

**Multisite Pilot Phase I HER-2 Pulsed DC Vaccine to Prevent Recurrence for
Patients with HER-2 Driven High Risk Invasive Breast Cancer**

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1.0 Purpose

We have demonstrated that administration of HER-2/neu pulsed DC1 in patients with DCIS and early invasive breast cancer (IBC) results in strong immunologic responses as well as apparent clinical responses during 4 and 6 week neoadjuvant vaccination. Patients with locally advanced breast cancer that have significant nodal disease have significant risk of systemic recurrence. In addition, those patients with recurrence after chemotherapy and trastuzumab have significant risk of additional local or systemic recurrence and may be resistant to trastuzumab. Those patients with HER-2 2+ that are FISH negative that are often ER positive with significant nodal disease are also at high risk for recurrence despite chemotherapy and anti-estrogen therapy. These patients are not classically treated with trastuzumab but may still respond to HER-2 directed therapy but also have diminished immunity to HER-2. All these patients have little additional therapy options. We hypothesize that adding DC1 vaccines pulsed with HER-2, will reduce the risk of recurrence and diminish deaths in these high risk patients. The primary goals of this trial will be to determine the safety and immune activity of HER-2 pulsed DC1 vaccine in patients with high risk HER-2^{POS} breast cancer with significant risk of systemic recurrence. We will also explore the possibility of determining whether circulating tumor cells can be used as surrogate to assess response to vaccination.

2.0 Duration of Study

This study will enroll 18 evaluable subjects over 1.5 years averaging 10 subjects per year. Evaluable implies that a patient received all 6 vaccinations and underwent leukapheresis after the vaccinations for immune response evaluation.

Fifteen subjects were enrolled at the Rowan Breast Center. Approximately 4 subjects will receive their treatment at follow up at Moffitt Cancer Center while remaining subjects will continue to be followed at Rowan Breast Center.

3.0 Subject Recruitment

Women with advanced breast cancer with ≥ 4 or more positive nodes with classic HER-2^{POS} or HER-2 2+ FISH negative, or those with local or systemic recurrence that are rendered NED by any means will be recruited from the clinical practices of the Rena Rowan Breast Center at the Hospital of the University of Pennsylvania and the Breast Center Moffitt Cancer Center.

4.0 Location of the Trial

The subjects will be recruited and followed in the Rowan Breast Center and the Moffitt Cancer Center. Mammograms will be performed at either site. Leukapheresis will be performed in the Apheresis Infusion Blood Donation Center on the 3rd Floor of Ravdin and at Moffitt Cancer Center. Elutriation will be performed in the FDA approved facility in the Blood Bank on 3 Ravdin and at Moffitt Cancer Center. The vaccines will either be prepared in the FDA approved facility, under the direction of [REDACTED], at the Abramson Family Cancer Research Institute on 6 Maloney, or the Vaccine Production Facility in the M2GEN building on the McKinley campus at Moffitt Cancer Center under the direction of [REDACTED] Vaccinations

Pilot Phase I HER-2 Pulsed DC Vaccine to Prevent Recurrence for Patients with HER-2 Driven High Risk Invasive Breast Cancer will be administered in the Clinical Research Center 1 Dulles Building at Penn or at Moffitt Cancer Center..

5.0 Background

5.1 The Problem

Most breast cancer patients, especially those with high-risk HER-2 positive or triple negative breast cancers, live with significant fear of recurrence-even when initial therapy appears successful. Patients with positive lymph nodes or patients with locally advanced HER-2 positive or triple negative cancer treated with neoadjuvant therapy that have residual disease found at surgery, both have significant risk of systemic recurrence⁵. Tragically, there is little to offer these patients in the way of recurrence risk reduction that does not come with significant toxicity or other deleterious side-effects. Even those patients with intermediate expression of HER-2 (2+) are at significant risk for late recurrence⁶. In addition, solid evidence now suggests HER-2 expressing stem cells and disseminated tumor cells can be found in the marrow and metastatic deposits in patients whose primary tumor does not express significant HER-2⁷. Even amongst HER-2 negative patients EGFR (HER-1) or HER-3 pathways are active and phosphorylated HER-2 proteins are identified⁸. These numerous patient populations are therefore in great need of new therapies to prevent outgrowth of distant disease, provided physicians and scientists can develop technologies to eliminate these remaining dormant cancer stem cells that will ultimately reactivate, leading to significant mortality.

Role of Receptor Tyrosine Kinases in Breast Development and Tumorigenesis

To explain why these molecules are important in breast cancer development is to understand their role in normal breast development. These RTK molecules are critically involved in multiple levels of breast development⁹. HER-1 and HER-2 are expressed in early breast bud development especially Her-1 (EGFR) which is required for duct elongation at puberty. HER-2 and HER-3 play particularly important roles in duct elongation and branching during pregnancy and invasion of mammary pads. Hence they are critically involved in normal breast development and lactation. Mutation or assault from carcinogens may cause these molecules to at later time become over-expressed or aberrantly expressed in breast tumor tissue. The expression of these molecules could be a particularly critical site for interactions with viruses, other pathogens, and chronic inflammation causing damage or mutation and over- expression of the HER members. There are examples of identified mutations in HER-1, HER-2 and HER-3 in all phenotypes of breast cancer^{10,11}.

Several murine models have been identified where over-expression of HER family members leads to invasive breast cancer development in mature mice¹². Over-expression of these molecules appears to be sufficient to cause breast cancer development. Evidence is fairly abundant in human tissues that these molecules are expressed in early human breast cancers. We have demonstrated that HER-2 expression in DCIS is associated with finding invasive disease suggesting HER-2 may be progression factor^{13,14}. HER-3 is also expressed in DCIS and we have found that its expression increases with grade of DCIS. HER-1 is least expressed in early breast cancer except in triple negative DCIS and few other instances. HER-1 is expressed in about 30% of the DCIS lesions in BRCA1 mutation carriers going along with its expression in triple negative breast cancer. Taken together these HER family members seem to play an important role in normal breast development and when mutated or over-expressed play a dominant role in early breast cancer development.

Immune Response Impacts Breast Cancer

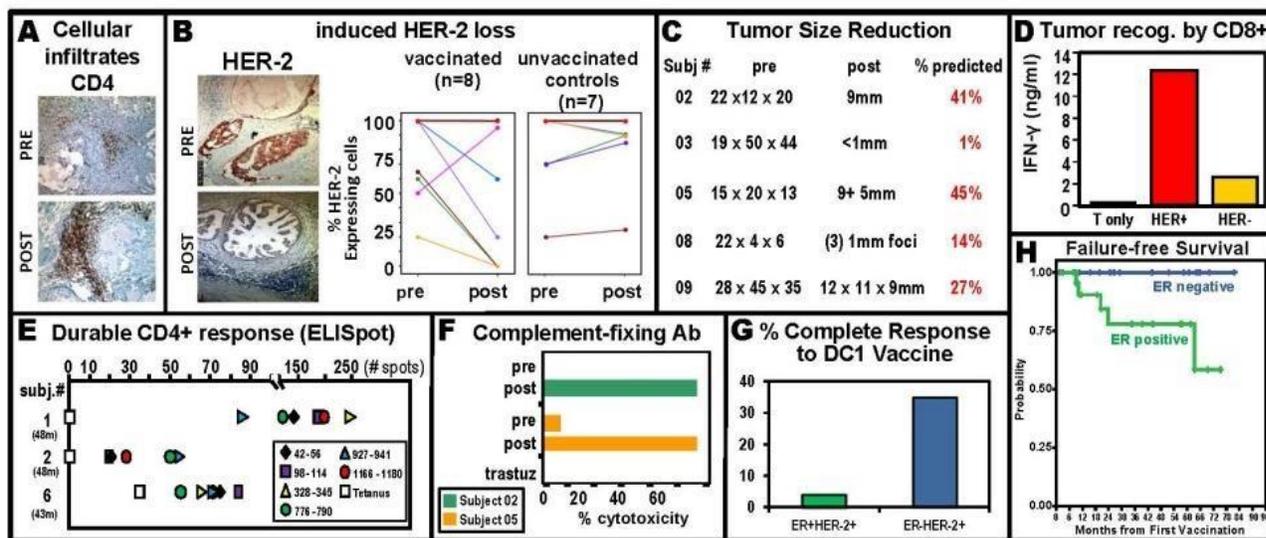
Evidence has demonstrated that infiltration of breast tumors with CD8 T cells results in prolonged survival¹⁵. There is also evidence that accumulation of CD4 and CD8 T cells in regional nodes is associated with better outcomes as well¹⁶. Counteracting this there is an accumulation of macrophages, immature myeloid suppressor cells and regulatory T cells that inhibit the immune response and promote growth and invasion of tumor cells^{17, 18}. Chemotherapies such as cyclophosphamide and docetaxol may eliminate some of the inhibitory cells enhancing immune responses¹⁹. There is evidence that combinations of chemotherapy and immune therapies with antibodies may increase responses such as seen with trastuzumab²⁰. Adding immune therapies especially through vaccines with chemotherapy may enhance the immune response as the inhibitory cells are diminished thus filling the immune space with tumor specific lymphocytes. We will take advantage of chemotherapy to open space in the immune repertoire and vaccinating to add specific anti-HER T cells to eliminate residual microscopic tumor deposits.

5.2 The Solution

The HER family of RTK provide critical signaling for development of breast cancer. HER-1 (EGFR) HER-2 and HER-3 have all been shown critical in normal breast development as well as breast tumorigenesis and invasion⁸. These molecules are over-expressed or mutated in a significant fraction of early and late breast cancer lesions^{13, 21}. These are particularly dominant in HER-2 and triple negative breast cancers²². There is evidence that therapeutic antibodies like trastuzumab and pertuzumab, combined with chemotherapy, can have dramatic impacts in eliminating these primary tumors²³. However, there is also evidence of the molecules being involved in resistance to treatments²⁴. Patients with locally advanced HER-2/neu and triple-negative cancers are at significant risk of systemic recurrence, especially when complete pathologic response is not obtained with neoadjuvant chemotherapy. We believe that targeting these pathways with a cellular immune response (particularly CD4 T cells) may prevent or eliminate dormant tumor cells remaining after therapy. Our preliminary data outlined below highlights an opportunity to potentially increase complete responses and help prevent recurrence in these patients with significant risk of systemic failure following standard therapy. Dendritic cell cancer vaccines combined with chemotherapy may increase complete responses giving breast cancer specific immune cells greater opportunity to function while the immune repertoire is being shifted by chemotherapy to anti-breast cancer response and offer the chance to test secondary prevention of breast cancer in high risk settings. There is a need to determine whether this ICAIT DC1 can activate CD4 and CD8 T cells prior to or in combination with chemotherapy with or without added trastuzumab. This would be a tremendous opportunity to improve outcomes for these high risk patients.

5.3 Preliminary Data

Experience with the vaccine to prevent recurrence: DC1 induces durable Th1-polarized anti-HER-2 responses with apparent clinical activity. A large body of novel and well-founded preclinical and clinical data justifies and supports our expandable platform for breast cancer prevention. We have completed a phase-I clinical trial with 27 subjects to vaccinate against HER-2/neu over-expressing early breast cancer (Ductal carcinoma in situ-DCIS) or DCIS with microinvasion^{3,4}, and are currently undertaking a second phase I/II study to extend these findings.



Subjects diagnosed with HER-2/neu^{pos} DCIS were vaccinated four times at weekly intervals prior to scheduled surgical resection of the tumor. The vaccines consisted of HER-2 peptide-pulsed autologous DC specially activated with IFN- γ and a clinical-grade toll-like receptor agonist (LPS), resulting in high-level IL-12 and Th1 chemokine secretion (DC1). The peptides used to pulse ICAIT DC consisted of 6 HLA class II promiscuous binders, as well as two HLA class I binding peptides (for HLA-A2.1^{pos} subjects). A summary of trial results is shown in the composite Figure 1 A-G. Briefly, Immunohistochemical staining and comparison of pre-vaccine biopsy with post-vaccine surgical specimen showed that vaccination induced heavy influx of CD4^{pos} helper-T cells around DCIS (Fig. 1A), as well as CD8^{pos} CTL and CD20 B cell (not shown). Immunohistochemical staining of HER-2/neu expression by DCIS in the pre-vaccine biopsy (“PRE”) compared with the post-vaccine surgical specimen (“POST”) indicated marked reduction of HER-2 expression in half of the subjects (Fig. 1B). The left panels of “B” show photo microscopy of stained DCIS sections from one representative subject (note loss of brown HER-2 staining in “POST” specimen) while the right panels show composite data of HER-2 reductions from 8 sample subjects as well as unvaccinated controls. No such reductions were observed in an unvaccinated contemporaneous control group (Fig. 1B) suggesting that vaccination could eliminate HER-2-overexpressing tumor cells. A comparison of estimated tumor size determined radiographically prior to vaccination with the actual tumor removed during surgery indicated reductions in the area of disease for about half of the subjects, with results for five of these clinically-responsive individuals shown (Fig. 1C). In fact, five of 27 subjects had no detectable DCIS at the time of surgery, suggesting vaccine-induced elimination of DCIS for some cases. For HLA-A2.1^{pos} subjects, we routinely demonstrated the capacity of post-vaccination CD8^{pos} T cells to directly recognize and secrete IFN- γ in response to HER-2^{pos} breast cancer cell lines *in vitro*. Results from a representative subject are shown (Fig.1D). This is important since CD4^{pos} and CD8^{pos} T cell infiltration in IBC is associated with long-term survival. The immunity generated by this vaccination

regimen proved to be extremely long-lived. Seventeen of twenty-seven subjects voluntarily provided additional blood samples 8-48 months post-vaccination. All subjects showed long-term CD4 T cell immunity to vaccine peptides by ELISpot. Anti-peptide results for subjects 1, 2 and 6 (taken 48, 48 and 43 month's post-vaccination, respectively) without prior stimulation are shown (Fig. 1E). Note that the long-term recall responses to vaccine peptides are often superior in magnitude to the recall response against tetanus toxoid. Despite the fact that this vaccine regimen was not optimized for antibody induction, we nonetheless demonstrated in some patients' sera a vaccine-induced increase in complement-dependent lysis of HER-2/neu^{pos} tumor lines in vitro (Fig. 1F), a property not seen with trastuzumab. Although antibody titers appeared fairly low (not shown), these data provide strong encouragement that antibody responses are present and ripe for dramatic improvement. Of utmost importance in this completed trial and a second follow-on trial now in progress is that we have a composite complete pathologic response rate of about 35% in ER^{neg} HER-2^{pos} (i.e. hormone-independent) DCIS patients compared with 4% for ER^{pos} HER-2^{neg} (Fig. 1G). Since pathologic CR in breast cancer is associated with decreased risk of recurrence and deaths, it is not surprising that we have observed no breast cancer events in the patients that are ER^{neg} HER-2^{pos} compared to the group that is ER^{pos} HER-2^{pos} (Fig. 1G) *we are highly confident that this Th1-polarizing DC vaccine will be associated with diminished secondary recurrence of DCIS and IBC.* We wish to determine whether these vaccines following chemotherapy can induce immune responses and ultimately decrease recurrence in high risk node positive patients.

Clinical experience with DC1 vaccines: The vaccines are readily produced from all 80 subjects thus treated with early breast cancer. There is abundant IL-12 production. There is 85-90% induction of CD4 and CD8 T cells that recognize HER-2 post vaccination. None of these patients had immediately had chemotherapy or trastuzumab. The side effects of vaccine included grade 1 and 2 fevers, chills, fatigue, injection site soreness, headache. There were <10% of patients with asymptomatic declines in MUGA scan all within normal range above 50%. There was no one that developed heart failure clinically or by cardiac ejection. Any grade 1 or 2 changes in cardiac ejection fraction were reverted to baseline in repeat scan 30 days later. Thus the vaccines were considered safe and well tolerated.

Evidence for CD4 T cells that recognize extracellular domain HER-1 and HER-3 in early breast cancer: The majority of DCIS patients even prior to vaccination have evidence of CD4 T cells that secrete INF- γ when stimulated with extracellular domain (ECD) HER-1 (Fig 2 left graph). Those patients that do not have anti-HER-1 response developed a response to HER-1 following vaccination against HER-2 (Fig 2 right graph). There were only a few patients with evidence of HER-3 CD4 T cell responses prior to vaccination but the majority developed suggestion of weak CD4 T cell responses beginning after vaccination (Fig 3). These data support that following HER-2 vaccination using DC1 there is evidence that immune responses against HER-1 and HER-3 can be generated and are ripe for the improvements proposed in this application.

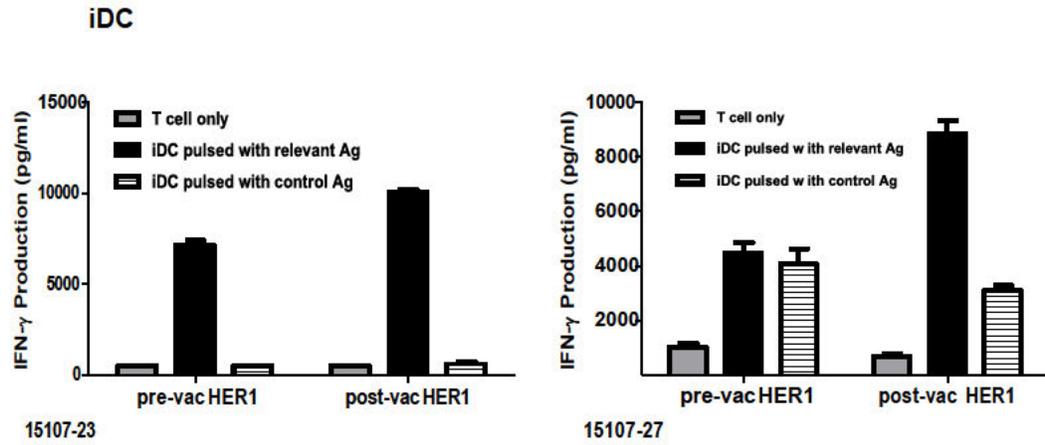
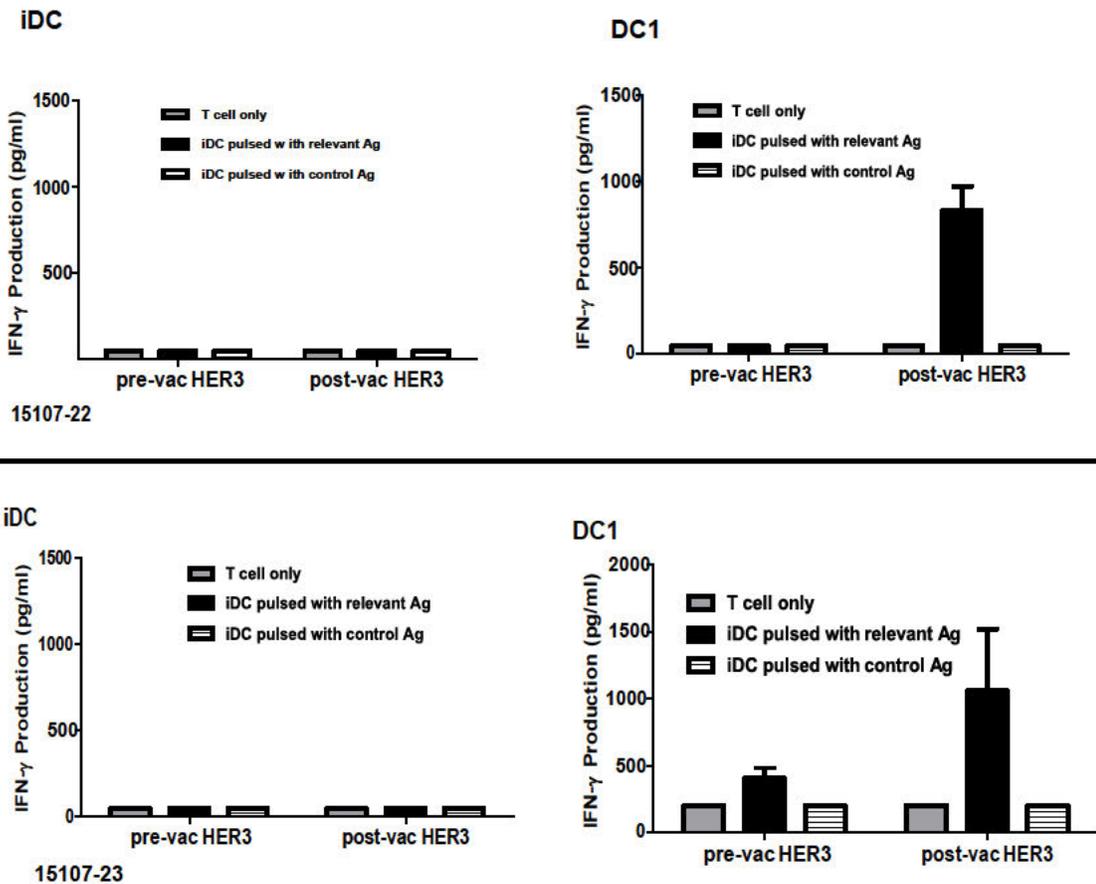


Fig 2. Patients CD4 T cells pre and post vaccine were co-cultured with extracellular domain HER-1 pulsed immature DC for 10 days. After 10 days the T cells were tested against IDC pulsed with HER-1 or irrelevant antigen. Left graph represents patients with pre-existing HER-1 responses and right graph only post vaccine is CD4 T cell responses seen against HER-1.

Fig 3. (Below) Pre and post vaccine CD4 T cells were co-cultured with immature or DC1 pulsed with HER-3 for 10 days. After 10 days they were tested against immature DC pulsed with HER-3 or irrelevant protein. Typically both patients demonstrate post-vaccine HER-3 reactivity when cells were stimulated with only DC1.



Activation of DC1 in patients with IBC: Several patients that were enrolled on the prior Phase I/II DC1 vaccine trials ultimately were discovered to have invasive cancers from 5 mm to 1.6 cm. We were able to prepare DC1 activated with IFN- γ and LPS inducing highly functional DC (not shown). In addition we have been able to prepare DC1 from patient with locally advanced HER-2 expressing IBC following treatment with chemotherapy and trastuzumab (Fig 4). Both Immature and DC1 pulsed with ECD HER-3 were able to stimulate autologous CD4 T to specifically secrete IFN- γ when primed by DC1. There was no evidence of immune response to HER-2 or HER-1 that could be generated by DC1 *ex vivo*. These results have been observed in other patients with IBC and suggest these immune responses to HER-1 and HER-2 can be substantially improved with DC1 vaccines.

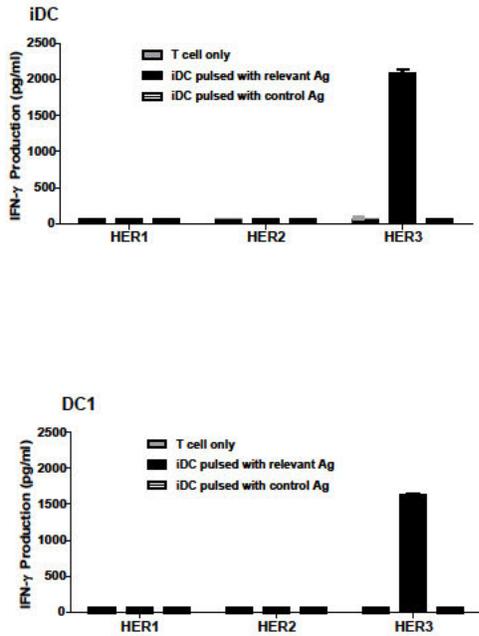


Fig. 4 Patient with HER-2 expressing IBC following treatment with chemotherapy and trastuzumab underwent leukapheresis and CD4 T cells were co-cultured with either immature or DC1 pulsed with extracellular domain HER-1, HER-2 or HER-3. After 10 days cells were tested against immature DC pulsed with HER-1, HER-2 or HER-3 or irrelevant control antigen. There is only evidence of existing HER-3 response there is no evidence of immune response to HER-1 or HER-2. Data shown are for interferon gamma production. Results suggest there is opportunity to improve immunity against HER-1 and HER-2. They also suggest patients can have an immune response against HER-3 with little evidence of adverse effects or autoimmunity.

We have treated 70 patients with this vaccine with only grade 1 and 2 toxicities including expected fever, chill, fatigue, aches, injection site soreness, and grade 1 and 2 decrease in cardiac output all in normal range no abnormal ejections. Repeat 30 days later return to baseline this is about 10% decrease in about 10% of patients treated.

Role of Anti-HER-2 CD4 T cells in Breast Tumorigenesis: We have tested CD4 T cells from healthy donors, benign breast biopsy patients (not shown), HER-2 expressing DCIS patients and patients with HER-2 expressing IBC. There is a progressive loss of anti-HER-2 CD4 reactivity to HER-2 from DCIS through early Stage I breast cancer (Fig 5). Note healthy donors and benign breast biopsy patients have similar results. In addition, IBC patients treated with trastuzumab and chemotherapy do not regain anti-CD4 T cell reactivity to HER-2, but vaccinated Stage I HER-2 expressing patients treated with HER-2 pulsed DC1 vaccines do (Fig 6) suggesting that these vaccines may correct underlying immune defect in CD4 T cells against HER-2.

Patient status

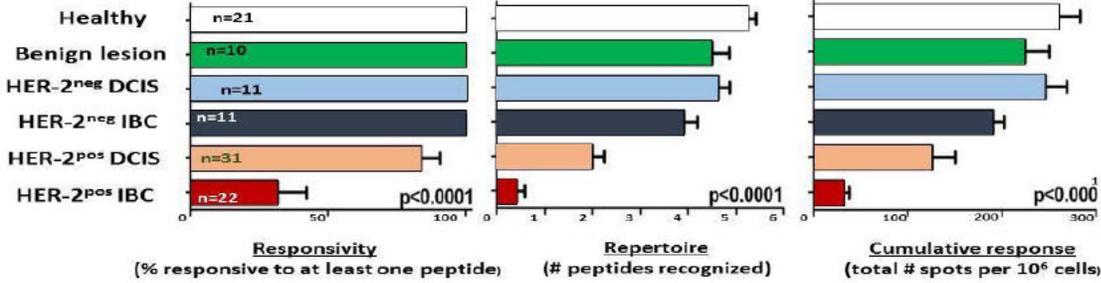


Figure 5. Loss of anti-HER-2 CD4 Th1 response from Healthy donors to HER-2+ DCIS and near complete loss in HER-2 positive IBC

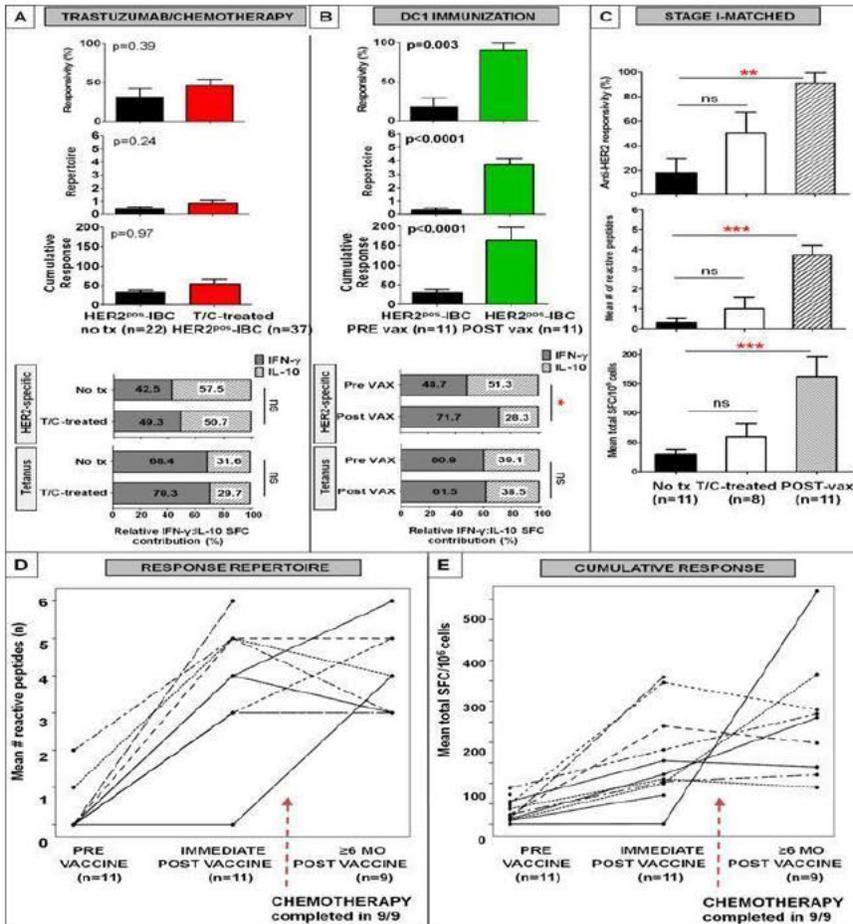


Figure 6. Chemotherapy and trastuzumab do not restore anti-HER-2 CD4 Th1 (A); DC1 Vaccines restore anti-HER-2 CD4 Th1(B); even matched for Stage I only DC1 vaccines restore immune response (C); Anti-HER-2 CD4 Th1 after activation with DC1 remain somewhat intact following chemotherapy (D,E).

6.0 Research Design

6.1 Consent Process

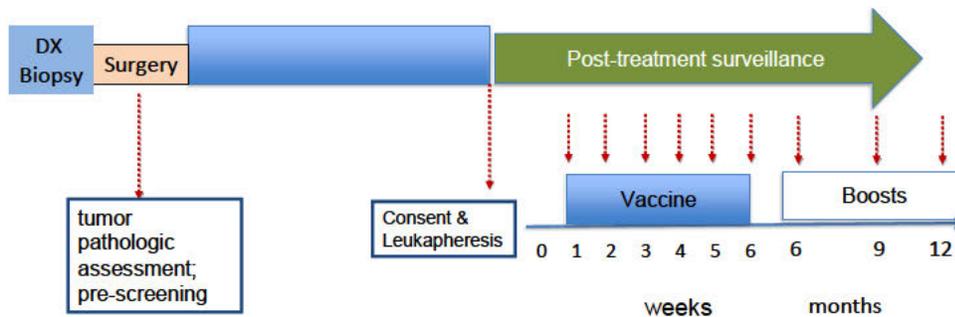
The PIs or their designees will be responsible for explaining all aspects of the initial informed consent to the subject. The person obtaining consent can have the subject sign the consent. The PI and protocol coordinator will review the data and deem the subject eligible. Once the consent is obtained the subject will be assigned a study number or identification.

6.2 Identification of subjects

Subjects with a diagnosis of **standard HER-2/neu-positive IBC** or **HER-2 2+ but FISH negative** with Stage IIIA \geq N2, or have **recurrent HER-2 positive metastatic breast cancer rendered NED** by any means that have completed chemotherapy and/or trastuzumab will be recruited from the Rowan Breast Center. Up to 18 patients will be enrolled in this Pilot Phase I trial. No exceptions to eligibility will be made.

Fifteen subjects were enrolled at Rowan Breast Center.

Study Schema: Adjuvant HER2+



Inclusion Criteria

1. Women \geq 18 years.
2. Subjects with Invasive Breast Cancer at least Stage IIIA \geq N2 ($>$ 4 positive nodes) or have recurrent metastatic breast cancer rendered NED by any means that are classic HER-2 3+ 10%, 2+ IHC and FISH positive or HER-2 2+ FISH negative that have completed chemotherapy and/or trastuzumab and have no evidence of disease.

3. Patients deemed to require anti-estrogen therapy for treatment of their breast cancer can continue anti-estrogen therapy during vaccinations
4. Women of childbearing age with a negative pregnancy test documented prior to enrollment.
5. Patients with ECOG Performance Status Score of 0 or 1 (Appendix D).

6. Women of childbearing potential must agree to use a medically acceptable form of birth control (medically accepted methods: birth control pills, condoms and spermicidal lubricants, intrauterine device, and diaphragm) during their participation in the study.
7. Subjects who have voluntarily signed a written Informed Consent in accordance with institutional policies after its contents have been fully explained to them.

Exclusion Criteria

1. Pregnant or lactating females.
2. Subjects with positive HIV or hepatitis C at baseline
3. Subjects with coagulopathies, including thrombocytopenia with platelet count <75,000, INR > 1.5 and partial thromboplastin time > 50 sec
4. Subjects with major cardiac illness MUGA < 50% EF.
5. Subjects with pre-existing medical illnesses or medications which might interfere with the study as determined by PI.

6.3 Pre-enrollment screening

A. Tumor Characteristics

1. A core biopsy or excisional biopsy demonstrating IBC.
2. A standard clinical HER-2/neu immunohistochemical stain and FISH test will be performed using the Hercept test (Dako) on specimens obtained by core biopsy material or resected tumor.
3. A standard clinical ER, PR test.

B. Baseline patient evaluation

1. All subjects will have a baseline history and physical exam.
2. Laboratory work-up will consist of:
 - a) CBC w/ differential
 - b) PT/PTT
 - c) electrolytes-Na/K/Cl/CO₂/BUN/Cr/glucose
 - d) liver function tests
 - e) urinalysis
 - f) Beta-HCG serum (for all pre and perimenopausal women)
 - g) EKG
 - h) HLA typing (to be obtained as part of study)
 - i) MUGA or Echocardiogram (Must have 50% EF)
 - j) Blood for immune analysis (6 tubes)
3. Baseline radiologic evaluation
 - a) Mammogram up to six months prior to consenting to participate in the study will be acceptable. (Unilateral or bilateral if bilateral mastectomies mammogram not needed)

- b) CT scans of the chest abdomen and pelvis or PET/CT within 60 days of enrollment

C. Performance of the Pre-screening tests

All of the pre-screening tests will be obtained by the patient prior to first treatment.

1. Histopathologic Testing of Tumor

Subjects will have undergone core or resection of primary tumor prior to enrollment in this protocol to confirm diagnosis of IBC. The Hercept (Dako) antibody will be used to analyze the expression of HER-2/neu on the IBC. **Conventional HER-2 3+ 10% of the cells will be considered classic HER-2 positive or 2+ with FISH amplification 2 fold increase in FISH amplicons. For HER-2 2+ FISH negative, ten percent of the cells staining at least 2+ will be considered positive for HER-2/neu expression.**

6.1 Preparation of the vaccine

Preparation of the vaccine is a multi-step process starting with leukapheresis and countercurrent centrifugal elutriation to obtain the relevant monocyte pool. This monocyte pool will then be treated with GM-CSF then pulsed with HER-2/neu peptides and finally matured with INF- γ , and LPS for conversion into type 1 dendritic cells (DC1). When occurring at University of Pennsylvania a study monitor will oversee quality assurance of vaccine production. When occurring at Moffitt Cancer Center, all standard institutional quality assurance policies will be followed.

6.1.1 Subject Leukapheresis

Subjects will first undergo leukapheresis to obtain lymphocyte and monocyte fractions. The leukapheresis will be performed in the pheresis facilities at the University of Pennsylvania or at Moffitt Cancer center by certified personnel. Subjects will be apheresed 15-20 liters whole blood on a Gambro Spectra or equivalent apparatus. We expect to be able to generate $1-5 \times 10^{10}$ PBMC. In the event a subject has poor peripheral access, the option of having an internal jugular line

placed by the interventional radiology division will be offered as an alternative. At the time of the apheresis collection, a tube of serum will be drawn and tested for a baseline anti-HER-2/neu response. This will then be compared with a specimen of serum that will be taken and tested post vaccine.

For subjects in whom leukapheresis did not yield sufficient number of cells to make all 6 vaccines and 3 booster vaccines (1.8×10^9 monocytes), an additional apheresis collection will be undertaken to yield enough cells to manufacturer the remaining vaccines. .

Subjects may additionally undergo leukapheresis (15-20 liters) at the completion of the first 6 vaccines to obtain adequate numbers of the T lymphocyte population for immunologic analysis, as well as to obtain lymphocyte and monocyte fractions to produce 3 booster vaccines. For subjects in whom the initial and second leukapheresis did not yield sufficient number of cells to make all 6 vaccines and 3 booster vaccines, an additional pheresis may be undertaken. At the completion of pheresis all IV access will be removed.

Subjects will have 5mL of peripheral blood drawn at multiple time points during the vaccine schedule (pre vaccine, post six vaccines, pre booster 1, pre booster 2, pre booster 3, and completion of all vaccinations) to monitor immunologic responses to the administered vaccines. Subjects will also have the option to undergo additional leukapheresis (10-15 liters) at the completion of the 6 vaccines or completion of the three boosters to obtain T lymphocyte population for additional immunologic analysis.

6.1.2 Elutriation

To separate the lymphocyte and monocyte fractions, the apheresis product obtained will then be subject to countercurrent centrifugal elutriation on a Model J-6M centrifuge (Beckman Instruments) equipped with a JE-5.0 elutriation rotor operating at 1725g and 20°C) or equivalent apparatus. The apheresis product is loaded onto the elutriator at 120cc/minute flow rate, then fractions are collected using stepwise increases in flow rates to obtain lymphocyte-rich (120 and 140 cc/minute), transitional (150 cc/minute) and monocyte-rich (180 and 190 cc/minute) fractions. A final monocyte-rich fraction is collected at zero rpm (rotor off). Lymphocyte- rich fractions are then further purified with density gradient centrifugation using Ficoll-Hypaque to remove the red blood cells.

The monocyte fraction is washed, counted and aliquoted out into portions for individual vaccines and cryopreserved for use at a later date.

6.4.3. Cell culture and preparation of dendritic cell vaccine (Table 1)

All elutriation will be performed in the FDA-approved facilities in the blood bank. All work here in the production of the monocytes will be performed following strict guidelines for sterility and will be done with Good Laboratory Practices. Ms. Mary Sell, Supervisor in the Cell Processing Center will oversee elutriation at University of Pennsylvania and Dr. Linda Kelly will oversee elutriation at Moffitt Cancer Center. The vaccine activation will take place in the FDA approved facility in the Clinical Cell and Vaccine Production Facility (CVPF) in Maloney 6 of the Hospital of the University of Pennsylvania, or at the Cell Therapy Facility (CTF) at Moffitt Cancer Center, under Good Manufacturing Practices. The CVPF and CTF are both accredited by the Foundation for the Accreditation of Cellular Therapy (FACT). [REDACTED], will oversee vaccine production and [REDACTED] will be responsible for release of vaccine for clinical delivery at University of Pennsylvania. [REDACTED] has 20 year's experience with preparing clinical grade DC vaccines. [REDACTED] will oversee DC1 vaccine manufacturing at the Moffitt Cancer Center.

For those subjects transferring from University of Pennsylvania and receiving vaccines at Moffitt Cancer Center, elutriated product will be shipped cryopreserved and monitored using standard shipping methods already in place at the Cell Therapy Facility at the Moffitt Cancer Center. Those elutriated monocytes will be stored cryopreserved and activated into DC as described below. Any subjects newly enrolled at Moffitt Cancer Center will have elutriated product generated in the Cell Therapy Laboratory at Moffitt Cancer Center. Any subject receiving vaccine or boost at Moffitt cancer center will be a registered patient of Moffitt Cancer Center.

Monocytes will be washed 2 times in Hanks Buffer. Monocytes will be cultured with GM-CSF, IL-4, IFN- γ , and LPS for conversion into DC1. Monocytes at concentrations of 3×10^6 cells/ml will be cultured in serum free medium (Gibco BRL, Grand Island, NY) with 50 ng/ml (250 IU/ml) rhGM-CSF, and IL-4 1000 u/ml (R&D Systems, Minneapolis, MN) and 20 ug/ml HER-2/neu peptides will be added to the culture on the morning of day 2. If, after thawing the cryopreserved cells, there is less than 85% cell viability, the exact number of cells needed for vaccine production will be cultured for one extra day in rhGM-CSF and vaccine administration will be moved back one day. Recombinant HER-2/neu peptides **3 ECD** p42-56 (HLDMLRHLYQGCVV), p98-114 (RLRIVRGTQLFEDNYAL), p328-345 (TQRCEKCSKPCARVCYGL) and **3 ICD** p776-790 (GVGSPYVSRLGICL), p927-941 (PAREIPDLLEKGERL) and p1166-1180 (TLERPKTLSPGKNGV) (American Peptide Co, Sunnyvale, CA) will be pulsed on day 2. The peptides will be pulsed in separate wells 2-3 wells per peptide to avoid competition for binding in the MHC. The evening of day 2 IFN- γ (1000 u/ml) will be added. Six – eighteen hours later LPS (10 ng/ml) will be added and the cells cultured an additional 2-8 hours. For HLA-A2+ subjects, one hour prior to harvest, pulse DC1 in addition to above with 50ug/ml of HER-2/neu class I binding peptides into one well of each ECD and ICD with two different peptides (369-377, 689-697). For HLA-A3+ subjects, one hour prior to harvest, pulse DC1 in addition to above with 50ug/ml of HER-2/neu class I binding peptide into one well of each ECD and ICD with one peptide (369-377). In the event that HLA-A tissue typing results that have not been completed in time for the HLA-A2 peptide addition, will have the peptides added to the culture under the principle that if said patient is not HLA-A2+ or HLA-A3+ the peptides will not be able to bind properly and be washed away during the harvest of the vaccine. The cells will then be harvested from the plates, washed three times with Hanks Buffer, counted and pooled. Viability will be determined by trypan blue exclusion. About 2 to 2.5 million DC1 from each peptide specificity will be pooled so that a total vaccine number of about $1.0-2.0 \times 10^7$ cells resuspended in 20cc sterile saline. From this 20cc 5cc will be removed for gram stain, sterility testing, endotoxin testing and FACS analysis. These tests will be performed with this 5cc solution. The remainder of the cells, about 10-20 million cells, will be centrifuged and the pellet resuspended in 1ml for administration. The final vaccine solution will be labeled with the subject's name, date and time prior to release. The vaccine will be administered shortly after preparation. Long term, > 4 hour, storage of the vaccine is not anticipated. Short term storage of the vaccine at 4⁰C will be accommodated for 4-6 hours after which the vaccine will be discarded if not used. The vaccine prepared will be stored in the administration syringe 1cc tuberculin syringe at 4⁰C and will be directly administered to the subject nodes. A 22 or 23 gauge needle will be used for injection to prevent cell lysis.

A sample of each vaccine will be sent for endotoxin testing, gram stain, and Bac Tec and broth sterility cultures. An aliquot of the clinical product will be submitted to the Department of Microbiology or to the Cell Therapy Quality Control Lab at Moffitt Cancer Center and they will prepare a smear for gram stain testing. In addition, two tubes (or BacTAlert blood culture bottles at Moffitt) will be inoculated and this will be sent to the Department of Microbiology or to the Cell Therapy Quality Control Lab for sterility testing A negative gram stain of the

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vaccine sample will be

required in order to clear the vaccine for subject administration. The endotoxin test will be performed according to Good Laboratory Practices in the CVPF. **The results of endotoxin testing and microbiology cultures will be checked when available and if not meeting specifications the vaccine will be discarded. An endotoxin level of less than 5 EU/kg body weight will be accepted.** Sterility testing will be performed in accordance with CFR 610.12 and a specification of no growth will be required. A final quality analysis certificate will be issued for each dose of vaccine administered. (Appendix B). Additionally, cell viability of the prepared vaccine will be determined using trypan blue staining. The results will be available prior to vaccine administration. Cell viability of >70% will be required for release of vaccine. If this cutoff is not met, then the vaccine will be re-made. In addition, a sample of each vaccine will also be removed for FACS analysis of cell surface markers to confirm conversion of monocytes to DC. Since the activity of the vaccine does not depend on phenotype the vaccine will not be held for phenotype but will serve as an internal evaluation of the process of vaccine preparation (see below).

The requirements for vaccine release then are:

Viability	>70%
gram stain result	no organisms
endotoxin test	<5EU/kg subject weight
culture and sensitivity	no growth final culture

Since the culture test will become available after vaccine administration the following contingency plan will be in place for a positive culture:

- Notification of both the primary attending physician and the subject.
- Identification of the responsible microorganism and sensitivity testing.
- A description of additional subject monitoring as required by the circumstances of the contamination incident.
- Initiate an investigation of the event and take corrective actions to prevent future recurrences. The scope of the investigation and proposed corrections should include:
 - Evaluation of your current procedures for collecting and processing the autologous peripheral blood product to determine the step at which contamination could be introduced.
 - Development of changes in the procedures that will assist in preventing sterility lapses in the future.
 - Development of appropriate testing to ensure that revised changes to the procedures produce a sterile product. This should include sterility testing at various points in the preparation of the product.
- Report the incident as an adverse event to the agency within 15 working days.

The first vaccine may be made from freshly collected and elutriated or cryopreserved cells. Subsequent vaccinations will be made from cryopreserved aliquots. At the University of Pennsylvania, once thawed, these cells will be washed with medium twice prior to their placement in culture. At Moffitt Cancer Center, an individual vial will be removed from liquid nitrogen and allowed to thaw to room temperature. Once vial is fully thawed, the vaccine will be transferred from the vial into a syringe for injection. The thawed vaccine will be stored at a cool temperature, around 4 degrees C.

6.4.4 Quality assurance of DC1

Surface markers on monocytes cells after treatment will be analyzed by FACS to in select products to determine whether they have converted to dendritic cells. After culture activation a small quantity of DC will be harvested for FACS analysis. Cells will be stained with FITC labeled antibodies against human cell surface markers CD14 and PE labeled antibodies CD80, CD86 and CD83. Two color analyses will be performed on a Beckman FACscan with appropriate isotype matched control antibodies. Dead cells will be gated using propidium iodide. This facility is available through the Abramson Cancer Center at the University of Pennsylvania. The presence of the monocytic marker CD14, costimulatory molecule B7.1 and B7.2, MHC Class I and II and the expression of accessory molecules, CD40 and CD54 will be assessed using fluorescent multicolor flow cytometry. The conversion from monocytes to dendritic cells will be confirmed if the cells are CD80, CD86 and CD83 positive and have downregulated CD14. Flow cytometry will not be available for analysis until after the vaccine administration. The most useful measure of DC1 function will be a sample and will be analyzed for IL-12 production by standard ELISA. We utilize ELISA reagents purchased from Pharmigen according to the manufacturer's recommendation to measure secreted IL-12.

Table 1: Preparation of Dendritic Cell Vaccines: Summary of Procedure

1. Suspend monocytes in serum free medium (SFM) to concentration of 3×10^6 cells/ml. Plate 1cc of cell suspension/well in 64 well plate for a total of 12-18 wells (60-80 at Moffitt). Cells plated in numbers higher than actual dose to compensate for 50-75% retrieval rate with harvesting.
2. If monocytes cryopreserved, thaw, add 20 cc Hanks Buffer and spin at 1200 rpm for 5 minutes at 22°C . Aspirate supernatant and resuspend pellet in 10 cc medium. Remove $20\mu\text{l}$ for cell count. Use trypan blue to exclude dead cells from cell count. Add 10 cc Hanks Buffer and spin again at 1200 rpm for 5 minutes at 22°C . Resuspend to concentration of 3×10^6 cells/ml in SFM and plate as above.
3. Add rhGM-CSF for final concentration of 50 ng/ml or 250 IU/ml and IL-4 1000 u/ml (see IV Preparation of Reagents). Culture overnight. If, after thawing the cryopreserved cells, there is less than 85% cell viability, the exact number of cells needed for vaccine production will be cultured for one extra day in rhGM-CSF and vaccine administration will be moved back one day.
4. The next morning, add HER-2/neu peptides for a final concentration of $20\mu\text{g/ml}$ 3 ECD - 56, p98-114, p328-345 and 3 ICD p776-790, p927-941 and p1166-1180 These will be pulsed in separate wells.

5. In the afternoon, add IFN- γ 1000 u/ml, culture cells at 37° C and 5% CO₂ overnight for 14 hours.
6. The following morning, add LPS (10 ng/ml)
7. Incubate 2-8 hours.
8. For HLA-A2+ subjects, one hour prior to harvest, pulse DC1 in addition to above with 50 ug/ml HER-2/neu class I binding peptides into one well of each ECD and ICD with two different peptides (369-377 and 689-697). For HLA-A3+ subjects, one hour prior to harvest, pulse DC1 in addition to above with 50ug/ml of HER-2/neu class I binding peptide into one well of each ECD and ICD with one peptide (369-377). In the event that HLA-A tissue typing results have not been completed in time for the HLA-A2 peptide addition, will have the peptides added to the culture under the principle that if said patient is not HLA-A2+ or HLA-A3+ the peptides will not be able to bind properly and be washed away during the harvest of the vaccine.
9. Harvest cells into 50cc Falcon tubes. Maximum 20 cc cell volume and add 20 cc Hanks Buffer and spin at 1200 rpm for 5 minute @ 22° C. Repeat this step, wash the cells again.
10. Aspirate supernatant. Remove 20 μ l for cell count to estimate % cell death. Pool 2-2.5 million cells per each peptide and respin cells at 1200 rpm, 22° C for 5 minutes. Resuspend in 20cc sterile saline. Remove 12-18 million cells, about 15cc, for vaccine preparation and from the 5cc remaining the tests of endotoxin, sterility testing, gram stain and FACS (see 6.5.4 Phenotypic Analysis of DC1) will be obtained. The vaccine component will be centrifuged and resuspended to 1cc with a total cell number 1.0-2.0 x 10⁷ cells.
11. Label with subjects name, date and time. Release for administration if cell viability >70% and gram stain no organisms seen Endotoxin testing must be less than 5 EU/kg body mass for administration. At University of Pennsylvania, some of the tested material will be reserved so that, in the event a contaminant is discovered in the process of sterility testing, we will have individual components to test so to determine the exact point of contamination.
12. Remake the vaccine if cell viability <70%, positive gram stain, or endotoxin level >5EU/kg. At Moffitt the vial will be removed from liquid nitrogen and allowed to thaw at room temperature. Once vial is fully thawed the vaccine will be removed from the vial and transferred into a syringe for injection. The vaccine will be kept at a cool temperature, around 4 degrees C, until administration

6.4.5 Pharmacologic Information and Preparation of Reagents

A. HER-2/neu ECD and ICD peptides

Recombinant HER-2/neu peptides **3 ECD** p42-56 (HLDMLRHLYQGCQVV), p98-114 (RLRIVRGTQLFEDNYAL), p328-345(TQRCEKCSKPCARVCYGL) and **3 ICD** p776-790 (GVGSPYVSRLGICL), p927-941 (PAREIPDLLEKGERL) and p1166-1180 (TLERPCTLSPGKNGV) will be made at American Peptide Co. Sunnyvale, CA. These peptides

will be >95% pure and certificate of analysis will be provided for each of the 15mers. The peptides will be stored lyophilized and reconstituted in sterile PBS for use. Sterility tests will be performed on a sample of each shipment of peptides delivered to the University of Pennsylvania.

B. HER-2/neu MHC class I binding peptides

Three MHC class I binding peptides 369-377, 971-979, and 688-695 will be synthesized at United Biochemical Research, Seattle, WA or BACHEM, Torrance, CA. These peptides will be >95% pure and certificate of analysis will be provided for each of the 14mers. The peptides will be stored lyophilized and

reconstituted in sterile PBS for use. These peptides will be used in addition to the above peptides for the subjects that are HLA-A2+ and HLA-A3+.

C. Cytokines

1. GM-CSF (NSC# 613795)

Granulocyte macrophage colony stimulation factor (GM-CSF, Leukine, Sargramostim, Immunex Co, Seattle, WA) is available as a sterile, white, preservative free lyophilized powder in vials containing 250 µg GM-CSF (1.25×10^7 Units) or 500 µg GM-CSF (2.5×10^7 Units) with 40 mg mannitol, USP, 10 mg sucrose, USP and 1.2 mg tromelhamine, USP. Each dosage form is supplied individually or in a package of 10 single use vials. GM-CSF should be reconstituted with 1cc sterile water, USP (without preservative). Reconstituted GM-CSF is a clear, odorless, isotonic solution, pH 7.4 ± 0.3 . The single use vials should not be reused.

GM-CSF is further diluted in 0.9% NaCl, USP (or Hanks, at Moffitt) to a concentration 1 µg/ml and used in cultures at a final dose of 10 ng/ml (50 IU/ml).

2. rhIFN- γ

rhIFN- γ will be purchased from Actimmune (Brisbane, CA) or R&D Systems, Minneapolis, and provided as a lyophilized powder. It will be stored according to manufacturer's recommendation, reconstituted in serum free media when appropriate, and diluted in sterile aliquots for addition to cultures.

3. LPS (NSC # 67801)

At University of Pennsylvania, Clinical Grade LPS will be obtained from Dr. Anthony Suffredini at the NIH supplied in sterile saline. It will be aliquotted in sterile containers and added to the cultures as specified. It will be stored at 4° C. At Moffitt Endotoxin Reference Standard LPS will be obtained from the USP and provided as lyophilized powder. It will be stored according to manufacturer's recommendation, reconstituted in sterile water, and diluted in sterile aliquots for addition to cultures.

4. IL-4

Recombinant human IL-4 will be purchased from R&D and will be stored frozen until aliquotted. A certificate of analysis will be obtained. It will be added to cultures at 1000 units /ml final concentration.

The Investigational Pharmacy at the University of Pennsylvania Hospital or the Cell Therapy Facility at Moffitt will order and aliquot and disperse all clinical reagents including peptides, GM-CSF, IL-4, INF- γ , and LPS.

6.4.6 Administration of the Vaccine

A. Selection of Lymph nodes for vaccination

Ultrasound will be used to identify non-pathologic lymph nodes in the groin as first choice or axilla as second choice. Non-pathologic nodes by ultrasound criteria have a fatty hilum and will

be selected; two nodes per vaccine preferentially 1 node per groin. If a patient has lymphedema or prior groin dissection on a single side then the contralateral groin will be used or alternatively the axilla. Often we only are able to identify 1-2 nodes in the groin by ultrasound. Nodes without a fatty hilum that have evidence of pathologic disease will be excluded from injection.

B. Administration of the initial Vaccine

Vaccines will be labeled (for example, with the subjects study ID, study initials, date and time) prior to release. The patient will be seen by the PI or designee, and Research Coordinator and a history and physical exam will be performed. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes.

C. Post vaccine assessment

Subjects will be administered the vaccine and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will be then discharged. The anticipated complication could be fever. If an adverse reaction is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the principal investigator or his/her designee.

D. Vaccine Schedule

The vaccine schedule is meant to be weekly; however the first 6 vaccines may be given anywhere between 8-21 days apart.

E. Vaccine Tolerance

DC vaccination protocols on humans to date have not demonstrated serious toxicities and we anticipate that subjects on this protocol will also tolerate vaccination without incident. Specifically, our own HER-2/neu peptide pulsed DC1 protocol using this same regimen has been well tolerated. We have therefore designed this vaccination trial as a largely out-patient based protocol. However, should significant toxicities be noted this trial as a result of the additional breast vaccinations, the trial will be amended to increase the period of patient observation accordingly.

F. Administration of Second Vaccine

Between 8 and 21 days post vaccine 1 the subject will return for the second vaccination. Again a history and physical exam will be taken. Any changes in history or physical will be documented.

Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes.

G. Post vaccine assessment

Subjects will be administered the vaccines and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained just prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the monitoring schedule will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged patient who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

H. Administration of Third Vaccine

Between 8 and 21 days post vaccine the patient will return for the third vaccination. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes.

I. Post vaccine assessment

Subjects will be administered the vaccine and observed All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately .A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment as necessary. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designees.

J. Administration of Fourth Vaccine

Between 8 and 21 days post vaccine the patient will return for the fourth vaccination. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist

or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes

K. Post vaccine assessment

Subjects will be administered the vaccines and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

L. Administration of Fifth Vaccine

Between 8 and 21 days post vaccine the patient will return for the fifth vaccination. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee.

Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes

M. Post vaccine assessment

N. Subjects will be administered the vaccines and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained just prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

Administration of Sixth Vaccine
Between 8 and 21 days post vaccine the patient will return for the sixth vaccination. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee.

Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes.

O. Post vaccine assessment

Subjects will be administered the vaccines and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designees.

P. Administration of Additional Boost Vaccines.

After the subject has completed 6 vaccines, they will undergo leukapheresis to obtain lymphocyte and monocyte fractions, if needed.

Q. Administration of First Boost Vaccine

Approximately 3 months from completion of the induction vaccines, subjects will receive 3 boost vaccines at approximately 3 month intervals. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes

R. Post vaccine assessment

Subjects will be administered the vaccine and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

S. Administration of Second Boost Vaccine

Approximately 3 months from completion of the first boost vaccine Subjects will be administered 2nd boost vaccine. Subjects will first have blood drawn for immune assessment of the boost this will be 5 green top and 1 serum tube. Again a history and physical exam will be

taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes.

T. Post vaccine assessment

Subjects will be administered the vaccines and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

U. Administration of Third Boost Vaccine

Approximately 3 months from completion of the second boost vaccine Subjects will be administered 3rd boost vaccine. Again a history and physical exam will be taken. Any changes in history or physical will be documented. Ultrasound (US) guided intranodal delivered vaccines will be administered in the NIH Clinical Research Center at Penn or the Clinical Research unit at Moffitt Cancer Center by a radiologist or sonographer experience in ultrasound guidance and the principal investigators or his/her designee. Each dose will consist of between $1.0-2.0 \times 10^7$ cells and will be injected into 1-2 different normal groin lymph nodes or axillary nodes

V. Post vaccine assessment

Subjects will be administered the vaccine and observed. All subjects with vaccine related AEs will be followed until the AE has resolved to baseline. Vital signs will be obtained prior to the vaccination and again 15 minutes after the dose is given. Subjects with no noted complications will then be discharged. The anticipated complication could be fever. If an Adverse Event is observed, the subject observation time will be revised appropriately. A physician familiar with the study will be readily available in the event of any complication and such subjects will be transferred to an appropriate care unit for further monitoring and treatment. Similarly any discharged subject who develops symptoms will be promptly re-evaluated and admitted for care if inpatient hospitalization is deemed necessary by the PI or his/her designee.

6.5 Post-Vaccine Procedures

A. In vitro Immunologic testing

Subjects will have 5mL of peripheral whole blood drawn at multiple time points during the vaccine schedule (pre vaccine, post six vaccines, pre booster 1, pre booster 2, pre booster 3, and completion of all vaccinations) to monitor immunologic responses to the administered vaccines. Subjects will also have the option to undergo additional leukapheresis (10-15 liters) at the completion of the 6 vaccines or completion of the three boosters to obtain T lymphocyte population for additional immunologic analysis. The leukapheresis will be performed in the pheresis facilities at the University of Pennsylvania by certified personnel or at Moffitt Cancer Center, if needed. Attempts will first be made to obtain peripheral access for the pheresis protocol. Subjects will be pheresed 15-20 liters whole blood on a Gambro Spectra or equivalent apparatus. We expect to be able to generate $1-5 \times 10^{10}$ PBMC.

B. Post vaccine laboratory studies

In addition to the blood which will be drawn for the above analyses, an extra tube of serum will be drawn to test the subject's anti-HER-2/neu response. After boost vaccines additional 5 tubes of whole blood will be obtained for immune analysis. These results will be compared with the immune response results that will have been obtained prior to vaccination. An alternative post vaccine leukapheresis can be performed as alternative to blood draws.

1. After receiving their vaccines and prior to receiving boost vaccine series, subject will have the following completed:
 - a) CBC w/ differential
 - b) PT/PTT
 - c) electrolytes-Na/K/Cl/CO₂/BUN/Cr/glucose
 - d) liver function tests
 - e) urinalysis
 - f) MUGA or Echocardiogram (Compare to first study)

In addition to the blood drawn for the above analyses, 5 tubes of whole blood will be obtained for immune analysis and tube of serum will be drawn to test subjects anti-HER-2/neu response. These results will be compared with the immune response results that will have been obtained prior to vaccination.

In the event of abnormal symptoms, laboratory monitoring may be done earlier. The laboratory studies and intervals at which they are done will be determined by the PI on a case by case basis.

If significant decline in EF was evident in pre/post vaccine MUGA scan or ECHO, another MUGA scan or ECHO may be performed 1 month post-vaccine, as determined by the PI. Subjects will be closely monitored by the PI, who will determine the need for additional MUGA scan(s) during the 6 month post-vaccine monitoring period on a case by case basis.

C. Long-term Follow-up

After completing the 6 month post-vaccine monitoring period, all subjects will be seen yearly. Mammograms will be obtained at yearly visits if patients have remaining breasts. For

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mastectomy subjects a yearly mammogram of the contralateral breast. This will continue for 5 years.

6.6 Clinical Monitoring

A. Monitor

At University of Pennsylvania, monitoring will be conducted by the PI and the Research Coordinators in accordance with all applicable regulations. Additionally, the PI and RC will meet with the Medical Monitoring Committee as needed to discuss the study and request additional monitoring support as necessary.

At Moffitt Cancer Center, regulatory documents and case report forms will be monitored internally according to Moffitt Cancer Center Monitoring Policies. Monitoring will be performed regularly by the MCC Clinical Monitoring Core for accuracy, completeness, and source verification of data entry, validation of appropriate informed consent process, reporting of SAEs, and adherence to the protocol, Good Clinical Practice (GCP) guidelines, and applicable regulatory requirements.

B. Initial Assessment Monitoring

This serves as early detection and will help identify trouble areas in the study, staff or site early enough in the study to allow corrections which will benefit the study long term. The PI and the RC will meet to review the study data and processes after each treatment (9 total) for the first two subjects. During the initial assessment monitoring, the PI will review all AE/SAE and lab abnormalities with the Radiologist, Medical Oncologist and/or Pathologist (as appropriate). AE/SAEs will be recorded on a study specific Adverse Event form and all SAEs will be reported to the IRB, DSMC, Safety Monitoring Committee, GCRC, FDA, and NIH in accordance with all applicable regulations. All errors will be addressed and corrected at that time and training and study design related issues will be resolved in a timely manner.

C. Ongoing Monitoring

During the course of the trial, safety and data quality monitoring will be performed in an on-going manner by the PI, RC and other applicable study staff. The RC will monitor the data quality every 6 week (s). This includes ensuring that all source documents exist for the data on the case report forms, ensuring all fields are completed appropriately, all corrections are done according to GCPs and any inconsistencies/deviation are documented. The PI will review the lab results for each patient on an on-going basis and will document his review by signing and dating the lab report. The PI will also review all Adverse Event forms in real-time to ensure appropriateness of the data and timeliness of reporting. All errors will be addressed at the time the monitoring takes place and when the PI reviews the safety data. The PI will also ensure that all renewals are received in a timely manner and all correspondences are documented. Issues related to staff training, protocol design, CRF design and monitoring frequency will be documented along with the appropriate corrective actions. Any issues uncovered will be addressed and corrected immediately.

Moffitt Cancer Center

Regulatory documents and case report forms will be monitored internally according to Moffitt Cancer Center Monitoring Policies. Monitoring will be performed regularly by the MCC Clinical Monitoring Core for accuracy, completeness, and source verification of data entry, validation of appropriate informed consent process, reporting of SAEs, and adherence to the protocol, Good Clinical Practice (GCP) guidelines, and applicable regulatory requirements.

D. Data Quality

At University of Pennsylvania the RC will make sure that all CRFs are completed, all changes to CRFs are made in accordance with regulations, source documents exist for all data points, the consent/re-consent process is clearly documented in the source records, investigational agent accountability is maintain proactively and response is clearly documented. Paper CRFs will be utilized until electronic CRFs are available. After the first 1/3 of the subjects are enrolled, the RC and PI will collectively review the charts of 3 randomly selected subjects to ensure compliance with regulations and this plan. The collective review will again occur with a review of 3 subjects drawn from the next 1/3 of accrual and 3 subjects drawn from the final 1/3 of accrual.

E. Safety Monitoring Committee

At University of Pennsylvania [REDACTED], in the Department of Gynecology, [REDACTED] [REDACTED] in the Department of Medical Oncology will be the Safety Monitoring Committee for this study. Neither [REDACTED] is directly involved in the trial. None of these physicians have any clinical trials in association with Dr. Czerniecki or [REDACTED]. The Safety Monitoring Committee will meet every six months. Two weeks prior to this meeting each committee member will receive a report of all AEs and safety data prepared by the research coordinator. The committee will review all AEs including grading, toxicity assignments, all other safety data and activity data observed in the ongoing clinical trial at this meeting. They may recommend reporting of adverse events and relevant safety data not previously reported and may recommend suspension or termination of the trial. The initial meeting of this committee will be six months after the first patient is enrolled. The final meeting will be six months after the final subject is vaccinated.

F. Study Changes

All changes to the protocol and informed consent will be submitted to the IRB, NIH, FDA and CTSRMC in the form of a formal amendment for approval prior to conducting the study under the proposed changes. If said changes potentially affect the subject's willingness to participate, each subject will be re-consented. Any changes to this monitoring plan will be submitted to the DSMC for approval prior to implementing the changes. In the event of an early study suspension, the PI will immediately notify the IRB, NIH, FDA and CTSRMC.

G. Monitoring Log

At University of Pennsylvania a monitoring log will be completed and maintained to document monitoring by the PI and RC. Moffitt Cancer Center will follow institutional procedures for monitoring of subjects that transfer care to the Center and are newly enrolled at the Center.

H. Auditing

This study will be audited by the Department of Compliance and Monitoring (DOCM) on behalf of the DSMC in accordance with the NCI approved Institutional Data Safety and Monitoring Plan (DSMP).

Moffitt Cancer Center Protocol Monitoring Committee (PMC);

The PMC meets monthly and reviews accrual, patterns and frequencies of all adverse events, protocol violations and when applicable, internal audit results.

Monitoring by the DSMC will be at the following milestones: within 2 weeks of the sixth, or final, vaccination. Two weeks after each boost vaccine and two weeks after completion of post vaccine studies. Monitoring will be conducted on the first three subjects enrolled. The frequency of monitoring of additional subjects (e.g. every other or every third) will be determined after the review of the first three subjects. The frequency of monitoring is subject to change if the DOCM feels it is necessary. The DOCM will be informed when each subject is enrolled, at which time the monitoring visits will be scheduled with the research coordinator.

Moffitt Cancer Center Protocol Monitoring Committee (PMC):

The PMC meets monthly and reviews accrual, patterns and frequencies of all adverse events, protocol violations and when applicable, internal audit results.

6.7 Toxicity and Adverse Event Reporting

A. General Toxicity

The HER-2/neu protein is expressed by the epithelial cells of normal breast, skin, gastrointestinal, respiratory, reproductive and urinary tissues. The HER-2/neu gene is not over-amplified in these normal cells and a level of protein expression is thus expected to be significantly lower than the gene over-amplification and protein over-expression seen in DCIS. In animal models of successful immunity to HER-2/neu protein using HER-2/neu ECD and ICD peptides, no evidence of autoimmunity has been noted. Additionally, in DC trials of melanoma and prostate, no clinical evidence of autoimmunity directed against normal tissues sharing the targeted tumor antigen has been discovered. Therefore, although the risk is presumed to be minimal, the possibility of autoimmune disease directed at these normal tissues cannot be entirely excluded.

This vaccine has not been previously administered to individuals who have been treated with Herceptin. Recent trials of Herceptin, the monoclonal antibody therapy to HER-2/neu ECD, have noted approximately 5% risk of cardiac toxicity leading to heart failure. The mechanism appears to be related to presence of HER-2/neu on cardiac cells. The levels of HER-2/neu expression by cardiac myocytes, however, are extremely low, detectable only by PCR analysis.

Subjects on this trial will have cardiac evaluation of MUGA or echocardiogram to determine their ejection fraction. Subjects will also be closely monitored for signs/symptoms of heart failure. In our first trial there was no evidence of clinically relevant cardiac failure. Less than 8% of subjects had asymptomatic declines in ejection fractions post-vaccine, though all EFs were still within normal range. There have been no long term cardiac toxicities noted as a result of this vaccine. Additionally, Herceptin does not cause long term cardiac toxicity issues. We do not expect the cardiac toxicity of prior Herceptin treatment and treatment with the vaccine to be additive.

It has been demonstrated that fetuses express HER-2/neu during the third trimester and there is the possibility of spontaneous abortion as a result of anti-HER-2/neu T cell responses against the

Pilot Phase I HER-2 Pulsed DC Vaccine to Prevent Recurrence for Patients with HER-2 Driven High Risk Invasive Breast Cancer fetus. Therefore women of childbearing years will be made aware of this possibility.

B. Toxicity Definitions

Toxicity will be graded according to the NCI-CTCAE version 4.02

C. Toxicity Definition for Leukapheresis

Grade 1: Requirement for oral calcium supplementation either during or just after procedure. Severe bruising requiring treatment such as warm compresses or anti-inflammatory medication for a brief 1 week duration.

Grade 2: Prolonged requirement for calcium administration greater than 24 hours, infection at the intravenous site requiring treatment or intervention with antibiotics or surgery.

Grade 3: Alteration in vital signs such as severe hypotension requiring hospitalization, hospitalization secondary to infection.

D. Dose Limiting Toxicity (DLT)

Since the agent being administered is a biologic agent prepared from the subjects own cells as opposed to a single drug we will lump the toxicity together as that caused by the process of preparation of the DC vaccine. A subject that develops any grade 3 or greater toxicity per the NIH Cancer Therapy Evaluation Program Common Toxicity Criteria version 4.03 except for fever greater than 40°C for <12 hours will be taken off protocol. A continuous monitoring rule for dose-limiting toxicity will be followed during the trial, as described in section 6.11 Statistical Considerations.

Grade 2 allergic reaction: generalized urticaria
Greater than or equal Grade 3 allergic reaction
Greater than or equal Grade 2 autoimmune reaction
Greater than or equal Grade 3 cardiac toxicity
Any Greater than or equal Grade 3 hematologic and non-hematologic toxicity.
Greater than or equal Grade 2 toxicity for leukapheresis

E. Adverse Events

Adverse events will be graded according to NCI Common Toxicity Criteria (version 4.03 see <https://evs.nci.nih.gov/ftp1/CTCAE/About.html>). A copy is enclosed in the Appendix.

1. Adverse Event Reporting

All adverse events will whether or not associated with the study agents be recorded on the CRF. Adverse Events will be reported to the IRB, Data Safety and Monitoring Committee (DSMC), Moffitt Cancer Center Protocol Monitoring Committee (PMC); Safety Monitoring Committee, GCRC, FDA, and NIH. The information collected includes:

- The time of onset of any new adverse event or worsening of a previous adverse event

- The specific type of adverse reaction in standard medical terminology
- The duration of the adverse event
- The grade of the adverse event
- The attribution of the adverse event to the study reagents
- Description of action taken in treating the adverse event and/or change in study administration of dose

NCI Adverse Event Reporting for Local, Investigator-Initiated Phase I Trials

UNEXPECTED EVENT		EXPECTED EVENT	
Grade 2-3 Attribution of Possible, Probable or Definite	Grades 4 and 5 Regardless of Attribution	Grades 1-3	Grades 4 and 5 Regardless of Attribution
Grade 2 – Expedited report to within 10 working days	Report by phone within 24 hrs. Expedited report to follow within 10 working days	Adverse Event Expedited Reporting is NOT required.	Report by phone within 24 hrs. Expedited report to follow within 10 working days.
Grade 3 – Report by phone/fax within 24 hrs. Expedited reports to follow within 10 working days.	NOTE: this includes deaths within 30 days of the last dose of treatment.		NOTE: This includes deaths within 30 days of the last dose of treatment.
Grade 1 – adverse event reporting not required			

Adverse events will be reported to the IRB in accordance with their SOPs.

2. Serious Adverse Event Reporting

Deaths that are unforeseen and indicate that other participants or others are at increased risk will be reported to the IRB at the University of Pennsylvania, the Data Safety and Monitoring

Committee (DSMC), Moffitt Cancer Center Protocol Monitoring Committee (PMC); Safety Monitoring Committee, GCRC, FDA, and NIH within 24 hours of the time the investigator becomes aware of the event. Deaths that occur that do not meet the above definition will be reported within 72 hours of the time the investigator becomes aware of the event.

Serious Adverse Events will be reported to the IRB, Data Safety and Monitoring Committee (DSMC), Safety Monitoring Committee, Moffitt Cancer Center Protocol Monitoring Committee (PMC) GCRC, FDA, and NIH.

SAEs will be reported to the IRB promptly within the IRB's guidelines

All on-site SAEs will be sent to Abramson Cancer Center's Data Safety and Monitoring Committee (DSMC) and Moffitt Cancer Center Protocol Monitoring Committee (PMC) according to the following guidelines: all grade 3 or higher events within ten days of knowledge, all unexpected deaths within 24 hours of knowledge, all others deaths within 30 days of knowledge.

All SAE's to the Safety Monitoring Committee and Moffitt Cancer Center Protocol Monitoring Committee (PMC) biannually in a written report including all AE's which will be distributed by intramural mail and email.

All SAE's observed at University of Pennsylvania will be reported to the GCRC promptly, but within three days with a written report of the incident via intramural mail and email.

Serious Adverse Events that are not "unexpected" will be reported to the FDA annually in the Annual Safety Report. SAEs that are drug-related and unexpected will be reported to the FDA within 15 calendar days of discovery of the event via IND safety report. SAEs that are drug-related, unexpected and life-threatening or fatal will be reported to the FDA within 7 calendar days of discovery of the event via IND safety report by telephone or fax.

SAEs will be reported to the NIH promptly, but within three days with a written report via email and mail.

6.8 Immunologic Testing

To ascertain the immunologic efficacy of the prepared vaccines, several parameters will be investigated at the initiation and then again at the conclusion of the four vaccine regimen. These include *in vitro* sensitization assays to determine the development of HER-2/neu CD4⁺ and CD8⁺ T cell precursors. For each subject, immunologic evaluations on pre and post vaccination samples will be conducted simultaneously to minimize discrepancies in technique and culture conditions.

To assess the *in vivo* immunologic effects of our DC vaccine, CD4⁺ T and CD8⁺ T cells will be assessed before and after the vaccine protocol for *qualitative* evidence of HER-2/neu sensitization. In addition T cells from the sentinel node will be tested post vaccination. If this

qualitative assay demonstrates sensitization of T cells to HER-2/neu then a quantitative precursor frequency will be determined by ELISPOT analysis in order to assess the number of HER-2/neu specific T cell precursors prior to and after administration of the DC vaccine.

A. *In Vitro* Sensitization of Autologous CD4⁺ and CD8⁺T Cells (Optional Alternative)

Preparation of Human Lymphocyte Subsets. Pooled cryopreserved 120-140 lymphocyte cell fractions will be thawed, and separated into CD8⁺ and CD4⁺ T cell subsets by negative selection. T cell subset columns (R&D, Minneapolis, MN) will be used to negatively select the appropriate T lymphocyte subset by single pass over a column of glass beads coated with anti-human Ig, anti-Fc receptor antibody (CD 64) and anti-CD8 (when selecting for CD4⁺ T cells) or anti-CD4 (when selecting for CD8⁺ T cells). This method reliably enriches 90-95% for the required T cell subset while depleting the opposite T cell fraction, NK cells, B cells, and monocytes.

***In Vitro* Sensitization Assay.** In a 24 well plate, protein- or peptide- pulsed monocytes will be plated in a 1:10 ratio with either CD4⁺ or CD8⁺ T cells and 7 days later T cell proliferation and cytokine production measured to determine if there is any primary HER-2/neu response. Macrophage Serum Free Medium (Gibco BRL, Grand Island, NY) will be used and all wells treated with GM-CSF 10ng/ml (50 IU/ml). To assess for evidence of CD4⁺ T cell sensitization, 100,000 CD4⁺ T cells will be plated alone, with an equal number of monocytes pulsed with HER-2/neu protein (20µg/ml), with 100,000 pigeon cytochrome c (PCC, 20µg/ml) pulsed monocytes and with 100,000 unpulsed monocytes. As a positive control, CD4⁺ T cells will also be incubated with tetanus pulsed monocytes. All groups will be run in triplicate. After incubation at 37⁰ C for 5 days, thymidine, approximately 1µCi, will be added to all wells. After overnight incubation, the cells will be harvested onto glass fiber filtermats and assayed in a betaplate liquid scintillation counter (Wallac, Gaithersburg, MD) to determine proliferation. Additionally, to assess the cytokine profile, CD4⁺ T cells will be plated in a 48 well plate together with HER-2/neu pulsed monocytes, at a ratio of 1:10 using 500,000 T cells and 50,000 monocytes. After 7 days of coculture, the CD4⁺ T cells will be harvested and replated in new 48well plates at a 1:1 ratio with unpulsed monocytes, or monocytes pulsed with HER-2/neu or pulsed with PCC. After 24 hours, the supernatants will be harvested and tested for production of IL-2, IL-5 (as a representative Th-2 cytokine) and IFN-γ (as a marker for Th-1 type cell) by ELISA (PharMingen, San Diego, CA). CD4⁺ T cells will be considered sensitized to HER-2/neu if the specific proliferation or cytokine production is at least twice that of background levels (i.e. to PCC pulsed monocytes or to unpulsed monocytes).

B. Determination of HER-2/neu specific CD8⁺ T cells

To evaluate CD8⁺ T cell sensitization the CD4⁺ T cell proliferation and cytokine experiments described above will be duplicated, however, with the following modifications. The monocytes will be pulsed with HER-2/neu protein 20 ug/ml and then cocultured with CD8⁺ T cells obtained pre- and post vaccination. The T cells will be cultured for one week and tested against autologous monocytes pulsed with HER-2/neu protein or peptides and irrelevant protein or peptides and IFN-γ production measured by ELISA. T cells generated by such DC1 recognize tumor it is highly likely that the CD8⁺ T cells able to recognize pulsed autologous monocytes can recognize endogenous HER-2/neu on tumor cells. In HLA.A2.1 subjects, which we expect

to be about 25% of the total subjects, monocytes will be pulsed with either the HER-2/neu peptide (369-77) or an irrelevant melanoma and colon peptides. Proliferation of CD8⁺ T cells to the flu peptide will be utilized as the positive control. We will further evaluate CD8⁺ T cells sensitization to HER-2/neu epitopes using HLA.A2.1 positive, HER-2/neu expressing tumor cell lines and negative controls. Proliferation assays and IFN- γ production by CD8⁺ T cells to HER-2/neu positive and negative tumor cell lines will be tested. Subjects not HLA-A2⁺ or HLA-A3⁺ will have their HLA class I typing and attempt to find matched breast cancer cell lines that are HER-2/neu over-expressing and non-expressing to demonstrate specific tumor recognition.

C. ELISPOT Analysis

To *quantitatively* determine whether the levels of circulating, HER-2/neu specific CD4⁺ and CD8⁺ T cells, precursors are increased with vaccination therapy, ELISPOT analysis will be performed on the subjects pre- and post vaccine T cells. As before, CD 4⁺ and CD8⁺ T cells will be obtained from subjects through leukapheresis prior to administration of vaccines and again after all six injections have been delivered and the assays run at the conclusion of the trial. As with the sensitization studies, monocytes are pulsed with protein or peptide and cocultured with T cells at 10:1 T cell to monocyte ratio. After 7 days the T cells will be harvested and restimulated with antigen presenting cells pulsed with relevant and irrelevant protein in ELISPOT plates.

The ELISPOT plate will be prepared by coating Millipore plates with nitrocellulose membranes with 10 ug/ml anti-human IFN- γ or IL-5. The plates will be coated at 4° C overnight and unbound antibody will be removed with washing with PBS. The plates will be blocked with RPMI/10% heat-inactivated human AB serum for 1 hour at 37° C. Harvested T cells 10⁵ will be added in triplicate and HER-2/neu pulsed and irrelevant pulsed monocytes will be added at 1:10 ratio with T cells in a final volume of 200 ul. Cells will be cocultured for 24 hours and cells will then be removed by washing six times with PBS/0.05% Tween 20. Captured cytokine will be detected by incubation for 2 hours at 37° C with biotinylated mAb anti-IFN- γ or anti-IL-5 in PBS/0.5% BSA. The wells will be washed again six times with PBS/Tween, then Avidan-Peroxidase Complex will be added 1 hour at room temperature. Unbound complex will be washed with 3 washes PBS/Tween then 3 washes PBS. Peroxidase staining will be performed with 3-amino-9-ethyl-carbozole for 4 minutes and stopped by placing the plates under tap water. Spot numbers will be determined with an automatic ELISPOT reader that is to be purchased and the accompanying computer software. Spots from well with relevant vs. irrelevant protein pulsed APC will be compared and precursor frequency will be estimated.

6.9 Statistical Considerations

6.9.1 Design. A single arm phase I trial of HER-2 pulsed DC1 vaccine in subjects with high risk HER-2^{pos} breast cancer with residual disease after neoadjuvant therapy. Subjects will receive 6 weekly DC1 vaccinations and may then be eligible to receive up to 3 boost vaccinations given every 3 months. This study will enroll 18 evaluable subjects. Evaluable implies that a subject received all 6 weekly vaccinations and underwent leukapheresis after the vaccinations for immune response evaluation. Subjects will be followed for 30 days after their *final* vaccination.

6.9.2 Objectives. The primary objectives are to determine feasibility and safety. The secondary objectives are to evaluate immunogenicity and anti-HER2 immunity.

6.9.3 Endpoints. Feasibility is defined as a subject's ability and willingness to complete the treatment regimen (6 weekly vaccinations). Safety is established by grading the observed toxicities using CTC Version 3.0. Cardiac toxicity due to trastuzumab will be monitored 2 weeks after the sixth weekly vaccination (week 8) and at EOS and scored separately from vaccine-related toxicity. Cardiac toxicity is defined as either asymptomatic change in MUGA (i.e., >20% decline in EF by repeat echo or EF <50%) or symptomatic EF drop or heart failure. Dose-limiting toxicity (DLT) due to vaccination is defined as: Grade 2 allergic reaction/generalized urticaria, \geq Grade 3 allergic reaction/other, \geq Grade 2 autoimmune reaction, \geq Grade 2 cardiac toxicity or as specified above, \geq Grade 3 hematologic and non-hematologic toxicity (except fever) and \geq Grade 2 toxicity due to leukapheresis. DLT rate will be defined by the weekly vaccination schedule. Toxicities will be scored separately for the maintenance boost schedule. Immunogenicity is evaluated with descriptive statistics and box plots for multiple CD4+ and CD8+ T cell metrics obtained at baseline, 2 weeks after the sixth weekly vaccination (week 8) and after each maintenance boost, as show in the table below. Immune response (IR) is defined as ≥ 2 fold increase relative to baseline. Anti-HER2 response will be a quantitated by dilution studies. Long-term anti-HER2 immunity will be assessed at follow-up visits (e.g., q 6 months).

Immune Parameter	Assay Method	Fold change	Response (>2 fold \uparrow)
Peptide specific reactivities	Tetramer	✓	✓
CD8+ T cell activation or tumor recognition	cytokine release	✓	✓
CD4+ T cell activation	ELISPOT	✓	✓
Overall immune response	(response to any of above)		

6.9.4 Monitoring Unacceptable Toxicity. Early termination rules for asymptomatic and symptomatic cardiac toxicity and vaccine-related dose-limiting toxicity have been defined for monitoring purposes. Toxicity data will likely be reviewed after groups of 3 women have been treated. Enrollment will be terminated if data indicate that the rate of 1) asymptomatic cardiac toxicity >20%, 2) symptomatic cardiac toxicity/heart failure >5% or 3) vaccine-related DLT rate >25%. For cardiac toxicity, we assume an uninformative beta (1, 1) prior and for vaccine-related DLT we assume a modestly informative beta (1, 3) prior since safety of our DC1 vaccine has been established in >50 women treated on our DCIS trials. Given the prior and the observed data, the Bayesian posterior probability that the toxicity rate exceeds the specified rate, is calculated. If the numbers of subjects with toxicity equals or exceeds the numbers (in shaded rows) in the table below, then it is likely that the toxicity rate is unacceptable, as noted by Bayesian posterior probabilities.

Bayesian Early Termination Rules for Cardiac Toxicity Rate				
Subjects treated	3	6	9	12
Subjects with asymptomatic cardiac toxicity	1	2	3	4
Bayesian Posterior Prob[tox rate >20%]	0.82	0.85	0.88	0.90
Subjects with symptomatic cardiac toxicity/heart failure	1	1	1	1
Bayesian Posterior Prob[tox rate >5%]	>0.9	>0.9	0.91	0.86

Subjects with vaccine-related DLT	2	3	4	5
Bayesian Posterior Prob[DLT rate >25%]	0.83	0.83	0.84	0.85
Action	Terminate accrual, re-evaluate trial			

6.9.5 Statistical Analyses and End of Trial Decision-making. Feasibility. A narrative of reasons why subjects could not complete treatment will be written. **Safety.** All graded toxicities will be tabled and described by frequency and percentage. Attribution to DC1 vaccine or trastuzumab will be assigned, if possible. All cardiac toxicities will be reported in detail. Toxicities observed during weekly vaccination will be tabled separately from toxicities observed during maintenance boosts. **Immunogenicity.** Immunogenicity will be evaluated by descriptive statistics, plots of pre- and post-treatment values and fold changes. Immune response rate and 95% exact confidence interval will be calculated. Anti-HER2 response will be quantitated as EOS/baseline fold change in dilution studies.

6.9.6 Sample Size Justification and Study Duration. This phase I trial will enroll 17 evaluable subjects over 1.5 years and will firmly establish feasibility and safety of DC1 vaccination. In addition to Bayesian posterior probabilities for toxicities, binomial probability statements will also inform our decision-making. For example, a treatment may be considered excessively toxic if ≥ 3 of 15 subjects exhibit unacceptable vaccine-related toxicity. This rule yields at least 0.76 probability of correctly judging the treatment as excessively toxic if the true toxicity rate is >25%, with no more than 0.18 probability of incorrectly judging the treatment as excessively toxic if the true toxicity rate is <10%.

6.10 Data Storage

Subject data from this trial will be kept in the offices of the Research Project Manager at University of Pennsylvania or at Moffitt Cancer Center. Data will be recorded on paper CRFs and kept in subject files. These records will be maintained for 2 years after the conclusion of this protocol, at which time they will be archived. Each subject will have a unique subject identifier number.

7.0 Potential Risks

A. Leukapheresis

Leukapheresis will be performed in the blood bank at the University of Pennsylvania by nurses experienced in the procedure or in the Cell Therapies Facility at Moffitt Cancer Center. We have performed the procedure numerous times without any untoward side effects. The risks include hematoma at the intravenous site, and transient hypocalcemia.

B. Vaccination

Administration of HER-2/neu pulsed DC may result in several potential complications. First the administration of activated DC may cause transient hypotension from release of cytokines. This is a theoretic possibility and has not been observed in mouse models or human trials administering DC alone without IL-2 or other cytokines. The second possible complication includes the induction of autoimmune reactions to normal epithelial cells expressing the HER-2/neu antigen. However, in animal models of successful immunity to HER-2/neu protein using HER-2/neu ECD and ICD peptides, no evidence of autoimmunity has been noted. Additionally, in DC trials of melanoma and prostate, no clinical evidence of severe autoimmunity directed against normal tissues sharing the targeted tumor antigen has been discovered. To date, in our own trial using peptide pulsed DC1s for vaccination in subjects with DCIS, the protocol has been well tolerated with toxicities of grade 1 fever, chills, headache, fatigue, and injection site tenderness.

Recent data from studies of Herceptin, a monoclonal antibody targeting HER-2/neu, have reported a small incidence of cardiomyopathy likely related to low level expression of HER-2/neu on cardiac cells. The risk of this complication is estimated at 5%. This toxicity is a result of an antibody and it is anticipated that with low level expression that T cells will be able to discriminate against the heart. In addition since the heart cells are MHC class II negative CD4+ T cells would not interact with these cardiac cells.

This vaccine has not been previously administered to individuals who have been treated with Herceptin. Subjects on this trial will have cardiac evaluation of MUGA or echocardiogram to determine their ejection fraction. Subjects will also be closely monitored for signs/symptoms of heart failure. In our first trial there was no evidence of clinically relevant cardiac failure. Less than 8% of subjects had asymptomatic declines in ejection fractions post-vaccine, though all EFs were still within normal range. There have been no long term cardiac toxicities noted as a result of this vaccine. Additionally, Herceptin does not cause long term cardiac toxicity issues. We do not expect the cardiac toxicity of prior Herceptin treatment and treatment with the vaccine to be additive.

It has been demonstrated that fetuses express HER-2/neu during the third trimester and there is the possibility of spontaneous abortion as a result of anti-HER-2/neu T cell responses against the fetus. Therefore women of childbearing years will be made aware of this possibility.

8.0 Consent Procedure

The nature of the study and the procedures to be performed will be reviewed with the subjects by the Principal Investigator or his/her designee. The subjects will be given ample time to ask questions and decide on participation in the study. A consent form will be signed and kept in the research file and a copy on the patient's chart. Any patient given vaccine or boost at Moffitt will be re-consented using Moffitt Cancer Center consent form.

9.0 Protection of Subjects

Subject records and data sheets will be kept in individual research files. The subject's chart will document participation in the study with the protocol name and number. Under no circumstance

will any medical record or donor information be issued to persons outside this study unless deemed medically indicated. Donor confidentiality will be maintained in any manuscripts or documents that are prepared for scientific publication. All routine safe medical practices applied to subjects with IBC will be used to keep this protocol as safe as possible for the subjects treated. Once IND officially moved to Moffitt Cancer Center and IRB approvals noted records of the trial and data will be transported to Moffitt Cancer Center using secured Aegis Fortress encryption drive to regulatory site at Moffitt Cancer Center.

10.0 Potential Benefits

For subjects enrolled in this trial there is no evidence to suggest any likelihood of benefit. The subjects enrolled have significant risk of systemic recurrence and targeting HER-2/neu may reduce the risk of subsequent recurrence and if this study is safe and generates immune responses would lead to additional trials to test for reducing recurrence.

11.0 Exceptions and Deviations

There will be no exceptions to eligibility, contraindicated treatment/therapies/interventions or safety tests.

An exception is a one time, intentional action or process that departs from the IRB and CTSRMC approved study protocol, intended for one occurrence. If the action disrupts the study progress, such that the study design or outcome (endpoints) may be compromised, or the action compromises the safety and welfare of study subjects, advance documented approval will be requested from the IRB, DSMC, Moffitt Cancer Center PMC and Safety Monitoring Committee.

A deviation is a one time, unintentional action or process that departs from the IRB and DSMC approved study protocol, involving one incident and identified retrospectively, after the event occurred. If the impact on the protocol disrupts the study design, may affect the outcome (endpoints) or compromises the safety and welfare of the subjects, the deviation will be reported to the DSMC and Safety Monitoring Committee within 5 business days and to the IRB within 10 business days.

12.0 Risk/Benefit Ratio

In breast cancer, there remain a significant proportion of subjects who will not benefit from the current adjuvant therapies and relapse with metastatic disease. Immunotherapy offers a new approach to adjuvant therapy and one that may even be applied in prevention of breast cancer. Therefore, the immunologic information gathered from studies such as this one have the potential to impact large numbers of subjects with breast cancer or at high risk for breast cancer. The recent DC trials for melanoma and prostate have demonstrated that such vaccines can be administered safely without adverse effects. Therefore, with minimal risk to the subjects enrolled in this study, information will be gathered that can determine the feasibility of a whole new approach for breast cancer care.

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Pilot Phase I HER-2 Pulsed DC Vaccine to Prevent Recurrence for Patients with HER-2 Driven High Risk Invasive Breast Cancer
Appendix 1 – Schedule of Assessments

Procedures	Baseline / Pre Vaccine	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	Vaccine 5	Vaccine 6	Booster 1	Booster 2	Booster 3	EOT
Consent	X										
Tumor Biopsy ¹	X										
Biomarkers ²	X										
Medical Hx	X	X	X	X	X	X	X	X	X	X	
Vital Signs	X	X ³									
Physical Exam	X	X	X	X	X	X	X	X	X	X	
Height	X										
Weight	X										
CBC w/diff	X							X			
PT/PTT	X							X			
CMP	X							X			
Urinalysis	X							X			
Pregnancy Test	X										
EKG	X										
HLA	X										
LVEF ⁴	X							X			
Immune Assays	X							X	X	X	
ECOG PS	X										
Mammogram	X										
CT/PET	X										
Ultrasound		X	X	X	X	X	X	X	X	X	
AE		X	X	X	X	X	X	X	X	X	X
Leukapheresis	X						X ⁵			X ⁵	

Procedures	FOLLOW UP					
	6 mos.	Y1	Y2	Y3	Y4	Y5
Consent						
Tumor Biopsy ¹						
Biomarkers ²						
Medical Hx	X					
Vital Signs	X					
Physical Exam	X					
Height						
Weight						
CBC w/diff	X					
PT/PTT	X					
CMP	X					
Urinalysis						
Pregnancy Test						
EKG						
HLA						
LVEF ⁴						
Immune Assays	X					
ECOG PS						
Mammogram		X	X	X	X	X
CT/PET						
Ultrasound						
AE						
Leukapheresis	X ⁵					

1. Core biopsy or excisional biopsy demonstrating IBC.
2. Biomarkers collected: ER, PR, HER-2/neu and FISH
3. Collect VS prior to injection and 15 (+/- 5 min) post injection
4. Subject is closely monitored and the need for additional LVEF during the 6 month post-vaccine monitoring period is determined on case-by-case basis.
5. Optional leukapheresis, done only if needed