 45 HER2-overexpressing stage II/IIIA breast cancer patients were treated with a standard anthracycline and taxane chemotherapy regimen combined with trastuzumab. Biopsy samples were taken at diagnosis, and all samples were profiled with Affymetrix U133A GeneChips. Normalized data ($\log^2$ intensity) was used. Hybridization probe sets were mapped to Entrez GeneID, using RefSeq and Entrez database version 2007.01.21. The AUC using receiver operating characteristic curves was used to assess the association of the given gene's expression level with pCR. The pCR is accepted to be an excellent surrogate for disease-free and overall survival in HER2-overexpressing breast cancer. This method avoids having to determine a cutoff to binarize a gene's expression level. Statistical analysis using gene expression data were performed with R version 2.5.1 and BioConductor version 1.8. The remaining statistical analyses were performed using SPSS 18.0 (SPSS Inc.).

In Vitro Proliferation Assays. For in vitro proliferation, 10^4 tumor cells per well were cultured in 96-well plates in complete media for 4 d with increasing doses of recombinant IFN-γ and/or 7.16.4 mAb. For the last 16 h, 10 μCi per well of tritiated thymidine was added. Cells were harvested using a cell harvester and radioactivity measured using a Chameleon beta-counter (Hidex Ltd.).

1. Esteva FJ, et al. (2007) CD40 signaling predicts response to preoperative trastuzumab and concomitant paclitaxel followed by 5-fluorouracil, epirubicin, and cyclophosphamide in HER-2-overexpressing breast cancer. *Breast Cancer Res* 9:R87.

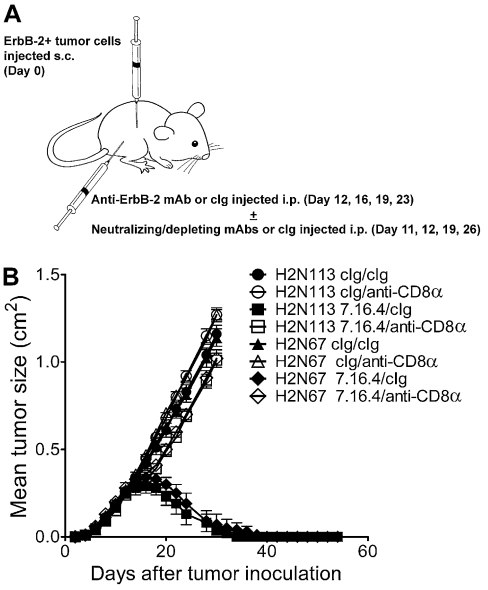

Fig. S1. (*A*) Experimental design. BALB/c mice were injected s.c. with ErbB-2+ tumor cells (derived from BALB/c transgenic mice expressing oncogenic ErbB-2), and treated on days 12, 16, 19, and 23 with anti–ErbB-2 mAb or control Ig (clg) injected i.p. To assess the contribution of specific immune pathways, gene-targeted mice were used or WT mice were injected i.p. with depleting or neutralizing mAbs on days 11, 12, 19, and 26. (*B*) CD8α+ cells are required for anti–ErbB-2 mAb therapy in different tumor models. Tumor cell lines (H2N113 and H2N67) derived from two distinct spontaneous tumors arising in BALB/c-MMTV-neu transgenic mice were injected s.c. (5×10^5 cells) into control-treated WT and CD8α+ cell-depleted (mAb 53.6.7; $^*P = 0.0097$ vs. 7.16.4 + clg) BALB/c mice, and treated with 100 μg anti–ErbB-2 mAb (clone 7.16.4) or control Ig injected i.p. on days 12, 16, 19, and 23. Depleting mAb was injected i.p. on days 11, 12, 19, and 26. Data are mean ± SE of five mice per group. Statistical analyses were performed using the Mann–Whitney test.

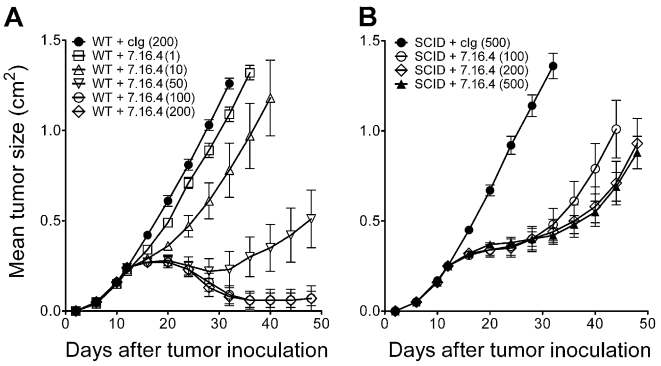

Fig. S2. Anti–ErbB-2 mAb therapy is abrogated in SCID mice. H2N113 tumor cells were injected s.c. (5×10^5 cells) into (*A*) WT and (*B*) SCID mice and treated with increasing doses (doses in parentheses are micrograms) of anti–ErbB-2 mAb (clone 7.16.4) or control Ig injected i.p. on days 12, 16, 19, and 23. Data are mean ± SE of five mice per group.

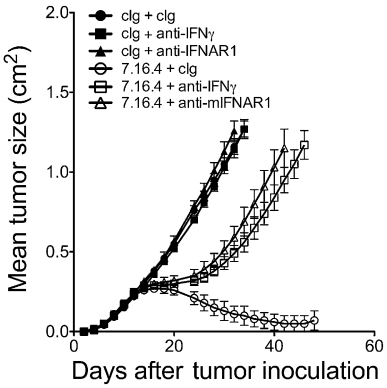

Fig. S3. IFNAR1 and IFN-γ are required for anti–ErbB-2 mAb therapy of H2N113 tumors. H2N113 tumor cells (5×10^5 cells) were injected s.c. into WT BALB/c mice and treated with 100 μg anti–ErbB-2 mAb (clone 7.16.4) or control Ig (clone MAC4) injected i.p. on days 12, 16, 19, and 23. Mice were injected i.p. with control Ig or treated with neutralizing mAbs to IFNAR1 (clone MAR1-5A3) or IFN-γ (clone H22). Data are mean ± SE of five mice per group. Neutralizing mAbs were injected i.p. on days 11, 12, 19, and 26.

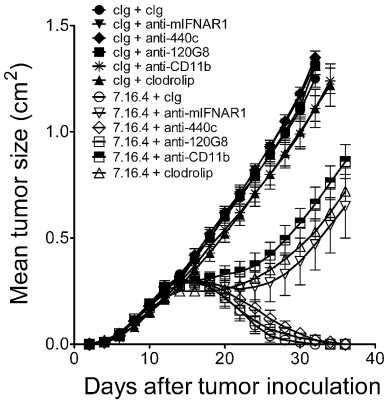

Fig. S4. Role of plasmacytoid DCs (pDCs) and monocytes/macrophages during anti–ErbB-2 mAb therapy. H2N100 tumor cells (5×10^5 cells) were injected s.c. into BALB/c mice and treated with 100 μg anti–ErbB-2 mAb (clone 7.16.4) or control Ig injected i.p. on days 12, 16, 19, and 23. Mice were injected i.p. with control Ig or neutralizing mAbs (100 μg i.p.) against pDCs (anti-440c and anti-120G8), IFNAR1 (clone MAR1-5A3), and CD11b+ cells (clone 5C6) on days 11, 12, 19, and 26 or with macrophage-depleting clodrolip (100 mg/kg i.p. on day 10 and 50 mg/kg i.p. on days 12, 14, 16, 18, 20, 22, 24, and 26). Data are mean ± SE of five mice per group.

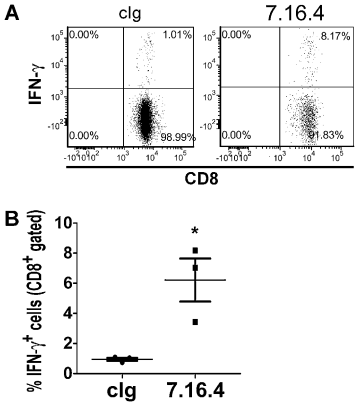

Fig. S5. Anti–ErbB-2 mAb therapy increased IFN-γ–producing CD8+ tumor-infiltrating lymphocytes. H2N100 tumor cells (5×10^5 cells) were injected into WT mice and treated with 100 μg anti–ErbB-2 mAb (clone 7.16.4) or control Ig (clone MAC4) injected i.p. on days 12 and 16. On day 20, tumors were harvested, pooled by treatment group, exposed to collagenase/DNase, single-cell suspensions enriched for CD8+ cells, and restimulated with plate-bound anti-CD3 mAb for 4 h before being analyzed for IFN-γ intracellular expression. (*A*) Representative flow cytometry dot plots on CD8+ gated events. (*B*) Percentage of IFN-γ+ CD8+ cells of triplicates, Data are SE. $P = 0.02$ by *t* test.

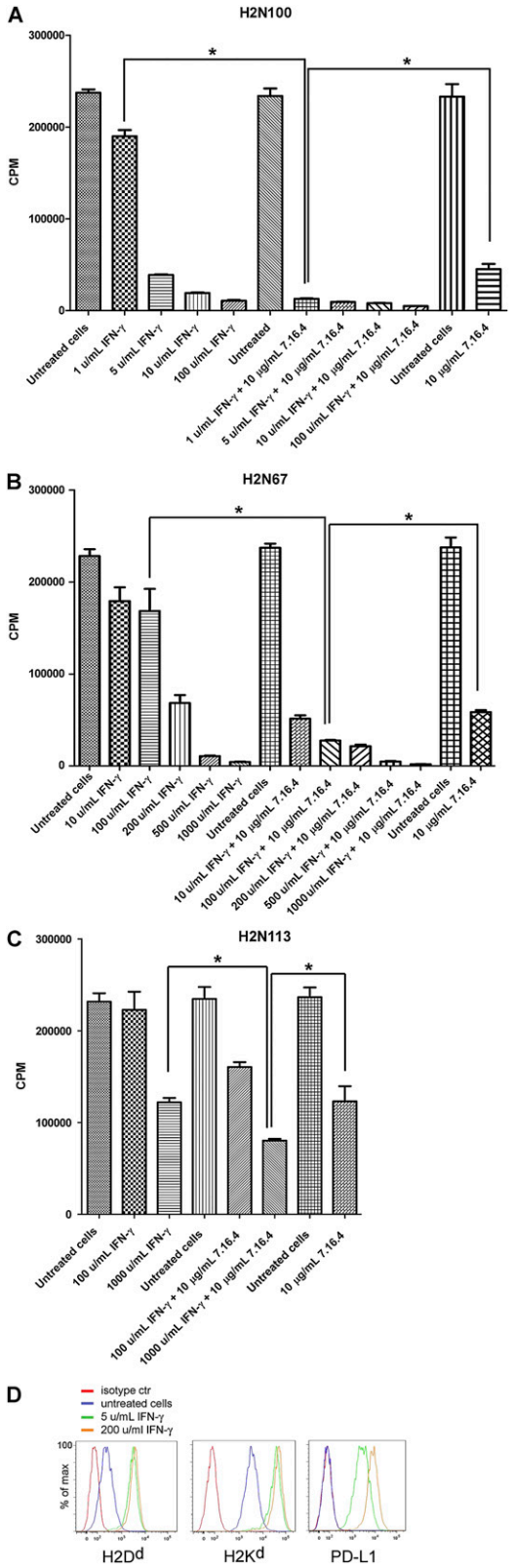

Fig. S6. IFN-γ inhibits proliferation of H2N tumor cells and up-regulates MHC class I and PD-L1 expression. (*A*) H2N100 tumor cells were cultured for 4 d with increasing concentrations of recombinant IFN-γ and/or 10 μg/mL anti–ErbB-2 mAb (clone 7.16.4). Tritiated thymidine was added for the last 16 h of culture and radioactivity measured using a beta-counter. (*B* and *C*) Same as *A,* except that H2N67 and H2N113 cells were used, respectively. Data are mean ± SE of triplicate samples of one experiment. *$P < 0.05$ by Student *t* test). (*D*) H2N100 cells were left untreated or were treated for 24 h with 5 U/mL or 200 U/mL recombinant IFN-γ, and analyzed by flow cytometry for H2Dd, H2Kd, and PD-L1 expression.

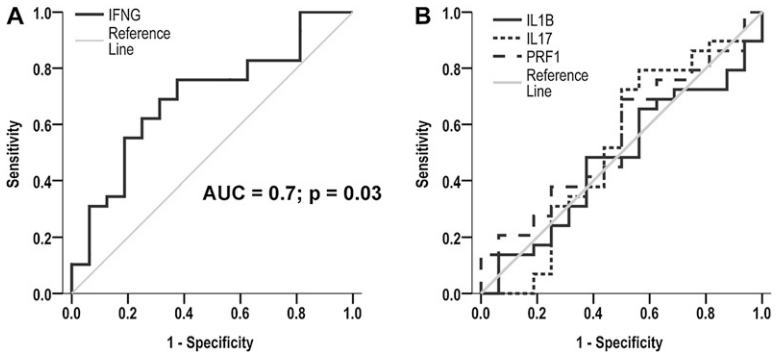

Fig. S7. IFN-γ levels are correlated with pathological complete response in HER2-overexpressing breast cancer. Microarray dataset consisting of 45 HER2-overexpressing stage II/IIIA breast cancer patients treated with trastuzumab combined with anthracycline and taxane chemotherapy was used to assess prediction of pCR (the complete disappearance of invasive tumor evaluated by pathologists) (1). Biopsy samples were taken at diagnosis (i.e., before treatment), and samples were profiled with Affymetrix U133A GeneChips. Area under the curve (AUC) of receiver operating characteristic (ROC) curves was used to assess the association with pCR after 4 mo treatment, where the null hypothesis is that the true AUC = 0.5. Higher AUC indicate correlation with pCR. (*A*) ROC curve for IFNG (AUC = 0.7; *P* = 0.03). (*B*) ROC curves for IL-1B (AUC = 0.5; *P* = 0.8), IL-17 (AUC = 0.5; *P* = 0.8), and PRF1 (AUC = 0.5; *P* = 0.5).