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## A Feasibility Study of Cyclophosphamide, Trastuzumab, and an Allogeneic GM-CSF-secreting Breast Tumor Vaccine for HER-2+ Metastatic Breast Cancer

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### Abstract

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor vaccines are bioactive, but limited by disease burden and immune tolerance. Cyclophosphamide (CY) augments vaccine activity in tolerant *neu* mice and metastatic breast cancer (MBC) patients. HER-2-specific monoclonal antibodies (MAb) enhance vaccine activity in *neu* mice. We hypothesized that CY-modulated vaccination with HER-2-specific MAb safely induces relevant HER-2-specific immunity in *neu* mice and HER-2<sup>+</sup> MBC patients. Adding both CY and the HER-2-specific MAb 7.16.4 to vaccination maximized HER-2-specific CD8<sup>+</sup> T-cell immunity and tumor-free survival in *neu* transgenic mice. We therefore conducted a single arm feasibility study of CY, an allogeneic HER-2<sup>+</sup> GM-CSF-secreting breast tumor vaccine, and weekly trastuzumab in 20 HER-2<sup>+</sup> MBC patients. Primary clinical trial objectives were safety and clinical benefit (CB), in which CB represents complete response+partial response+stable disease. Secondary study objectives were to assess HER-2-specific T-cell responses by delayed type hypersensitivity (DTH) and intracellular cytokine staining. Subjects received three monthly vaccinations, with a boost 6-8 months from trial entry. This combination immunotherapy was safe, with CB rates at 6 months and 1 year of 55% (95% CI:32-77%, p=0.013) and 40% (95% CI:19-64%) respectively. Median progression-free survival (PFS) and overall survival (OS) were 7 (95% CI:4-16) and 42 months (95% CI:22-70) respectively. Increased HER-2-specific DTH developed in 7/20 subjects (of whom 4 had CB (95% CI:18-90)), with a trend toward longer PFS and OS in DTH responders. Polyfunctional HER-2-specific CD8<sup>+</sup> T cells progressively expanded across vaccination cycles.

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Further investigation of CY-modulated vaccination with trastuzumab is warranted.  
([Clinicaltrials.gov](https://clinicaltrials.gov) identifier: NCT00399529)

## Keywords

vaccine; trastuzumab; cyclophosphamide; HER-2<sup>+</sup> breast cancer; immune response

## INTRODUCTION

The availability of multiple drugs specific for HER-2 (trastuzumab, lapatinib, pertuzumab, and ado-trastuzumab emtansine) has revolutionized the treatment of HER-2<sup>+</sup> metastatic breast cancer (MBC) (1, 2). However, not all patients benefit and survival gains could be further enhanced. Innovative therapies that complement and synergize with these drugs are urgently needed to improve disease outcomes.

Immune-based therapies, including vaccines and immune checkpoint blockade, have several unique properties (3). First, immune-based therapies recruit the host tumor-specific immune response rather than targeting the tumor directly. Second, the host immune system can recognize an unlimited number of overexpressed and/or mutated targets differentially expressed by tumor cells relative to normal tissue. Third, immune-based therapies confer a durable treatment response due to immunologic memory. Although the evaluation of immune checkpoint blockade has been limited in breast cancer patients, multiple vaccines have been tested. Despite a good safety profile, immune responses have been modest and inconsistently associated with clinical benefit. Most of these vaccination regimens were limited by some combination of immune tolerance, established disease burdens, or the use of suboptimal therapeutic targets and/or vaccination regimens.

Cytokine-modified tumor cell vaccines that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce robust T cell-dependent immunity that clears established tumors in preclinical models (4). Multiple clinical studies have demonstrated their single agent safety and bioactivity in cancer patients, but clinical benefit remains unproven (5, 6). This lack of success is probably because vaccination alone is not potent enough to induce immune responses sufficiently robust to overcome immune tolerance and suppression, and lyse bulky disease. More recently, clinical trials combining GM-CSF-secreting vaccines with the immune checkpoint agent ipilimumab demonstrated enhanced T-cell activation associated with survival benefit in prostate and pancreas cancer (7, 8). These trials provide clinical proof of principle for combination immunotherapies.

Standard cancer drugs may also augment immune-based therapies, either at approved doses or at an immune-modulating dose and schedule (9). We showed that low dose cyclophosphamide (CY) enhances a HER-2<sup>+</sup> GM-CSF-secreting vaccine in both immune tolerant *neu* transgenic mice (10), and in MBC patients (11). Low dose CY relieves the suppressive influence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) in *neu* mice, allowing the recruitment of potent, tumor-specific CD8<sup>+</sup> T cells (12, 13). The monoclonal antibody (MAb) trastuzumab also has immunomodulatory activity (14). We showed that HER-2-specific MAbs can augment HER-2-specific CD8<sup>+</sup> T-cell responses and tumor-free survival

in vaccinated, tumor-bearing *neu* mice (15); further, the trastuzumab-like MAb 7.16.4 enhances immune priming, augmenting primary and memory CD8<sup>+</sup> T-cell responses in vaccinated *neu* mice (16). In breast cancer patients, trastuzumab-based chemotherapy induces T-cell responses within both the peripheral blood and tumor microenvironment (17, 18).

We therefore evaluated the immunologic and antitumor activity of a GM-CSF-secreting, HER-2<sup>+</sup> whole cell breast cancer vaccine given in sequence with immune-modulating doses of CY and weekly HER-2-specific MAb in tolerant *neu* mice, and in MBC patients. The clinical study was designed to assess the safety, clinical benefit, and immunologic activity of this combination immunotherapy regimen in patients with measurable or evaluable HER-2<sup>+</sup> MBC.

## METHODS

### Preclinical Study Design

**Mice**—*Neu-N (neu)* mice were derived from the colony of Muller (19), bred to homozygosity as verified by Southern blot (20), and maintained at Johns Hopkins University. Experiments were performed with 8-10 week old mice using AAALAC-compliant protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

**Cell lines and media**—The GM-CSF-secreting vaccine cell lines GM (mock) and neuGM (neu-specific), the NT2.5 neu-expressing tumor cell line, and the NT2.5B7.1 antigen-presenting cell line were derived and grown as previously described (4, 10). GM-CSF levels were verified by ELISA, and neu and B7.1 levels were verified by flow cytometry biannually. All cell lines used were regularly tested and validated to be mycoplasma-free; no other authentication assay was performed.

**Antibody purification**—Hybridoma cells secreting the anti-rat neu MAb 7.16.4 (21) were grown in athymic nude mice, and ascites collected (Harlan Bioproducts for Science). 7.16.4 was purified over a T-Gel column (Pierce Biotechnology) using the Biologic LP purification system (Bio-Rad), dialyzed into PBS, and >90% purity determined by SDS-PAGE analysis using Criterion 4-15% Tris-HCl Pre-cast Gels and the Criterion Cell (Bio-Rad). Nonspecific isotype-matched antibody served as the control. Antibodies were confirmed endotoxin-free by the Limulus ameocyte lystate test (Bio Whittaker).

**Murine immunotherapy experiments**—*Neu* mice were challenged with  $5 \times 10^4$  NT2.5 tumor cells, and vaccinated 3 days later. Vaccine cells were irradiated prior to subcutaneous (SQ) injection in both hind limbs and the left forelimb. CY (Bristol-Myers Squibb) at 100 mg/kg and 7.16.4 at 100  $\mu$ g were injected intraperitoneally (IP) 1 day prior to vaccination; 7.16.4 was then given IP weekly. The tumor and vaccine cell doses, and the CY and MAb dose and schedule have been optimized previously (10, 20, 22). Mice were monitored for tumor onset and growth twice weekly, and tumors were measured in 2 perpendicular dimensions with calipers.

**ELISPOTS**—CD8<sup>+</sup> splenic lymphocytes were purified by negative selection (DynaL Biotech, Invitrogen). 10<sup>5</sup> CD8<sup>+</sup> T cells were incubated in duplicate with 10<sup>4</sup> target cells (NT2.5B7.1 cells stimulated with IFN $\gamma$  for 2 days) at 37°C overnight (22) on pre-coated IFN $\gamma$ -specific ELISPOT plates and developed according to the manufacturer's protocols (R&D Systems). IFN $\gamma$ -secreting CD8<sup>+</sup> T cells were enumerated using the Immunospot counter (Cellular Technology, LTD). The number of spots in control wells was averaged and subtracted from the number of spots in each well containing both CD8<sup>+</sup> T cells and targets.

**Statistical analysis**—A Student's *t* test was applied to determine the statistical significance of differences between treatment groups, with  $P < 0.05$  being significant. Analyses were performed using GraphPad Prism, version 3.0a for Macintosh (GraphPad Software). All experiments were repeated at least twice, with 5-10 mice per group.

### Clinical Study Design

The clinical study was an open label, single arm feasibility study to evaluate the safety and clinical benefit associated with the administration of a fixed dose of allogeneic GM-CSF-secreting breast tumor vaccine cells given in sequence with low dose CY and weekly trastuzumab. A Simon two-stage design (23) was used to evaluate 20 vaccinated research subjects.

The clinical study was conducted in accordance with the principles of Good Clinical Practice and the ethical principles stated in the Declaration of Helsinki. It was approved by the Johns Hopkins University School of Medicine Institutional Review Board, the National Institutes of Health Recombinant DNA Advisory Committee, and the US Food and Drug Administration Center for Biologics Evaluation and Research.

### Patient Selection

Twenty-two patients with HER-2<sup>+</sup> MBC were enrolled at the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center between November 14, 2006 and July 13, 2009; 20 were treated on study. Eligible patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1, and a histologic diagnosis of HER-2<sup>+</sup> breast cancer by IHC 3+ staining or gene amplification  $> 2.0$  by FISH. Prior chemotherapy must have been completed 28 days before vaccination; concurrent endocrine and/or bisphosphonate therapy was allowed. Other requirements included a cardiac ejection fraction  $\geq 45\%$ , adequate end-organ function, and negative HIV and pregnancy tests. Stable treated central nervous system disease was allowed. Key exclusion criteria included past/current autoimmune disease, non-protocol-specific treatment or parenteral steroids within 28 days of vaccination, and past/current second malignancy (except superficial non-melanoma skin cancer, bladder cancer, tamoxifen-related endometrial cancer cured by hysterectomy, and cervical carcinoma *in situ*).

### Study Plan and Intervention

**Eligibility Determination**—Written informed consent was obtained from each research participant. Baseline studies included computed tomography of the chest, abdomen, and

pelvis, bone scans, complete blood count with differential, chemistry profile, absolute eosinophil count, and echocardiogram or multiple gated acquisition scan.

**Treatment Plan**—Subjects received three monthly vaccinations, with a boost 6-8 months from trial entry. During active vaccination cycles, patients received CY 300 mg/m<sup>2</sup> on D<sup>-1</sup> and 5×10<sup>8</sup> vaccine cells on D0, on a backbone of weekly trastuzumab given at the standard dose (2 mg/kg, with a 4 mg/kg loading dose as necessary); trastuzumab was given on D<sup>-1</sup> the week of vaccination. During the interim period between cycles 3 and 4, trastuzumab could be given weekly (2 mg/kg) or every 3 weeks (6 mg/kg). CY-modulated vaccination was given every 4-6 weeks for three cycles, with a fourth cycle 6-8 months after the first cycle. Patients with disease progression were taken off study.

**Vaccinations**—Vaccine development and manufacturing has been published (24). Briefly, the parent cell lines T47D (HER2<sup>low</sup>) and SKBR3 (HER2<sup>high</sup>) were genetically modified by plasmid DNA transfection to secrete GM-CSF. Clinical lots were prepared from two subcloned cell lines secreting bioactive levels of GM-CSF, 2T47D-V and 3SKBR3-7. On D0, serum-free, cryopreserved, irradiated vaccine cells were thawed and mixed to create a HER2<sup>+</sup> vaccine that secreted GM-CSF levels of about 300 ng/10<sup>6</sup> cells /24 hours. Vaccine cells were injected intradermally, evenly distributed over 3 lymph node areas. Anesthetic lidocaine cream was applied to the injection sites before vaccination.

## End Points

**Toxicity assessment**—Toxicities were graded using the National Cancer Institute's Clinical Trials Common Terminology Criteria for Adverse Events Version 3.0 (CTCAE v. 3.0). Toxicity monitoring included clinical assessment and complete blood counts on days 3 and 7; chemistry profiles were measured before and after each cycle and on day 7.

**Clinical Benefit Rate and Survival Outcomes**—The primary endpoint of clinical benefit (CB) was defined as complete response+partial response+stable disease (CR+PR +SD) at 6 months; CB at 1 year was also determined. Exploratory endpoints of progression-free survival (PFS) and overall survival (OS) were defined as time to first disease progression or death from the eligibility date.

**Analysis of serum cytokine levels**—Serum was collected at baseline and after each vaccination, and on days 0, 1, 2, 3, 4, 7, and 14 of each cycle. Serum was separated from whole blood by centrifugation, and frozen in 1-mL aliquots at -80°C. Serum GM-CSF levels were measured as described previously (11). Serum TGF-β was measured by Luminex pre- and post-vaccination according to the manufacturer's protocols.

**Assessment of delayed-type hypersensitivity (DTH) using HER-2 peptides**—One hundred ug each of two MHC class II HER-2 epitopes (p369 and p776) (25), with mutated *k-ras* as a negative control, were injected intradermally on the back as previously described (11). Erythema and induration were assessed 2-3 days after injection.

DTH was considered positive if induration increased by >5mm from baseline.

**Collection, preparation and cryopreservation of peripheral blood**

**mononuclear cells (PBMC)**—200cc whole blood was collected in heparin tubes pre- and post-vaccination; 20cc whole blood was collected on days 0, 1, 2, 3, 4, 7, and 14. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation; interface cells were harvested and washed twice. PBMCs were resuspended in AIM-V medium (Invitrogen) with 10% dimethylsulfoxide, frozen at  $-80^{\circ}\text{C}$  for 1-2 days, and stored in  $\text{LN}_2$  until analysis (8).

**Assessment of Treg and myeloid-derived suppressor cells (MDSC)**—Treg and MDSC analyses were performed with modifications to published methods (26-28). Briefly,  $10^6$  PBMCs were stained with for 30 minutes at  $4^{\circ}\text{C}$  with: Pacific blue-CD4 (BD Pharmingen), FITC-CD25 (BD Pharmingen), PE-Cy7-CD45RA (eBioscience), PerCP-Cy5.5-CCR7 (BD Pharmingen), APC-CTLA-4 (BD Pharmingen), FITC-Lineage cocktail (Biolegend), APC-HLA-DR (BD Pharmingen), PE-Cy7-CD33 (eBioscience) and/or PE-CD11b (eBioscience). For Treg analysis, cells were fixed for 40 minutes at  $4^{\circ}\text{C}$  and permeabilized with Transcription Factor Buffer Set (BD Pharmingen), followed by staining with PE-FoxP3 (BD Pharmingen) for 30 minutes at  $4^{\circ}\text{C}$ . Data were acquired by a Gallios cytometer (Beckman Coulter) and analyzed using FlowJo V7.6.5 software (TreeStar, Inc.).

**Assessment of peripheral HER-2-specific CD8<sup>+</sup> T cells**—T-cell analyses were conducted with modifications to published methods (29, 30). Monocytes were isolated using the MACS CD14 isolation kit (Miltenyi Biotec), and cultured in 6-well plates ( $2 \times 10^6$  cell/mL) in RPMI-1640 medium containing 5% pooled human serum (PHS), 50 ng/mL GM-CSF (Miltenyi Biotec) and 50 ng/mL IL-4 (Miltenyi Biotec). Cells were cultured for 48 hours and subsequently incubated for 18 hours with 10 ng/mL IL-1 $\beta$  (Miltenyi Biotec), 10 ng/mL IL-6 (Miltenyi Biotec), 10 ng/mL TNF- $\alpha$  (Miltenyi Biotec), and 0.5-1  $\mu\text{g/mL}$  PGE<sub>2</sub> (Sigma-Aldrich). The quality of monocytic dendritic cells (MoDC) was assessed by staining with PE-Cy7-CD80 (Biolegend), FITC-CD83 (Biolegend), Pacific blue-CD86 (Biolegend), APC-HLA-DR (BD Pharmingen), and/or APC-IL-12 (BD Pharmingen). Overlapping peptide mixtures were used to stimulate CD8<sup>+</sup> T cells (JPT Peptide Technologies). PepMix HER-2 (extracellular and intracellular domains) is a pool of 15-mer peptides that span the entire HER-2 protein and overlap by 11 amino acids; PepMix CEF Pool and PepMix HIV served as positive and negative controls, respectively. Peptides were resuspended in DMSO, stored at  $-80^{\circ}\text{C}$ , thawed the day of assay and diluted to the required concentration.

Isolated, activated MoDCs were mixed with peptides at 1  $\mu\text{g/ml}$  in RPMI-1640 medium containing 5% PHS and incubated at  $37^{\circ}\text{C}$  for 2 hours. CD8<sup>+</sup> T cells were purified from PBMCs using the Dynabeads Untouched CD8 T cell kit (Invitrogen). HER-2-specific CD8<sup>+</sup> T cells were indistinguishable from background by flow cytometry directly *ex vivo*, and were therefore stimulated *in vitro* by co-culturing peptide-loaded MoDCs with autologous CD8<sup>+</sup> T cells at a ratio of 1:3 in the presence of IL-7 (R&D Systems) and IL-2 (R&D Systems) at concentrations of 10 ng/ml and 10 IU/ml, respectively. On days 8 and 15, T cells were re-stimulated with MoDCs. The cells were re-fed with IL-7 and IL-2 every 3 days. The cells were harvested on day 15 after 4 hours of re-stimulation and assayed by ICS using FITC-CD8 (eBioscience), PE-IFN- $\gamma$  (Biolegend), APC-TNF- $\alpha$  (Biolegend), and PE-

Cy7-IL-2 (BD Pharmingen). Sample data were acquired by a Gallios cytometer (Beckman Coulter) and analyzed using FlowJo Version 7.6.5 software. The proportions of polyfunctional CD8<sup>+</sup> effector T cell subsets were classified by cytokine secretion.

### Statistical Considerations and Data Analysis

The trial database was closed on October 16, 2013. A Simon two-stage design was used (23). Nine research subjects were to be enrolled and treated in the first stage. At the end of the first stage, if 2 or more research subjects demonstrated CB, 11 additional research subjects were to be enrolled and treated. This design had an alpha level of 0.08 with a power of 0.86 to detect a clinical benefit (CR+PR+SD) of 0.45 from a null hypothesis rate of 0.20. At the end of the second stage, if 7 or more patients demonstrated CB, the null hypothesis was rejected. Based on this study design, there was a 44% chance of early stopping if the true response rate was 0.20, and a 4% chance of early stopping if the true response rate was 0.45.

The study met criteria for full accrual, and data from 20 patients were used in the analysis. Tumor responses (CR, PR, and SD) were determined by RECIST 1.0. CB was estimated using the binomial distribution along with the 95% confidence interval (CI). Continuous data were summarized using mean and standard deviation. Categorical data were represented using percent or proportion. Survival probability was estimated using the Kaplan-Meier method (31). The CI of median survival time was constructed by the method of Brookmeyer-Crowley (32). Paired and unpaired Student's t tests were used for pre- and post-vaccine comparisons, and between group comparisons. All P values are reported as two-sided, and all analyses were conducted using SAS software (version 9.2 SAS Institute, Cary, NC).

## RESULTS

### Preclinical Modeling

*Neu* mice exhibit pre-existing immune tolerance specific for rat HER-2 (*neu*), and are a stringent model for developing clinically relevant cancer immunotherapies (10, 20, 22). We first evaluated whether the trastuzumab-like MAb 7.16.4 could augment the activity of neu-specific vaccination alone or sequenced with immune modulating doses of CY in these mice. Treating tumor-bearing *neu* mice with 7.16.4 and mock vaccination delayed tumor growth relative to neu-targeted vaccination alone, which is ineffective in this model (Figure 1). Adding CY or 7.16.4 to neu-specific vaccination also delayed tumor outgrowth, increasing tumor-free survival from 0% (vaccine or 7.16.4 alone) to 10% (vaccine + CY) and 30% (vaccine + 7.16.4) (Figure 1A and 1B). Adding both CY and 7.16.4 to neu-targeted vaccination increased tumor-free survival to about 60% (Figure 1C). This relative antitumor efficacy was reflected in the numbers of tumor-specific IFN $\gamma$ -secreting CD8<sup>+</sup> T cells by ELISPOT, where the highest levels were detected in the setting of CY + 7.16.4 + vaccine (Figure 1D).

## Clinical Trial

**Patient Characteristics**—Based on this preclinical modeling, we conducted a clinical trial testing CY-modulated vaccination with trastuzumab in patients with HER-2<sup>+</sup> MBC. The intervention and data collection schedules are shown in Figure 2. Twenty eligible patients were vaccinated, with an age range of 34 to 69 years (Table 1). All had HER-2<sup>+</sup> metastatic disease, 13 had ER-positive and/or PR-positive disease. The mean disease-free interval to relapse from first diagnosis was 32 months (range, 0 to 130 months); 7 (35%) patients presented with MBC at diagnosis. Eighteen patients (90%) received prior trastuzumab for MBC, while 3 received prior trastuzumab as adjuvant therapy. Eleven (55%) were on concurrent endocrine therapy, and the majority (70%) received concurrent bisphosphonate therapy for skeletal metastasis. The mean and median sums of the longest tumor diameter at study entry were 40.7 mm and 24.5 mm, respectively.

All patients (100%) received at least one vaccination, 15 (75%) received at least three vaccinations, and 8 (40%) received all four vaccinations. Two patients came off-study for reasons unrelated to disease progression, one after 3 vaccine cycles due to re-location, and another after 2 vaccine cycles due to study demands. A third off-study event was related to both a vaccine-related serious adverse event and simultaneous disease progression. All other off-study events before cycle 4 were due to breast cancer progression.

**Toxicity**—The most common adverse events were local vaccine site reactions, including erythema, induration, pruritus, and/or discomfort (Table 2). These self-limited local reactions occurred in all individuals, and increased in intensity but not duration with subsequent vaccination cycles. The most common systemic adverse events were fatigue, urticaria, pruritus, and fever. Seven patients (35%) developed urticaria distant from the vaccine site; of these, 3 (15%) developed Grade 2-3 urticaria that required parenteral intervention with diphenhydramine and/or steroids. Of these, one was a vaccine-related serious adverse event requiring overnight hospitalization and steroid treatment. No evidence of clinically significant autoimmunity was detected. Despite the use of trastuzumab, no decrement in cardiac function was detected (**data not shown**).

**Serum GM-CSF Pharmacokinetics**—Serum GM-CSF levels were measured as an indicator of the vaccine's life span post-injection. Serum GM-CSF levels peaked at 48 hours, with peak levels persisting across all four cycles as previously observed (11). Trastuzumab did not alter the timing of the peak or the kinetics of GM-CSF decay (**data not shown**).

**Clinical Outcomes**—The CB rates of weekly trastuzumab with CY-modulated vaccination at 6 months and 1 year were 11/20 (55%, 95% CI:32-77%,  $p=0.013$ ) and 8/20 (40%, 95% CI:19-64%), respectively. One PR and no CRs were observed. Overall median PFS and OS were 7 months (95% CI:4-16) and 42 months (95% CI: 22-70), respectively (Figure 3A). The 5-year survival rate was 6/20 (30%, 95% CI:12-54).

**Immunologic Analyses**—We previously demonstrated the feasibility of using DTH responses specific for HER-2-derived peptides and HER-2-specific antibody responses by



ELISA as immune response biomarkers for this cell-based vaccine (11). Both because our preclinical data demonstrate a correlation of HER-2-specific CD8<sup>+</sup> T cells with tumor-free survival in vaccinated *neu* mice, and because trastuzumab confounds the assessment of HER-2-specific antibody responses, we assessed HER-2-specific DTH and measured HER-2-specific CD8<sup>+</sup> T cells by ICS as biomarkers of vaccine-induced immunity in this study. HER-2-specific DTH developed in 7/20 subjects (35%, 95% CI:15-59%); 4 of these (57%, 95% CI:18-90%) had CB. There was a trend toward longer PFS and OS for patients who developed HER-2-specific DTH post-vaccination (Figure 3B and 3C), though this was not statistically significant due to the small sample size of this study. Median PFS for DTH positive versus DTH negative subjects was 9 (95% CI:4-46) versus 4 months (95% CI:3-14,  $p=0.58$ ). Median OS for DTH positive versus DTH negative subjects was 70 (95% CI: 40-70) versus 29 months (95% CI:13—not reached,  $p=0.11$ ). Larger randomized studies are required to rigorously evaluate the possible relationship between DTH and survival.

HER-2-specific CD8<sup>+</sup> T cell function pre- and post-vaccination was assessed *in vitro* by ICS using commercially available peptide mixtures containing overlapping epitopes spanning the entire HER-2 protein, a representative analysis is shown in Figure 4A. Both the average level of CD8<sup>+</sup> T cells secreting IFN $\gamma$  0.501% versus 0.785%, ( $p<0.0001$ ), interleukin-2 (IL-2) (0.567% versus 0.683%,  $p=0.0006$ ) and tumor necrosis factor-TNF-0.751% versus 1.525%, ( $p<0.0001$ ), and the proportion of polyfunctional CD8<sup>+</sup> T cells secreting more than one cytokine (0.023% versus 0.735%,  $p<0.0001$ ) increased across the vaccination cycles (Figure 4B and 4C). In exploratory analyses, increased polyfunctional CD8<sup>+</sup> T cells were possibly associated with longer PFS (HR=0.44, 95% CI:0.2-0.98,  $p=0.04$ ) and OS (HR=0.43, 95% CI:0.13-1.36,  $p=0.15$ ).

We also assessed peripheral markers of immune suppression by FACS (Treg and MDSC) and Luminex (TGF- $\beta$ ). Though an association between decreased TGF- $\beta$  levels and immunity in patients treated with a HER-2 peptide vaccine and concurrent trastuzumab was reported previously (33), we found no difference in serum TGF- $\beta$  levels pre- and post-vaccination (43,125 versus 46,936 pg/ml,  $p=0.21$ ). Tregs decreased across all vaccine cycles, and both overall and CTLA-4<sup>+</sup> Treg decreased from baseline to post-vaccine cycle 1; MDSCs also decreased from baseline to post-vaccine cycle 1 (Figure 5). Exploratory analyses suggested that neither baseline nor decreased levels of peripheral Tregs were associated with survival benefit. However, decreased numbers of peripheral MDSC were possibly associated with both longer PFS (HR=0.5, 95% CI:0.29-0.85,  $p=0.010$ ) and OS (HR=0.52, 95% CI:0.31-0.89,  $p=0.016$ ) baseline levels of MDSC were not. To further explore possible associations between immunologic biomarkers and survival, a subgroup analysis of 12 patients was performed comparing 6 individuals with OS  $\geq$  24 months to 6 individuals with OS  $<$  60 months. Both decreased MDSCs and increased polyfunctional T cells post-vaccination were possibly associated with OS, with  $p$  values of 0.012 and 0.028, respectively.

## DISCUSSION

This study of a HER-2<sup>+</sup> GM-CSF-secreting breast tumor vaccine with low dose CY and weekly trastuzumab supports the following seven conclusions. First, in the clinically

relevant *neu* transgenic mouse, adding both CY and 7.16.4 (a trastuzumab-like MAb) to vaccination increases tumor-free survival and IFN $\gamma$ -secreting tumor-specific CD8<sup>+</sup> T cells relative to adding either CY or 7.16.4 alone to vaccination. Second, up to four sequential CY-modulated vaccinations are safe and well tolerated in patients with HER-2<sup>+</sup> MBC receiving weekly trastuzumab. Third, this immunotherapy was associated with a 6-month CB rate of 55%, and median PFS and OS of 7 and 42 months. This study met its primary endpoint, as the target therapeutic outcome was a 6-month CB rate of 45%. Fourth, this combination immunotherapy induced new or increased HER-2-specific T-cell immunity in HER-2<sup>+</sup> MBC patients as measured by HER-2-specific DTH and CD8<sup>+</sup> T cells by ICS. There was a trend toward longer PFS and OS in patients who developed HER-2-specific DTH relative to those who did not. Fifth, polyfunctional HER-2-specific CD8<sup>+</sup> T cells progressively expanded across the vaccination cycles. Sixth, both Tregs and MDSCs decreased across the vaccination cycles. Finally, hypothesis-generating analyses suggest that both decreased MDSCs and a greater proportion of polyfunctional HER-2-specific CD8<sup>+</sup> T cells may be associated with longer PFS and OS. A comparative trial powered for survival will be required to rigorously evaluate these associations.

Trastuzumab has revolutionized the management of HER-2<sup>+</sup> breast cancer, and remains the major backbone of therapy for early and late stage disease (1, 2, 14). However, intrinsic and acquired trastuzumab resistance remains a significant limitation to its clinical utility. In patients, trastuzumab-based chemotherapy is associated with increased levels of HER-2-specific antibodies and T cells (17), and intratumoral lymphoid nodules develop in the setting of neoadjuvant trastuzumab-based chemotherapy (18). These observations suggest that leveraging the ability of trastuzumab to activate adaptive immunity could increase its efficacy. One clinical study combined a peptide vaccine with weekly trastuzumab, demonstrating increased levels of HER-2-specific T cells, epitope spreading, and decreased serum levels of the immune suppressive cytokine TGF- $\beta$  (33). We showed that HER-2-specific MAbs alone induce low levels of *neu*-specific T cells in *neu* transgenic mice. The addition of HER-2-specific MAbs to *neu*-specific GM-CSF-secreting vaccination more effectively augments *neu*-specific CD8<sup>+</sup> effector T-cell function and prolongs tumor-free survival in these mice (15). We further showed that the trastuzumab-like MAb 7.16.4 augments locoregional immune priming through binding the Fc receptor of dendritic cells in similarly vaccinated *neu* mice, augmenting CD8<sup>+</sup> T-cell proliferation, cytokine secretion, and central memory development (16). Here we build on these prior studies to show that the combination of low dose CY, trastuzumab, and a GM-CSF-secreting HER-2<sup>+</sup> allogeneic cell-based vaccine is safe, bioactive, and associated with CB in patients with HER-2<sup>+</sup> MBC.

A significant challenge in optimizing combination tumor cell vaccine-based immunotherapy is the lack of clear biomarkers for assessing immune response and drug-drug interactions. We previously demonstrated the feasibility of using DTH responses specific for two MHC Class II HER-2-derived peptides and HER-2-specific antibody responses by ELISA pre- and post-vaccination as immune response biomarkers for this cell-based vaccine (11). Because the use of trastuzumab here confounds the assessment of HER-2-specific antibody responses, we measured HER-2-specific DTH and peripheral HER-2-specific CD8<sup>+</sup> T cells by flow cytometry. We found that HER-2-specific DTH developed in 7/20 subjects (35%); 4 of these patients had CB. In addition, there was a trend toward longer PFS and OS in

patients who developed HER-2-specific DTH relative to those who did not. These data are consistent with other small clinical trials that have shown an association between enhanced tumor-specific DTH and survival (34-37). Cytokine-secreting CD8<sup>+</sup> T cells also increased with vaccination in MBC patients. While vaccine-induced CD8<sup>+</sup> T cells from *neu* mice could be detected by ELISPOT directly *ex vivo*, vaccine-induced CD8<sup>+</sup> T cells from MBC patients were detected by flow cytometry only after *in vitro* stimulation. This differential sensitivity in detecting vaccine-induced CD8<sup>+</sup> T cells is likely explained by differences in the host (transgenic *neu* mice are genetically homogenous whereas MBC patients are genetically and biologically heterogeneous), the vaccine (the murine vaccine specifically targets HER-2, whereas the human vaccine delivers multiple tumor antigens where responses to HER-2 are used as a sentinel measure of vaccine activity), the T cells (assayed fresh from mice as opposed to frozen from MBC patients), and the assays (ELISPOT is more sensitive than flow cytometry). We used ICS by flow cytometry to rapidly evaluate HER-2-specific CD8<sup>+</sup> T cells that secrete one or more cytokines in MBC patients.

Polyfunctional antigen-specific T cells may be indicators of disease-control and/or survival in patients with chronic infections and cancer (38-40). Polyfunctional T cells secrete multiple relevant cytokines (IFN $\gamma$ , TNF- $\alpha$ , IL-2) that reflect the quality of the immune response as a more informative correlate of sustained protective immunity than T cells that secrete only a single cytokine (IFN $\gamma$  or TNF- $\alpha$ ). Durable T-cell polyfunctionality correlates with long-term disease control in HIV (41) and malaria patients (42), but has been less studied in vaccinated cancer patients. A telomerase peptide vaccine given with temozolomide to metastatic melanoma patients induced polyfunctional T cells (43). Here we demonstrate the progressive expansion of polyfunctional HER-2-specific T cells across vaccination cycles, where a greater proportion of polyfunctional CD8<sup>+</sup> T cells may be associated with longer PFS and OS. A comparative clinical study powered for survival is required to formally evaluate this hypothesis.

Tregs and MDSCs are significant mediators of immune tolerance and suppression in cancer patients. We showed previously that low dose CY depletes Tregs in *neu* mice, facilitating the vaccine-mediated recruitment and activation of latent, high avidity tumor-specific T cells and tumor rejection (10, 12, 13). Here, we demonstrate a decline in peripheral Tregs and MDSCs in vaccinated MBC patients. Several clinical trials have explored depleting Tregs in the setting of vaccination for cancer, with enhancement of antigen-specific immune responses to vaccination (11, 44-46). Notably, a randomized Phase II study evaluating a multi-peptide renal cell carcinoma vaccine alone or sequenced with low dose CY demonstrated that CY-modulated vaccination reduced Tregs and induced T-cell responses specific for multiple peptide antigens, increasing the OS of vaccinated patients (46). An association between levels of circulating MDSCs and immune response or clinical outcomes has been reported in several immunotherapy trials, and these results are consistent with our findings (46-49).

This combination immunotherapy was safe. Given the known cardiac toxicity of trastuzumab (14), cardiac function was assessed every 3 months on study. No patient developed a significant drop in left ventricular ejection fraction. These data suggest that the addition of CY-modulated vaccination to weekly trastuzumab did not increase cardiac

toxicity, though the sample size is limited. Notably, urticaria was observed in a significant number of patients in this study; three patients required parenteral diphenhydramine and/or steroids. It remains unclear if urticaria may be associated with higher levels of immunity or clinical benefit. We observed CB rates of 55% at 6 months and 40% at one year. The median PFS in this study was 7 months, the median OS was 42 months, and the 5-year survival rate was 30%. Although the sample size is small, these clinical results compare favorably to other trials of HER-2-directed therapy for metastatic HER-2<sup>+</sup> breast cancer that do not include cytotoxic chemotherapy. The seminal trials of single agent trastuzumab for the first line and second/third line treatment of patients with metastatic disease showed CB rates of 48% and 56% at 6 months (50, 51); median OS in these seminal trials was 24 and 13 months. More recently, novel MAb-based HER-2-directed therapies were approved for metastatic HER-2<sup>+</sup> breast cancer progressing on prior trastuzumab therapy (2). Concurrent pertuzumab and trastuzumab therapy is associated with a CBR of 50%, and a median PFS of 5.5 months (52) and ado-trastuzumab emtansine therapy is associated with a median PFS and OS of 9.6 months and 30.9 months (53).

In conclusion, this allogeneic GM-CSF-secreting breast tumor vaccine is safe, bioactive, and confers clinical benefit when sequenced with low-dose CY and standard trastuzumab in HER-2<sup>+</sup> MBC patients. It induces HER-2-specific immunity as measured by DTH and polyfunctional CD8<sup>+</sup> T cells, two measures of a high quality immune response. We observed a CB rate of 55% at 6 months, with PFS and OS of 7 and 42 months. Since this study began, two additional MAb-based therapies with survival benefit were approved for metastatic HER-2<sup>+</sup> breast cancer (pertuzumab and ado-trastuzumab emtansine). The data presented here are encouraging relative to results with other HER-2-directed combination therapies, including these new agents. Further investigation of GM-CSF-secreting vaccines in combination with low dose CY and HER-2-specific MAb therapies is warranted.

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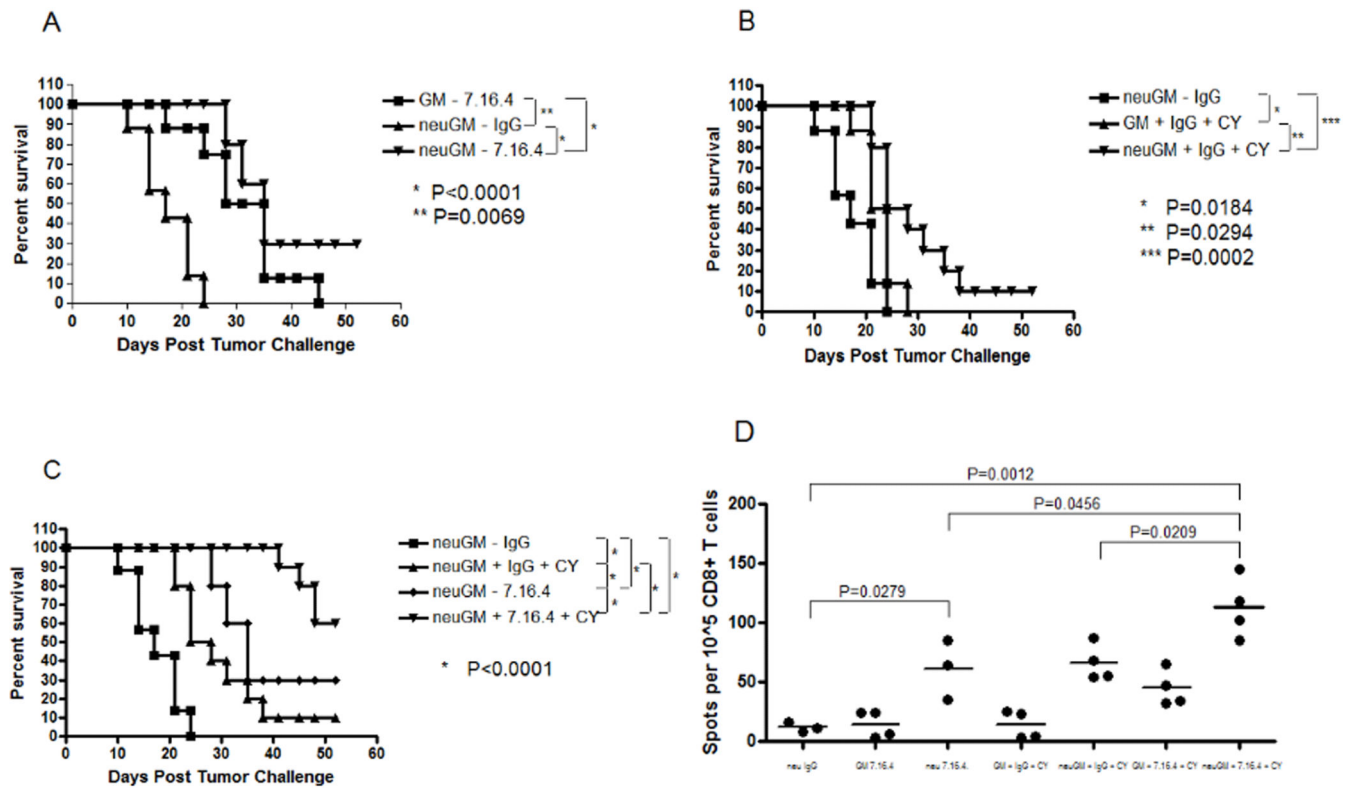
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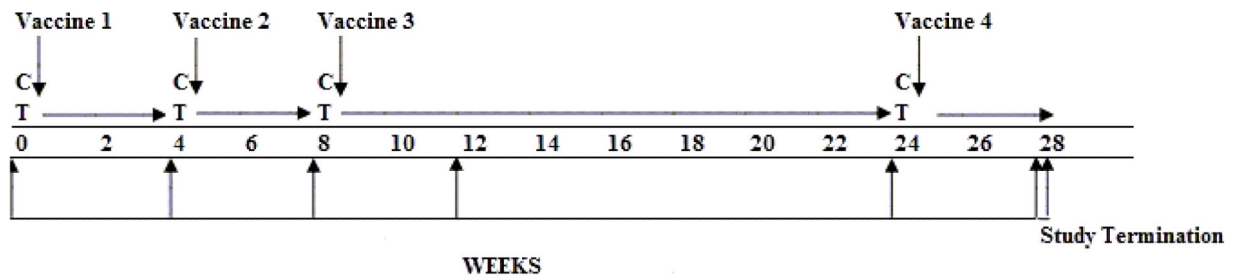


**Figure 1. HER-2-specific MABs combined with HER-2-specific vaccination augment tumor-free survival and HER-2-specific CD8<sup>+</sup> T cell levels in *neu* mice**

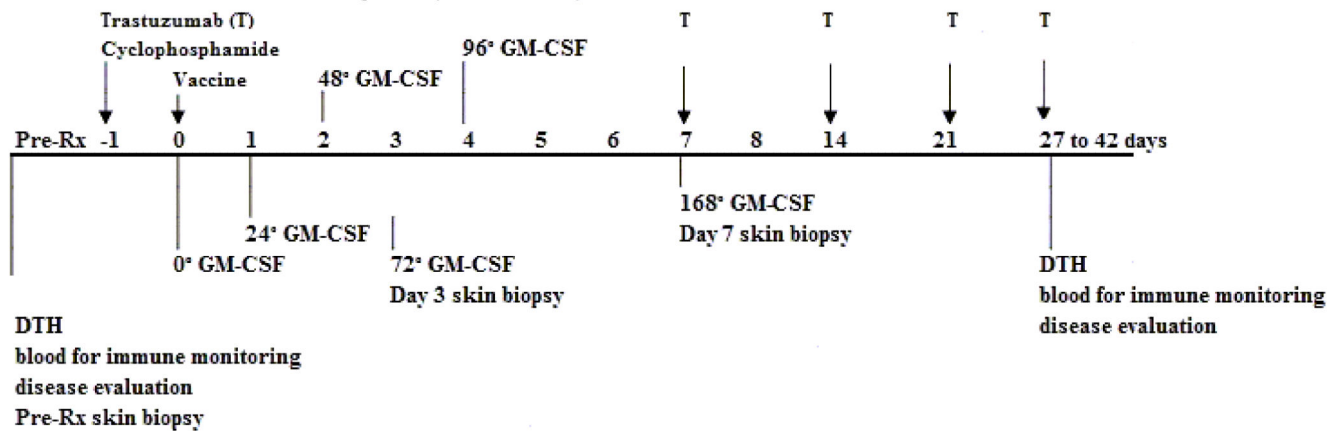
*Neu* mice (10 per group) were challenged with NT-2.5 tumor cells, and vaccinated 3 days later. A single dose of cyclophosphamide (CY) was given one day prior to vaccination in selected mice; weekly HER-2-specific MAb (7.16.4) or nonspecific IgG was also started one day prior to vaccination. (A) Vaccination + 7.16.4. (B) Vaccination + CY. (C) Vaccination + CY + 7.16.4. (D) Splenic T cells were isolated from *neu* mice at the end of treatment (3-5 per group). CD8<sup>+</sup> T cells were isolated and incubated with NT2.5B7.1 target cells for IFN $\gamma$  ELISPOT analysis. Circles represent individual mice; bars represent the average value. neuGM=3T3 cells that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) and express rat HER-2 (neu vaccine); GM=3T3 cells that secrete GM-CSF (control vaccine).



### A. Overall Trial Schema

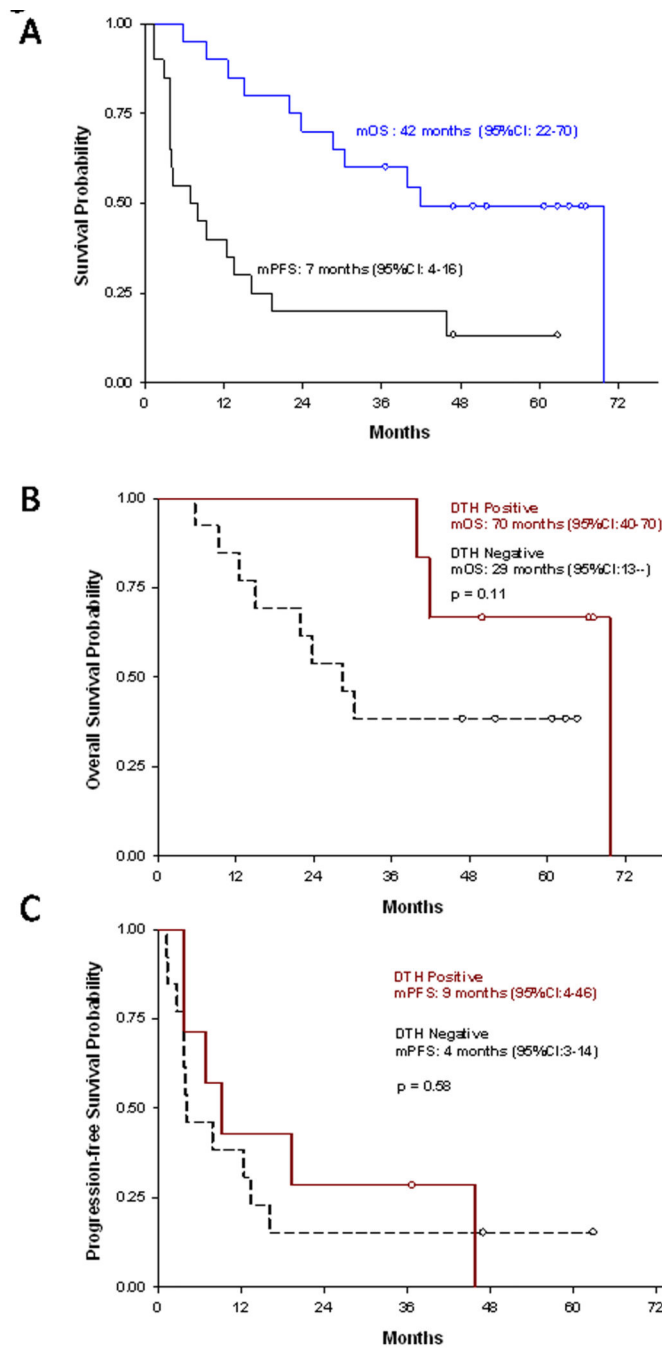


### B. Schedule for Treatment Cycles (not to scale)

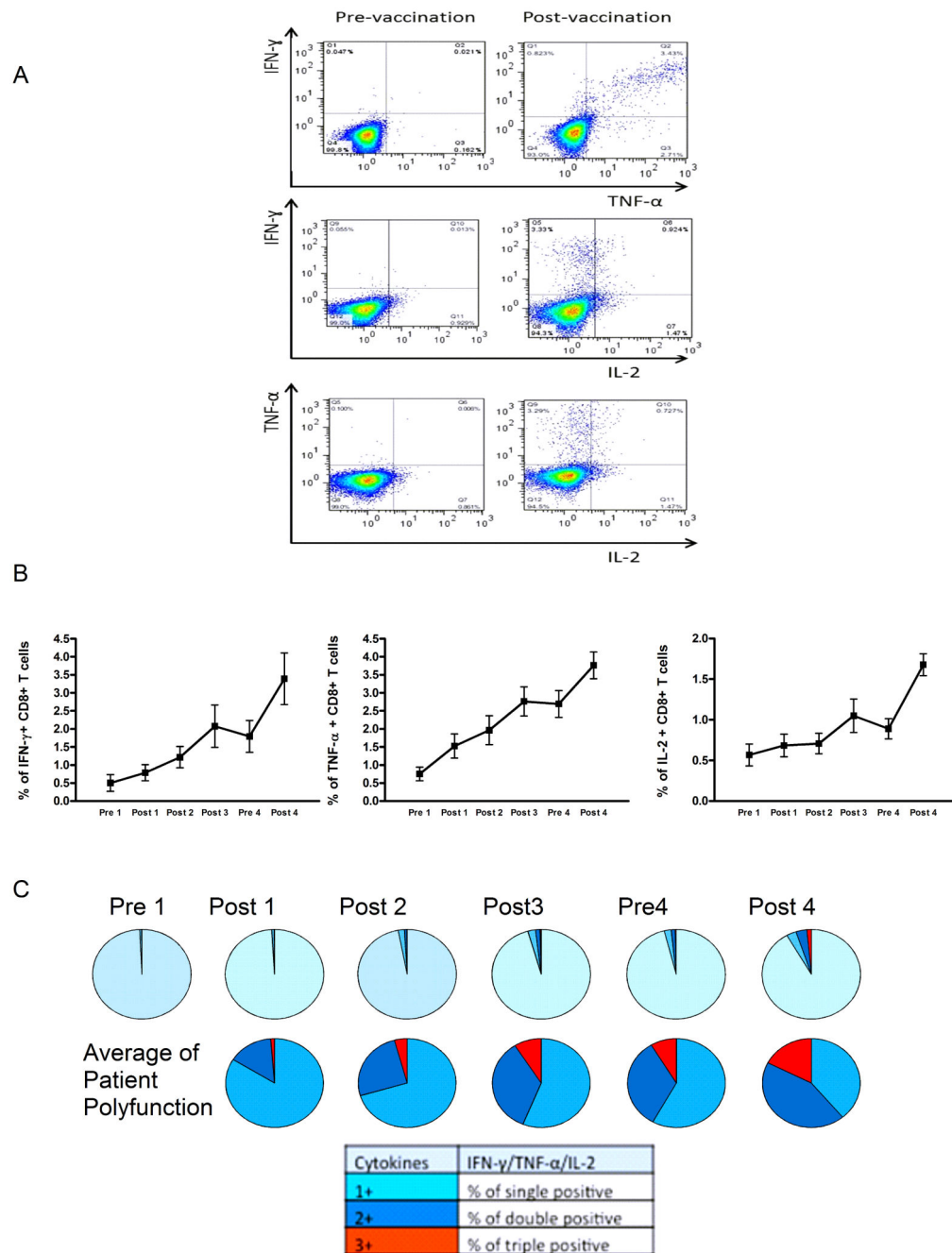


**Figure 2. Study schema**

Patients received up to four vaccination cycles. Cyclophosphamide (CY) was given on day -1, and vaccine on day 0. Trastuzumab (T) was given weekly during active vaccine cycles, and timed to coincide with CY administration on day -1. During the interim period between cycles 3 and 4, trastuzumab could be given weekly or every 3 weeks as per the standard of care. GM-CSF levels and immunity were measured as indicated. CY=cyclophosphamide; T=trastuzumab; DTH=delayed-type hypersensitivity to HER-2-derived peptides. Skin biopsies on days +3 and +7 were from the largest vaccine site.

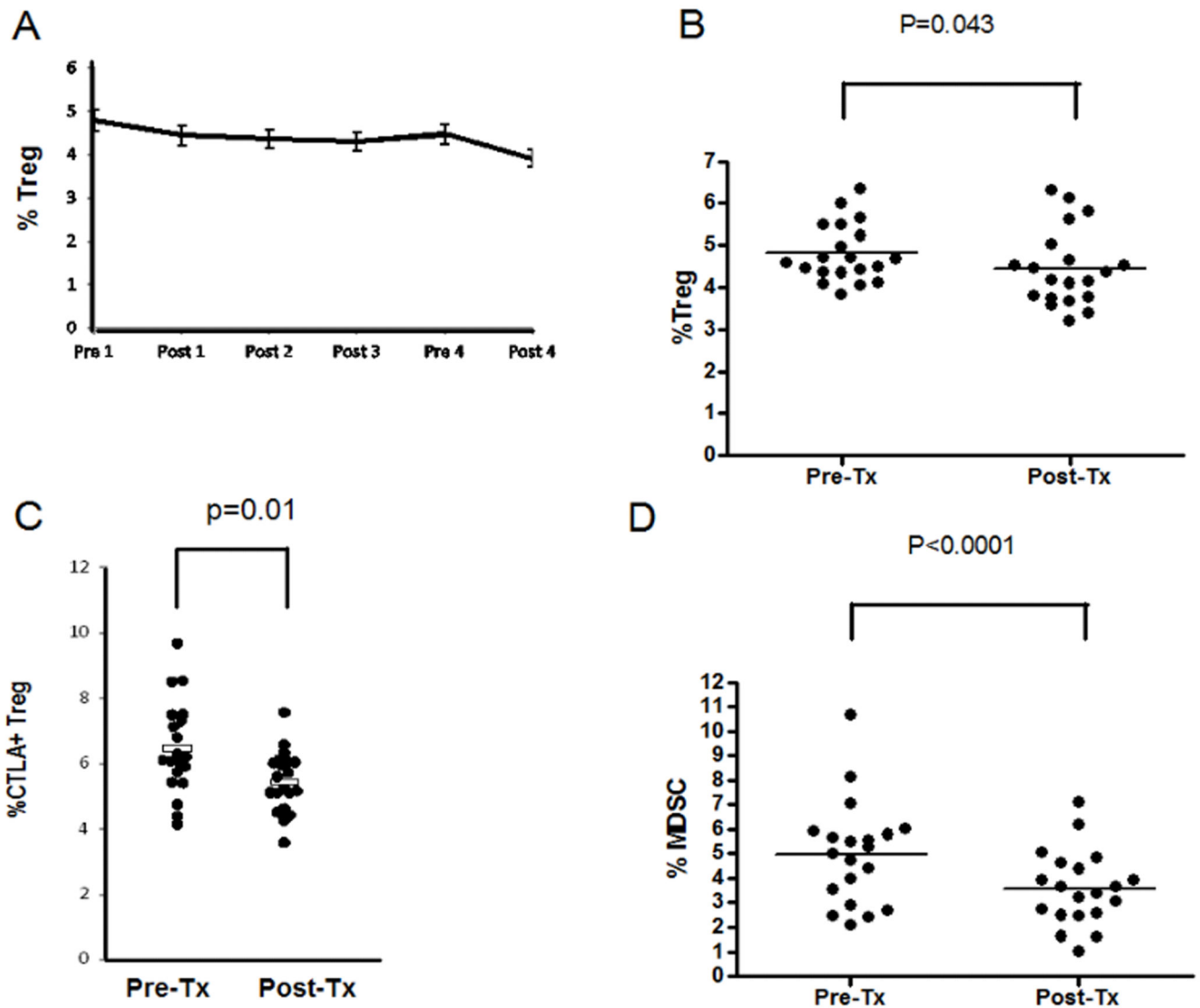


**Figure 3. Progression-free survival (PFS) and overall survival (OS) of vaccinated patients** (A) Kaplan-Meier curves of the percent PFS and OS in months from the time of eligibility determination (n=20). (B) Kaplan-Meier curves of PFS stratified by DTH-positive and DTH-negative research subjects. (C) Kaplan-Meier curves of OS stratified by DTH-positive and DTH-negative research subjects. DTH=delayed-type hypersensitivity.



**Figure 4. Vaccination elicits HER-2-specific CD8<sup>+</sup> T-cell responses in MBC patients as measured by intracellular cytokine staining (ICS)**

(A) HER-2-specific CD8<sup>+</sup> T cells that secrete IFN $\gamma$ , tumor necrosis factor- $\alpha$ (TNF- $\alpha$ ), or interleukin-2 (IL-2) are detectable by ICS in vaccinated patients. A representative patient is shown. (B) The percentage of HER-2-specific CD8<sup>+</sup> T cells that secrete IFN $\gamma$ , TNF- $\alpha$ , or IL-2 increase across the vaccination cycles. The connected points represent the mean and standard deviation of values across the patient population, with three replicates per patient. (C) The proportion of polyfunctional HER-2-specific CD8<sup>+</sup> T cells that secrete more than one cytokine increases across the vaccination cycles.



**Figure 5. Markers of immune suppression in the peripheral blood decrease after vaccination** (A)  $CD4^+FoxP3^+$  Tregs decrease across the vaccination cycles. (B)  $CD4^+FoxP3^+$  Tregs decrease from baseline after one vaccination cycle,  $p=0.043$ . (C)  $CD4^+FoxP3^+CTLA-4^+$  Tregs decrease from baseline after one vaccination cycle,  $p=0.01$ . (D)  $HLA-DR^{neg}/lin^{lo}/CD33^+/CD11b^+$  MDSCs,  $p<0.0001$ .

Table 1

## Patient Characteristics

Characteristic	Number of Patients	%
Total number of patients	22 enrolled 20 vaccinated 20 evaluable	
Age, years		
Median	52	
Range	34-69	
ER-positive or PR-positive tumor	13	65
HER-2-positive tumor	20	100
Metastatic disease at diagnosis	7	35
DFI to relapse (months)*		
Mean	32	
Median	28	
Range	0-130	
Sites of distant metastasis		
Bone	14	70
Liver	5	25
Lung	4	20
Lymph node (distant)	3	15
Brain	1	5
Sum of the longest tumor diameter at study entry		
Mean	40.7 mm	
Median	24.5 mm	
Prior lines of chemotherapy for metastatic disease		
Median	1	
Range	0-6	
Mean	1.25	
Prior trastuzumab therapy		
Adjuvant	3	15
Metastatic	18	90
Prior lapatinib therapy	2	10
Concurrent endocrine therapy	11	55

Characteristic	Number of Patients	%
Concurrent bisphosphonate therapy	14	70

Abbreviations: ER=estrogen receptor; PR=progesterone receptor; HER-2=human epidermal growth factor receptor-2; DFI=disease-free interval

\* 13 patients who did not present with metastatic disease at diagnosis

**Table 2**  
**Summary of Treatment-Related Adverse Events**

Adverse Event	All Grades		Grade 3 or 4	
	# Patients	%	# Patients	%
<b>Local vaccine site reactions</b>				
Erythema/induration	20	100	0	0
Pruritus	20	100	0	0
Pain/soreness/tenderness	17	85	0	0
Local rash at/near vaccine site	7	35	1	5
Blister formation	5	25	0	0
Hyperpigmentation	4	20	0	0
Echymosis (bruising)	3	15	0	0
Local edema	2	10	0	0
Vaccine site flare	2	10	0	0
Groin tightness	1	5	0	0
<b>Systemic toxicities</b>				
Fatigue	8	40		
Urticaria	7	35	2	10
Pruritus (distant from vaccine site)	6	30	1	5
Fever	5	25		
Flu-like symptoms/myalgia	4	20		
Lymphadenopathy	4	20		
Abdominal pain	3	15	1	5
Rash (distant from vaccine site)	3	15	1	5
Achiness/malaise	3	15		
Chills	3	15		
Dizziness	2	10		
Anorexia	1	5		
Erythema (distant from vaccine site)	1	5		
Headache	1	5		
Nausea	1	5		
Arm pain	1	5		
Cancer site pain	1	5		
Leg tenderness	1	5		

NOTE: Data are given as any incident per patient, for a maximum of 20 counts per event.