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Phase I/II Study of Combination Immunotherapy for the Generation of HER-2/neu (HER2) Specific Cytotoxic T Cells (CTL) *in vivo*

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SYNOPSIS

Title	Phase I-II Study of Combination Immunotherapy for the Generation of HER-2/neu (HER2) Specific Cytotoxic T Cells (CTL) *in vivo*
Study Population	Patients with HER2 overexpressing Stage IV breast cancer who are on maintenance trastuzumab alone after being treated with chemotherapy and trastuzumab until there is no evidence of disease (NED) or stable disease. Stage IV HER2 overexpressing ovarian cancer patients who are on maintenance trastuzumab alone or after being treated with chemotherapy and trastuzumab until there is no evidence of disease (NED) or stable disease. Patients must be HLA-A2.
Rationale	Data from tumor vaccine studies now indicate there might be survival advantages for patients who have received tumor-antigen specific vaccinations. Our group has demonstrated a potential survival advantage for patients with advanced stage HER2 overexpressing breast cancer immunized with a HER2 peptide based vaccine after being treated to maximal response or complete remission with standard therapy. Recent studies have demonstrated that "sensitization" of HER2 overexpressing tumor cells with trastuzumab, *in vitro*, will enhance the function of CTL specific for HER2. Theoretically, the mechanism of trastuzumab's enhancement of a HER2 specific CTL response might be the internalization of the HER2 receptor, degradation of the HER2 protein, and increased MHC-peptide presentation with a resultant increase in CD8+ HER2 specific CTL function. Thus, combination of trastuzumab with HER2 peptide based vaccine designed to elicit CTL in the context of HLA-A2 may even further enhance the generation of a HER2 specific CTL response and potentially translate into improved survival for advanced stage breast and ovarian cancer patients when used in the adjuvant setting. This proposal outlines a clinical trial designed to utilize the potential synergistic effect between trastuzumab and a HER2 CTL generating peptide based vaccine (HER2 CTL vaccine) in order to increase CTL precursor frequencies that target HER2 specific epitopes. Patients will be treated to NED or stable disease with chemotherapy and trastuzumab or trastuzumab alone and while on maintenance trastuzumab receive vaccinations with a HER2 CTL vaccine.
Objectives *Primary*:	1. To evaluate the safety of administering a HER2 CTL peptide-based vaccine to Stage IV breast and ovarian cancer patients receiving maintenance trastuzumab. 2. To quantify and characterize antigen specific T cells directed against HER2 in PBMC of patients after vaccination with a HER CTL peptide-based vaccine administered while receiving maintenance trastuzumab.

Secondary:

1. To evaluate overall survival (OS) in Stage IV breast cancer patients who complete a vaccination series with a HER2 CTL peptide-based vaccine while receiving maintenance trastuzumab.

Study Design

This will be a single arm phase I-II single institution clinical trial in patients with HER2 overexpressing Stage IV breast and ovarian cancer who are on maintenance trastuzumab alone after being treated with chemotherapy and trastuzumab or trastuzumab alone to NED or stable disease. Patients will receive a monthly vaccination for 6 months with a HER2 CTL peptide-based vaccine.

Number of Patients

Twenty subjects will be enrolled. This will provide statistically adequate numbers of subjects for gathering (1) safety data and (2) immunologic response data.

Outcome Measures

Primary Endpoints:

1. Safety will be assessed using NCI common toxicity criteria.

2. Immune response will be defined by cytokine flow cytometry (CFC). Specifically, a positive immune response will be defined as a post-vaccination HER2 antigen specific CD8 or CD4 precursor frequency measured by CFC of $\leq$1:20,000. For subjects who have measurable precursors by CFC at baseline, a positive response will be defined as a 2-fold increase in either T cell subset after vaccination.

HLA-A2 MHC tetramer analysis will be used to demonstrate the specificity of the T cells for the HER2 HLA-A2 defined epitopes. IFN-gamma secreting PBMC precursor frequency will also be measured by ELIspot in order to determine the development of lower precursor frequencies not detected by CFC. ELIspot measurements will not define response.

Secondary Endpoints:

1. Overall survival for all patients will be followed. Survival for the Stage IV breast cancer patients will be compared to historical control. This study will not enroll sufficient numbers of subjects to give statistical power to this endpoint. However, a large difference observed between the treatment group and historical control would give additional impetus for a phase II study of efficacy. Although the study will enroll both breast and ovarian cancer patients, from our extensive experience in combined breast and ovarian cancer vaccine studies for HER2, we anticipate approximately 80% of the subjects enrolled will be Stage IV breast cancer patients.

Appendices

Appendix A ECOG Performance Scale

Appendix B Laboratory Assays for Measuring Immune Response

Appendix C Data Safety Monitoring Plan

Appendix D Supplemental HER2 CTL Peptide-Based Booster Vaccine Study

Abbreviations:

APC	antigen presenting cells
BUN	blood urea nitrogen
CBC	complete blood count
CFC	cytokine flow cytometry
CTL	cytotoxic T lymphocytes
DC	dendritic cells
DLN	draining lymph node
DLT	Dose limiting toxicity
DSMP	Data Safety Monitoring Plan
ECD	extracellular domain of HER2
FISH	fluorescent *in situ* hybridization
GM-CSF	granulocyte macrophage colony stimulating factor
HER2	HER-2/neu
ICD	intracellular domain of HER2
id.	intradermal
IFN	interferon
IHC	immunohistochemistry
LC	Langerhans cells
LV	left ventricular
MHC	Major Histocompatability Complex
NED	no evidence of disease
OS	overall survival
PBMC	peripheral blood mononuclear cells
SI	stimulation index
sc	subcutaneous
Th	T helper cells
UA	urinanalysis
UPN	unique patient number

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1. Introduction

The HER-2/neu (HER2) oncogenic protein is a well defined tumor antigen in patients with breast cancer. Monoclonal antibody therapy with agents such as trastuzumab, targeting the HER2 protein, has demonstrated a survival benefit for patients with advanced stage breast cancer. We have completed a phase I study of a HER2 peptide-based vaccine designed to elicit both T helper (Th) and cytotoxic T cell (CTL) responses. In an evaluation of 20 patients the vaccine was safe, was not associated with toxicity, particularly autoimmune toxicity, and was immunogenic. Patients immunized developed T cell immunity directed against HER2. Specifically, HER2 specific CTL from patients post-vaccination lysed HLA-matched HER2 overexpressing tumors. Moreover, the immunization strategy was designed to augment both HER2 specific CD4+ and CD8+ immune responses and, indeed, long-lived immunologic memory was created in a substantial number of the patients vaccinated. Since this initial study, standard of care for advanced stage HER2+ breast cancer patients includes trastuzumab administered with chemotherapy. Furthermore, patients with Stage IV HER2 overexpressing ovarian cancer have received trastuzumab as part of their therapy. For patient's who achieve a complete remission (CR) or stabilization of disease, treatment with trastuzumab alone continues for several months to a year or until progression. For this reason, a HER2 specific vaccine aimed at preventing relapse in stage IV breast cancer patients after standard therapy will have to be given concurrently with trastuzumab. The concurrent administration of trastuzumab with a HER2 CTL vaccine may actually make the vaccine more effective in eliciting HER2 specific CTL responses. The internalization of the HER2 receptor after binding to the antibody would potentially increase presentation of processed HER2 proteins in MHC class I molecules. Indeed, recent studies have shown that pretreatment of HER2 overexpressing tumor cells with trastuzumab makes them more susceptible to lysis by HER2 specific CTL T cell clones. (1)

The proposed clinical trial is a phase I/II study of combination immunotherapy for the generation of HER2 specific CTL *in vivo*. Trastuzumab will be given concurrently with a HER2 peptide based vaccine composed of Th epitopes that encompass within their natural sequence HLA-A2 binding epitopes of HER2. The strategy is to administer a vaccine that will stimulate both CD4+ and CD8+ responses simultaneously. The primary objectives of the study are to evaluate the safety of administering a HER2 CTL peptide-based vaccine to Stage IV breast and ovarian cancer patients receiving trastuzumab as well as to quantify and characterize antigen specific T cell subsets specific to HER2 in PBMC of patients after vaccination. A secondary objective is to evaluate time to overall survival (OS) in patients who complete a vaccination series with a HER2 CTL peptide-based vaccine while receiving trastuzumab.

2. Background

A. HER2 is a tumor antigen in human adenocarcinomas. The HER2 oncogenic protein consists of a cysteine rich extracellular ligand binding domain, a short transmembrane domain, and cytoplasmic protein tyrosine kinase domain. (2,3) Binding of ligand to the extracellular domain (ECD) leads to dimerization that stimulates the intrinsic tyrosine kinase activity of the receptor and triggers autophosphorylation of specific tyrosine residues within the intracellular cytoplasmic domain (ICD). These phosphorylated residues then serve as anchoring sites for signaling molecules involved in the regulation of intracellular signaling cascades (2) and, thus, cell growth. HER2 is a self-protein expressed in a variety of tissues of epithelial origin and plays a fundamental role in cellular proliferation and differentiation during fetal development. In adults, the HER2 gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression is seen in various cancers including breast, ovarian, colon, uterine, gastric, prostate, and adenocarcinoma of the lung. Furthermore, the overexpression of HER2 is implicated in the malignant transformation of breast cancer (4-6) and is a biologically relevant protein in the pathogenesis of several other epithelial-based tumors, for example leading to the development of hormone resistance in prostate cancer. (7) Relapse is a significant clinical problem for breast cancer patients whose tumors overexpress HER2. (8)

HER2 is also a tumor antigen. Patients whose tumors overexpress the HER2 protein can have both low level antibody and T cell immunity directed against the protein. (9) Generating an active immune response directed against the HER2 protein has several potential clinical advantages. Vaccination, if effective, would stimulate immunologic memory and could result in the prevention of relapse after standard therapy.

B. Patients with HER2 over expressing tumors can be safely immunized with HER2 derived peptides and generate a HER2 specific CTL response. The CTL immune response has been considered the primary effector arm involved in mediating an anti-tumor immune response. Experimental vaccination strategies designed to stimulate tumor specific CTL responses *in vivo* vaccinate individuals with tumor cells or viruses recombinant for tumor antigens that can infect viable cells so that proteins are expressed inside the cell, processed and presented in the class I MHC antigen processing pathway. An alternative strategy uses soluble peptide that is identical or similar to naturally processed peptides present in class I MHC molecules as the vaccine along with an adjuvant. HLA-A2 binding peptide p369-377, derived from the protein sequence of HER2 ECD, has been used extensively in clinical trials. In an initial clinical study, HLA-A2 positive patients with metastatic HER2 overexpressing breast, ovarian, or colorectal carcinomas were immunized with 1 mg of p369-377 admixed in incomplete Freund's adjuvant (IFA) every 3 weeks. (10) Peptide specific CTL were isolated and expanded from the peripheral blood of patients after 2 or 4 immunizations. The CTL could lyse HLA matched peptide pulsed target cells but could not lyse HLA matched tumors expressing the HER2 protein. More recently, a similar study was performed immunizing patients with p369-377 using GM-CSF as an adjuvant. (11) GM-CSF is a recruitment and maturation factor for skin dendritic cells (DC), Langerhans cells (LC) and theoretically provides more efficient presentation of peptide epitopes than standard adjuvants such as IFA. HLA-A2 patients with HER2-overexpressing cancers received 6 monthly vaccinations with 500 mcg of HER2 peptide, p369-377 peptide, admixed with 100 mcg of GM-CSF. The patients had either stage III or IV breast or ovarian cancer. Immune responses to the p369-377 were examined using an IFN-gamma ELIspot assay. Prior to vaccination, the median precursor frequency, defined as precursors/10^6 PBMC, to p369-377 was not detectable. Following vaccination, HER2 peptide-specific precursors developed to p369-377 in just 2 of 4 evaluable subjects. The responses were short-lived and not detectable at 5 months after the final vaccination. Immunocompetence was evident as patients had detectable T cell responses to tetanus toxoid and influenza. These results demonstrate that HER2 MHC class I epitopes can induce HER2 peptide-specific IFN-gamma-producing $CD8^+$ T cells. However, the magnitudes of the responses were low, as well as short-lived. Theoretically, the addition of $CD4^+$ T cell help would allow the generation of lasting immunity.

Interestingly, immunizing patients with putative T-helper epitopes from HER2 that have HER2 HLA-A2 binding motifs embedded in their natural sequence resulted in the generation of peptide-specific CTL capable of lysing HER2 overexpressing tumor and immune responses were durable. (12) In that trial, 19 HLA-A2 patients with HER2-overexpressing cancers received the vaccine, twelve patients developed both HER2 specific CD4+ and CD8+ T cell responses, and the level of HER2 immunity was similar to immunity to KLH, a foreign protein used as a control immunization (Fig. 1). Importantly, the immune responses were detectable for greater than 1 year after the final vaccination in selected patients. These results demonstrate that HER2 MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER2-specific IFN-gamma-producing CD8 T cells. Indeed, when a comparison was made between the clinical trial that used a HER2 HLA-A2 binding peptide alone vs. immunizing to achieve CTL using a class II peptide with a class I peptide embedded in the natural sequence, the latter immunization strategy was necessary to maintain both high magnitude and long lived CD8+ T cell immunity (Fig. 2). We will evaluate this vaccine, composed of HER2 T helper epitopes which encompass HLA-A2 CD8+ epitopes of HER2 in combination with trastuzumab to augment HER2 specific CTL T cell immunity in a combination immunotherapy approach.

C. Immunization with a HER2 peptide based vaccine, designed to elicit CTL, is associated with the development of immunologic memory and epitope spreading. Epitope spreading was first described in autoimmune disease (13) and has been associated with both MHC Class I- and MHC Class II-restricted responses. An additional finding of the study described above (12) as well as a larger clinical trial vaccinating patients with a variety of putative class II binding HER2 epitopes (14) was that epitope spreading was observed in 84% of patients and significantly correlated with the generation of HER2 protein-specific T cell immunity ($p=.03$). Epitope spreading represents the generation of an immune response to a particular portion of an immunogenic protein and the natural spread of that immunity to other areas of the protein or even to other antigens present in the environment. (15,16) In this study, epitope spreading reflected the extension of a significant T cell immune response to portions of the HER2 protein that were not contained in the patient's vaccine (Fig. 3). Theoretically, a broadening of the immune response may represent endogenous processing of

antigen at sites of inflammation initiated by a specific T cell response or "driver clone". (17) The initial immune response can create a microenvironment at the site of the tumor that enhances endogenous immune effector cells present locally. These immune cells, e.g. APC and T cells present antigen and create a CTL response to tumor antigen present in the body. It may be that the development of epitope spreading is a better predictor of vaccine efficacy than the magnitude of the immune response generated. (18)

We have recently begun to assess the presence of long term immunity in previously immunized patients. Figure 4 demonstrates persistent detectable HER2 specific immunity and epitope spreading present in a patient over 4 years after receiving her last immunization with the HER2 CTL vaccine. This is one of the first demonstrations of persistent long term immunity developing in a cancer patient after the administration of a cancer vaccine. The mechanisms responsible for the maintenance of memory T cells are not entirely known. Studies have shown that memory cells are more metabolically active than naïve T cells suggesting that memory cells may be continuously signaled by extrinsic factors. Such signaling most likely involves some degree of contact with persistent antigen. As most of the patients in our vaccine trials are advanced stage patients who have been treated to maximal response and invariably have minimal residual disease, it may be that this persistent microscopic tumor stimulates the persistence of immunity. Overall survival in patients with HER2+ Stage IV breast cancer is well defined. (19) A recent trial randomized over 400 stage IV breast cancer patients to receive chemotherapy alone vs. chemotherapy plus trastuzumab as primary therapy for their disease. The median overall survival for the patients receiving chemotherapy alone was 20 months and for those receiving the combination antibody and chemotherapy was 29 months (Fig. 5). Patients from the HER2 peptide based vaccine studies (12) have now been followed a median of 5 years (Fig. 6). Six of the 9 (67%) patients who completed immunization with the CTL vaccine are still alive. The 74% overall survival (OS) Kaplan-Meyer statistic on this subset is limited because of the small number of patients, however, the historical control data for stage IV patients with distant metastasis suggests a benefit to vaccination. Although HER2 overexpressing ovarian cancer patients treated with trastuzumab alone are eligible for this study, based on our previous trials enrolling breast and ovarian cancer patients we anticipate over 80% of patients enrolled will be Stage IV breast cancer patients. Thus, immunization of patients with advanced stage disease who have been optimally treated to prevent relapse is a clinical goal of vaccination with the HER2 CTL vaccine.

D. Addition of trastuzumab to active immunization with a HER2 CTL peptide based vaccine may result in augmented immunity and the generation of a more vigorous CTL response. Treatment of advanced HER2 overexpressing breast cancer has evolved since our first study was initiated. A common regimen for relapsed stage IV HER2 overexpressing breast cancer is treatment with trastuzumab and chemotherapy for 4 months or until maximal response and trastuzumab monotherapy continued for a year. Patients with *de novo* stage IV disease often receive a longer cycle of chemotherapy with continued trastuzumab for 1 year. For this reason, vaccines given in the setting of stage IV disease need to be administered concurrent with trastuzumab if patients are to be immunized in a minimal residual disease state. Trastuzumab, when administered prior to or concurrent with doxorubicin results in a higher incidence of cardiac toxicity than what is anticipated with doxorubicin alone. (20) For patients with minimal prior exposure to anthracycline the risk of cardiac dysfunction, defined as decrease in left ventricular ejection fraction (LVEF), was 1%. For patients with more extensive prior doxorubicin exposure the risk of cardiac dysfunction with trastuzumab was 7%. If trastuzumab was given concurrent with doxorubicin the risk of cardiac dysfunction was 29%. The etiology of the cardiac dysfunction related to trastuzumab administration is unknown, but the temporal relationship to concurrent doxorubicin exposure suggests a link with anthracycline induced myocardial injury. As this will be the first study evaluating the concurrent administration of trastuzumab and active immunization targeting HER2 we will evaluate any potentially toxicities closely with particular emphasis on cardiac status.

There is a good rationale to suggest that trastuzumab concurrent to vaccination may actually augment HER2 specific CTL immunity. Recent studies, by our collaborator, Dr. Helga Bernhard, have demonstrated that "sensitization" of HER2 overexpressing tumor cells with trastuzumab, *in vitro*, will enhance the function of CTL specific for HER2. Lytic activity of HER2 specific CTL derived from unprimed patients has been low, in part, because HER2 is a self antigen and T cells may display a low affinity T cell receptor due to induction of tolerance. (21-23) On this basis, the lytic potential of HER2 specific CTL could, theoretically, be improved by

further increasing the number of HLA class I bound peptides on tumor cells with the help of trastuzumab, an inhibitory antibody against HER2. Upon binding of trastuzumab, the HER2 receptor is internalized and degraded, subsequently inhibiting HER2 mediated signal transduction and tumor cell growth. (24,25) As antibody-induced degradation of HER2 is likely to be accompanied with increased numbers of HER2 peptides presented with HLA molecules, Bernhard and colleagues questioned whether trastuzumab-treated tumor cells were more susceptible to CTL-mediated lysis. HER2 reactive CTL clones lysed class I-matched, HER2 overexpressing tumor cells more efficiently after treatment with trastuzumab (Fig. 7). (1) Thus, combination of trastuzumab with the HER2 CTL vaccine may even further enhance the generation of a HER2 specific CTL response and potentially translate into improved survival for advanced stage breast and ovarian cancer patients when used in the adjuvant setting.

3. Rationale

Data from a phase I study of a HER2 CTL peptide-based vaccine demonstrated the safety of the vaccine when administered as a single immunotherapeutic agent. Moreover, in addition to patients developing antigen specific T cell responses after vaccination, 5-year follow-up of the Stage IV breast cancer patients enrolled on study suggests a potential survival advantage for the patients immunized. Recent studies have demonstrated that "sensitization" of HER2 overexpressing tumor cells with trastuzumab, *in vitro*, will enhance the function of CTL specific for HER2. Thus, a combination of trastuzumab with the HER2 CTL vaccine may even further enhance the generation of a HER2 specific CTL response. Before moving forward with an efficacy study, however, safety of concomitant administration of vaccine and trastuzumab must be demonstrated. This proposal outlines a phase I-II study of a combined immunotherapeutic approach for the generation of HER2 specific CTL *in vivo*.

4. Objectives

A. Primary objectives

1. To evaluate the safety of administering a HER2 CTL peptide-based vaccine to Stage IV breast and ovarian cancer patients receiving maintenance trastuzumab.
2. To quantify and characterize antigen specific T cell subsets specific to HER2 in PBMC of patients after vaccination with a HER2 CTL peptide-based vaccine while receiving maintenance trastuzumab.

B. Secondary objectives

1. To evaluate overall survival (OS) in patients who complete a vaccination series with a HER2 CTL peptide-based vaccine while receiving maintenance trastuzumab.

5. Vaccine Preparation

A. HER2 CTL peptide-based vaccine

Synthetic peptides will be used. The peptides are 14-18 amino acids in length with an amino acid sequence identical to the normal HER-2/neu sequence. These peptides have been evaluated in a previous study (see Background) and are covered by BB IND 6524, Sponsor: Mary L Disis, M.D. The peptides are described below:

Table 1. *Peptides composing the HER2 CTL vaccine*

HER2 CTL vaccine peptide	Sequence	Sequence and location of embedded HLA-A2 binding epitope
p369-384	KIFGSLAFLPESFDGDPA	KIFGSLAFL (p369-377)
p688-703	RRLLQETELVEPLTPS	RLLQETELV (p689-697)
p971-984	ELVSEFSRMARDPQ	ELVSEFSRM (p971-979)

B. Peptide synthesis and characterization

Peptides are manufactured by Multiple Peptide Systems, San Diego, CA. using standard solid phase synthesis techniques employing Boc chemistry. Amino acids and resins for synthesis were purchased from Bachem California and the amino acids were analyzed by TLC and polarimetry prior to use. The peptides were cleaved from the solid support using anhydrous hydrogen fluoride and conventional techniques. Each peptide was

purified by HPLC using a Waters Delta-prep system with a gradient of acetonitrile (containing 0.1% trifluoroacetic acid) in water (also containing 0.1% trifluoroacetic acid). The peptides were lyophilized to dryness before being passed over an anion exchange column to exchange the trifluoroacetate for acetate. GMP procedures were followed for the manufacture of these sequences.

The peptides were characterized by mass spectrometry to confirm their identity, by HPLC for purity using two different elution buffers, and by amino acid analysis for identity and peptide content. The water content of each peptide was determined by the Karl Fischer procedure and the data obtained used to verify the mass balance of the peptides.

C. Peptide formulation and stability

Prior to formulation, the solubility of each peptide in 10 mM sodium acetate buffer (pH 4.0) was determined. Three peptides were made up in a 10 mM sodium acetate buffer (pH 4.0). The stability of each individual peptide in the acetate buffer, as well as the peptide mixtures, is monitored by HPLC and mass spectrometry. The dissolved peptides are stored at -20˚C prior to use. Microbial and sterility testing are conducted to ensure safety.

D. GM-CSF

rhuGM-CSF (Sargramostatin, LEUKINE) will be used as the vaccine adjuvant. GM-CSF is a growth factor that supports the survival, clonal expansion, and differentiation of hematopoietic progenitor cells including dendritic antigen presenting cells. The use of GM-CSF in humans has been associated with minor toxicity. GM-CSF has been given s.c. at the same dose and schedule without noted apparent differences in toxicities. In some studies the toxicity profile for s.c. administration has been more favorable than for i.v. For the current study GM-CSF will be used at a total injection dose of 100 mcg mixed prior to injection with HER2 CTL vaccine and injected i.d. at the time of immunization. Local effects at the injection site are not expected with this dose of GM-CSF. Recombinant GM-CSF (rhuGM-CSF) is generally well tolerated when administered i.v. or s.c. in doses ranging from 50-500 mcg /m^2/day. Severe toxicity is extremely rare in subjects treated with rhuGM-CSF.

Diarrhea, asthenia, rash, and malaise were the only events observed in more than 5% of the rhuGM-CSF group compared with the placebo group in phase III controlled studies of subjects undergoing autologous bone marrow transplantation. In uncontrolled phase I/II studies for various indications, the adverse effects report most frequently were fever, chills, nausea, vomiting, asthenia, headache and pain in the bones, chest, abdomen, joints, or muscles. Most of these systemic events were mild to moderate in severity and rapidly reversed by the administration of analgesics or antipyretics. Other events reported infrequently were dyspnea, edema, local injection site reactions, and rash. Thrombosis and cardiac arrhythmia have also been reported and there have been infrequent reports of tissue sloughing, leukemia progression, congestive heart failure, hepatomegaly, intracranial bleeding, and isolated reports of Guillain-Barre syndrome and increased histiocytes. Neutropenia, although usually a pre-existing condition in the subjects receiving rhuGM-CSF, has been reported in association with administration. There was an increased frequency of severe thrombocytopenia in subjects receiving concurrent chemotherapy and radiotherapy with rhuGM-CSF. Eosinophilia and other blood abnormalities may occur. There have been rare reports of: 1) sequestration of granulocytes in the lungs with respiratory symptoms; 2) a syndrome characterized by respiratory distress, hypoxia, flushing, orthostatic hypotension, and partial loss of consciousness; 3) peripheral edema, pericardial or pleural effusions, and capillary leak of fluid; and 4) serious allergic or anaphylactic reactions.

Administration of rhuGM-CSF may aggravate fluid retention in subjects with pre-existent edema, capillary leak syndrome, or pleural or pericardial effusions. In some subjects with pre-existing renal or hepatic dysfunction, elevation of the serum creatinine or bilirubin and hepatic enzymes has occurred during the administration of rhuGM-CSF. Dose reduction or interruption or rhuGM-CSF administration has resulted in a decrease to pretreatment values. Occasional transient and reversible supraventricular arrhythmia has been reported in uncontrolled studies, particularly in subjects with a previous history of cardiac arrhythmia. Stimulation of marrow precursors with rhuGM-CSF may result in a rapid rise in white blood cell count. Dosing should be

stopped if the ANC exceeds 20,000/cm^3. rhuGM-CSF may stimulate the growth of myeloid malignancies, therefore caution must be exercised in its use in the malignancies or myelodysplastic syndromes.

rhuGM-CSF is a sterile, white, preservative-free lyophilized powder in 250 mcg vials. Reconstituted rhuGM-CSF will be admixed with the vaccine product, HER2 CTL vaccine, and injected at a dose of 100 mcg (0.2 ml rhuGM-CSF).

E. Final vaccine preparation

The vaccine is vialed and stored at a concentration of 1.9 mg/ml peptide in a volume of 1.1 ml/vial (2.1 mg total). The peptides have been reconstituted in 10mM sodium acetate with 17.8% (v/v) DMSO. The vaccines are stored at -20°C prior to use. Prior to administration, 150 mcg of rhuGM-CSF (0.3 ml) is added to a single vial of the CTL vaccine bringing the total volume in the vial to 1.4 ml. Thus, the vaccine concentration after the addition of adjuvant is 2.1 mg/1.4 ml. The final dose of peptide vaccine to be delivered is 1.5 mg (500 mcg/each peptide) with 100 mcg GM-CSF. The vaccine will be administered as three intradermal injections, 0.33 ml each, within a 5 cm radius in the same draining lymph node basin.

6. Subject Selection

A. Inclusion criteria

1. Subjects must have either Stage IV breast or ovarian cancer in remission or with stable disease on trastuzumab monotherapy.
2. HER2 overexpression by immunohistocytochemistry (IHC) of 2+ or 3+, in the primary tumor or metastasis. If overexpression is 2+ by IHC, then patients must have HER2 gene amplification documented by FISH.
3. Subjects must be HLA-A2 positive.
4. Eligible subjects must have completed appropriate treatment for their primary disease and be off cytotoxic chemotherapy and any immunosuppressive agents such as systemic steroids for at least 30 days prior to enrollment. Patients should continue trastuzumab monotherapy throughout the course of this protocol. Concurrent hormonal and biphosphanate therapies are allowed.
5. Subjects must have an ECOG Performance Status Score = 0 or 1 (Appendix A)
6. Male subjects must agree to contraceptive use during the study period (7 months) and non-menopausal female subjects must agree to contraception for the remainder of their childbearing years. (HER2 is overexpressed in normal fetal tissues. Therefore, induction of long-term immunity to overexpressed HER2 could affect the viability of a future fetus.)
7. Subjects must have a hematocrit $\geq$ 30, a platelet count $\geq$ 100,000, and a WBC $\geq$ 3000/µl. Laboratory tests should be performed within 60 days of enrollment.
8. Age >18 years
9. Stable creatinine $\leq$ 2.0mg/dL, or creatinine clearance $\geq$ 60ml/min. Laboratory tests should be performed within 60 days of enrollment.
10. Serum bilirubin < 1.5 mg/dl. Laboratory tests should be performed within 60 days of enrollment.
11. SGOT < 2x ULN. Laboratory tests should be performed within 60 days of enrollment.
12. Subjects must have recovered from major infections and/or surgical procedures and, in the opinion of the investigator, not have a significant active concurrent medical illness precluding protocol treatment or survival.
13. Patients must have a baseline LVEF measured by MUGA equal to or greater than the lower limit of normal for the radiology facility and if there are two consecutive MUGAS performed while on trastuzumab from the same radiology facility, there cannot be a decrease in LVEF of > 15% from the original MUGA scan.

B. Exclusion criteria

1. Subjects cannot be simultaneously enrolled on other treatment studies.
2. Any contraindication to receiving GM-CSF based vaccine products.
3. Cardiac disease, specifically restrictive cardiomyopathy, unstable angina within the last 6 months prior to enrollment, New York Heart Association functional class III-IV heart failure on active treatment with normalized LVEF on therapy, and symptomatic pericardial effusion.
4. Active autoimmune disease.
5. Subjects cannot have an active immunodeficiency disorder, e.g. HIV

Subjects must meet all of the listed criteria in order to be eligible for study.

While on study, patients will be followed for disease progression by their primary oncologist according to conventional practice standards. Should patients show evidence of progression, they should receive appropriate treatment for their disease through their primary oncologist. They will be removed from the study if their treatment conflicts with the eligibility criteria (e.g., concurrent administration of chemotherapy, immune modulators, steroids).

7. Experimental Design

A. Study design

This will be a single institution, single arm phase I-II study designed to examine both the safety and the immunogenicity of a HER2 CTL peptide-based vaccine when administered concomitantly with trastuzumab to patients with Stage IV breast and ovarian cancer.

B. Sample size

The number of subjects traditionally required to gather preliminary data on the safety of a dose in a phase I study is less than the number needed to evaluate immunologic responses. Since phase I tumor vaccine studies, to date, have proven to be relatively free of serious toxicity, the defining endpoint that would move a study forward to a more extensive phase II efficacy trial will likely be immunogenicity of the vaccine. In order to have meaningful immunologic data we will accrue a target of 20 subjects unless there is excessive toxicity as defined in section 9.0 (see Statistics, Section 11). Up to 5 additional subjects can be enrolled to replace patients who withdraw from study for reasons not associated with toxicity.

C. Outcome measures

Chemical and clinical parameters will be evaluated at baseline, prior to each vaccination and 1 month following the last vaccination to evaluate systemic toxicity (See section 8 for evaluation and management of adverse events and section 10 for stopping rules based on adverse events). Toxicity grading will be evaluated according to NCI common toxicity criteria (http://ctep.cancer.gov/forms/CTCAEv3.pdf).

All subjects will undergo immunologic response evaluation by cytokine flow cytometry (CFC) on PBMC derived from blood drawn (200 cc) at beginning of study, prior to vaccine # 3, 1 month, 3 months, 6 months and 12 months following the 6^{th} vaccination or until disease progression (as long immune response is detectable by CFC). Immune response will be defined by CFC. Specifically, a positive immune response will be defined as a post-vaccination antigen specific CD8 or CD4 precursor frequency measured by CFC of $\leq$ 1:20,000. For subjects who have measurable precursors by CFC at baseline, a positive response will be defined as a 2-fold increase in either antigen-specific T cell subset after vaccination. HLA-A2 MHC tetramer analysis will be used to demonstrate the specificity of the T cells for the HER2 specific HLA-A2 epitopes embedded within the longer immunizing peptides. IFN-gamma secreting PBMC precursor frequency will also be measured by ELIspot in order to determine the development of low level precursor frequencies ($\geq$ 1:20,000) not detected by CFC. ELIspot measurements will not be used to define response. All patients will be vaccinated with tetanus toxoid at enrollment to study. This immunization with a foreign antigen (tt) will serve as a measurable vaccinated response for each patient thus confirming immune competence.

Overall survival will be followed and compared to historical control. This study will not enroll sufficient numbers of subjects to give statistical power to this endpoint. However if large difference are observed between the treatment group and historical control, it will give impetus for a sufficiently powered phase II study.

8. Plan of Treatment

The following section outlines the schedule for evaluation and treatment of subjects prior to and during the study. All blood for laboratory evaluations must be drawn before each immunization.

A. Prescreen

Potential subjects will be screened for eligibility by telephone interview with the clinical research staff member to insure that their tumor is HER2 positive as described in eligibility and that they are HLA A-2. Verification that the potential subject meets all eligibility criteria will be made by the P.I. prior to scheduling the subject for a visit.

B. Initial evaluation

1. Sign consent form for the vaccine study entitled "Phase I-II Study of Combination Immunotherapy for the Generation of HER-2/neu (HER2) Specific Cytotoxic T Cells (CTL) *in vivo*"
2. Medical history and complete physical examination. Clinical evaluation, including vital signs, symptom assessment and ECOG scoring. Pregnancy testing will be performed on female subjects with childbearing potential, i.e. B-HCG.
3. Evaluation of UA (urinalysis by dipstick), CBC (complete blood count with differential and platelet count), serum chemistries (electrolytes, AST, ALT, alkaline phosphatase, bilirubin, creatinine and blood urea nitrogen (BUN)).
4. Obtain 10 cc of peripheral blood for the evaluation of a pre-existent HER2 antibody response. 200cc peripheral blood to obtain PBMC for T-cell immunity studies.
5. Tetanus toxoid immunization.
~~7~~6. Patients will have a CXR (PA and lateral) as well as an ECG to serve as baseline. (These tests will not be used to determine eligibility and will only be repeated should subjects develop pulmonary or cardiac medical problems during the course of study.)

C. Monthly immunizations

1. Post immunization monitoring: The subjects will be observed for at least 60 minutes post immunization. Vital signs and local reactions will be assessed by the staff before discharge and at least 60 minutes after vaccination.
2. Monthly vaccination for 6 months. Vaccinations will be administered i.d. on the upper arm. If a subject has undergone complete axillary node dissection the vaccine will be administered to the contralateral side. If the subject has undergone bilateral axillary node dissections, the vaccine will be administered on the anterior or lateral thigh. As much as possible each dose of vaccine will be administered within the same draining lymph node site.
3. UA, serum chemistry and CBC.
4. 210 cc blood draw prior to vaccine #3 to evaluate HER2 immune responses.

D. Evaluation after the final vaccine

1. One month after the final vaccination subjects will undergo a leukapheresis. If the patient cannot undergo leukapheresis for any medical reason, they will undergo a 210 cc blood draw (same as at baseline and pre-vaccine #3). Additionally, patients will undergo a blood draw for clinical monitoring of CBC (7 cc) and serum chemistries (7 cc).
2. Three months after the final vaccine subjects will undergo a MUGA and a 210 cc blood draw to obtain PBMC for T cell immunity studies.
3. Six and 12 months after the final vaccine, 210 cc of blood will be drawn for the evaluation of persistent antigen specific antibody and T cell immune responses.
4. After the final visit with us, patients may ask their regular physician to send us blood samples (about one cup) 3, 6 and 12 months after the final vaccine to test for the persistence of an immune response. If this cannot be arranged, they will need to come to the University of Washington for follow-up blood draws.
5. We may ask subjects to undergo leukapheresis at follow-up visits in lieu of a follow-up blood draw. This will done to recover more lymphocytes for immune function analysis. This will be determined on a case-by-case basis by the PI. Subjects will have the option to choose or refuse leukapheresis without affecting their continued participation in the study.

E. Long term follow-up

The patient's primary oncologist will be sent a questionnaire regarding relapse yearly for 5 years after completion of study. The median TTP for stage IV breast cancer patients receiving trastuzumab is approximately 25 months. We will follow patients for OS for 5 years from enrollment. A comparison of OS to historical control will be made for patients with Stage IV breast cancer.

9. Evaluation and Management of Toxicity

Subjects will be asked to report local reactions (to include pain, tenderness, erythema and induration) at the injection site and to report systemic reactions to include but not limited to fever, malaise, myalgia, nausea and headache. Monthly evaluation for toxicity will also include: CBC, UA, serum chemistries and a limited physical examination. Toxicity grading will be evaluated according to the NCI criteria; http://ctep.cancer.gov/forms/CTCAEv3.pdf and the Data Safety Monitoring Plan described in Appendix C.

If a grade 3 or 4 toxicity related to the vaccination is observed in a subject, no further immunizations will be administered to that subject. Grade 2 allergy will not be defined as an excessive toxicity unless the reaction has lasted greater than 48 hours.

If the toxicity observed is related to the immune response generated, a regimen of corticosteroids will be administered. The following dose schedule will be used:

Day 1:	Intravenous Solu-Medrol at 1 mg/kg
Day 2:	Intravenous Solu-Medrol at 1 mg/kg
Day 3-4:	Prednisone at 30 mg BID PO q day
Day 4-5:	Prednisone at 15 mg BID PO q day
Day 5-6:	Prednisone at 10 mg BID PO q day
Day 6-7:	Prednisone at 10 mg PO q day
Day 8-9:	Prednisone at 5 mg PO q day

All subjects will be monitored closely until the toxicity resolves. If a patient develops a toxicity that requires steroid treatment, no further vaccinations will be administered.

10. Dose modifications

There will not be any modifications to doses. There may be allowances for the timing of the administration of vaccine to accommodate patient schedules or special circumstances. For example, if a subject has an acute viral illness, the administration of their vaccination may be delayed by as much as 3 weeks. Such variations in the timing of monthly vaccinations will be decided by the Principle Investigator on a case-by-case basis. Subsequent vaccinations will be scheduled 30 days from when the previous vaccine was actually administered, not when it should have been administered.

11. Accrual and criteria for premature study termination

Accrual will be terminated if dose limiting toxicity (DLT) is observed. DLT is defined as a 5% incidence of grade 4 toxicity or a 15% incidence of grade 2 allergy >48 hours or grade 3 or worse toxicity. Operationally, the 5th subject enrolled will be a minimum of 1 month from their last vaccination and will be evaluated for toxicity with no DLT before further accrual of the next 15 patients.

Stopping rules will become operational if evidence suggests that the true toxicity rate exceeds 5% Grade 4 or 15% Grade 3 toxicity, where sufficient evidence will be taken to be observed rates of toxicity that correspond to one-sided 80% confidence intervals with lower limits greater than 5% or 15%, respectively. Thus, an incidence of Grade 4 toxicity in 1/4 or 2/15 subjects or Grade 3 or worse toxicity in 1/1, 2/5, 3/10 or 4/15 subjects would constitute sufficient evidence that the toxicity rates exceed 5% Grade 4 or 15% Grade 3 toxicity. Tables 2 and 3 summarize the operating characteristics of these stopping rules, where the probability of stopping the study is estimated from 5,000 Monte Carlo simulations.

Table 2. *Probability of Stopping Trial for Grade 4 Toxicity*

Number of Patients	True Rate of Event	Probability of Stopping
5	15%	0.39
10	15%	0.55
15	15%	0.72
5	25%	0.61
10	25%	0.81
15	25%	0.93

Table 3. *Probability of Stopping Trial for Grade 3 or Worse Toxicity*

Number of Patients	True Rate of Event	Probability of Stopping
5	5%	0.02
10	5%	0.03
15	5%	0.03
5	25%	0.37
10	25%	0.54
15	25%	0.64
5	35%	0.56
10	35%	0.77
15	35%	0.87

12. Evaluation of immune response (see Appendix B)

13. Statistical Considerations

A. Study statistics

This is a phase I study designed to evaluate (1) the toxicity of a HER2 HLA-A2 specific peptide-based vaccine administered concomitantly with trastuzumab and (2) the immunogenicity of HER2 HLA-A2 specific peptide-based vaccine administered concomitantly with trastuzumab.

The first objective will be achieved by assessing baseline, the type and grade of toxicities and any adverse events noted by the investigator. These data will be tabulated according to the affected body system. Descriptive statistics will be used to summarize changes from baseline.

The second objective will be achieved by evaluating the immune response to HER2 in a quantitative fashion based on experimental data obtained using methods described in section 12. A significant immune response in an individual patient will be defined as either the development of, in patients with no measurable precursors at baseline, precursor frequencies > 1:20,000 as measured by CFC; or as a 2-fold increase in baseline precursor frequency in those patients who have a pre existent immune response. The number of patients who achieve a significant immune response after vaccination will be used to assess the immunogenicity of the vaccine. Fourteen or more patients out of 20 would need to be successfully immunized for the vaccine to be considered immunogenic. This criterion is based on our hypothesized immunization rate of at least 70% against the HER2 antigen, which would justify conducting a Phase II study, designed to evaluate the ability of the immune response to protect against recurrence of HER2 overexpressing tumor. Table 4 shows the probability of observing 14 or more successes among 20 subjects for several true rates of success.

Table 4. *Probability of observing at least 14 successes in 20 patients*

True rate of success	Pr (≥14 successes)
50%	0.06
60%	0.25
70%	0.61
80%	0.91
90%	1.0

Regarding secondary objective, OS in the Stage IV breast cancer patients will be compared to the rate in a cohort of historical controls. This study was not designed to be sufficiently powered for any particular hypothesis testing, since time-to-event analyses generally require larger sample sizes than simple two-way comparisons. However, if a large difference is observed between the treatment group and the historical controls, then it may give additional impetus for a larger phase II study.

B. Projected gender and ethnic distribution

The ethnic and gender distribution chart below (Table 5) reflects estimates of race and gender of the population to be included in this study. These estimates are based on the following general statistics of the population in the Pacific Northwest and the characteristics of the diseases to be studied. The population pool from which subjects will be drawn is 76% Caucasian, 11% Asian American, 5% African American, 1% American Indian or Alaska Native, 6.5% other and 0.6 % Native Hawaiian or other Pacific Islander. The number of Hispanics available for study is 2%. Patients with HER2 positive cancer will be eligible regardless of their ethnic or racial origin. Although men with breast cancer are not excluded from this study, less than 1% of all breast cancers occur in men. Ovarian cancer is found exclusively in women. Children are also excluded from the study as the long term effects of immunity against a self-antigen, HER2, is unknown. Furthermore, the incidence of breast and ovarian cancer in children is < 1%.

Table 5. *Projected composition of patient population for Protocol 118*

TARGETED/PLANNED ENROLLMENT: Number of Subjects			
	Sex/Gender		
Ethnic Category	Females	Males	Total
Hispanic or Latino	0	0	
Not Hispanic or Latino	20	0	20
Ethnic Category Total of All Subjects*	20	0	20
Racial Categories			
American Indian/Alaska Native	0	0	
Asian	0	0	
Native Hawaiian or Other Pacific Islander	0	0	
Black or African American	1	0	1
White	19	0	19
Racial Categories: Total of All Subjects *	20	0	20

14. Administrative Considerations

A. Institutional review board

In accordance with federal regulations, an Institutional review board that complies with the regulations in 21CFR 56 must review and approve this protocol and the informed consent form prior to initiation of the study. In addition the study cannot be instituted without FDA approval of the vaccine formulations.

B. Consent

The investigators or their associates must explain verbally and in writing the nature, duration and purpose of the study and possible consequences of the treatment. Subjects must also be informed that they may withdraw from the study at any time and for any reason without jeopardizing their future treatment. In accordance with Federal regulations (21 CFR 312) all subjects must sign the IRB approved consent form in the presence of a witness.

C. Reporting adverse events

Adverse events will be reported using the terminology and grading scale of the NCI's Cancer Therapy Evaluation Program (CTEP) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. A copy of the CTCAE version 3.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov). Guidelines for adverse events reporting to the FDA and the NCI are described in the DSMP, Appendix C. The Monitoring Plan (as mandated by the NIH/NCI) Policy and Procedure for this phase I study is described in Appendix C.

D. Specimen Handling

Upon entry into the study each subject will be assigned a unique patient number (UPN). All materials collected on that subject will be labeled with their UPN. Lymphocytes, sera and tissue collected will be stored in a liquid nitrogen freezer in the laboratory of the Principal Investigator.

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Appendix A

ECOG Performance Status Scale

Performance status: Patients will be assessed according to the current ECOG performance scale.

Grade	Scale
0	Fully active: able to carry on all pre-disease performance without restriction.
1	Restricted in physically strenuous activity but ambulatory (can walk) and able to carry out work of a light or sedentary (sitting) nature, e.g. light housework, office work.
2	Ambulatory and capable of self-care but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	Capable of only limited self-care: confined to bed or chair more than 50% of waking hours.
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead

Appendix B

Laboratory Analysis

A. Antigens used in the immunologic assays

Antigen	Designation	Assay	Concentration
No antigen	Background control	All	NA
CMV lysate	Positive control	CFC*	2.5 μg/ml
CMV HLA-A2 pp65 peptide	Positive control	CFC and MHC tetramer	10 μg/ml
tt protein	Positive control	CFC	1 μg/ml
HER2 ICD overlapping peptide pools	Experimental	CFC	0.7 μg/ml per peptide
HER2 ECD overlapping peptide pools	Experimental	CFC	0.7 μg/ml per peptide
Individual HER2 class II peptides	Experimental	CFC	10 μg/ml
Individual HER2 class I peptides	Experimental	CFC and MHC tetramer	10 μg/ml
CEA overlapping peptide pool	Experimental (for epitope spreading)	CFC	0.7 μg/ml per peptide
CEA CAP-1 class I peptide	Experimental (for epitope spreading)	CFC and MHC tetramer	10 μg/ml
PHA	Mitogen	CFC	2.5 μg/ml

* All CFC antigen would be operative for ELIspot analysis

B. Immunologic analysis of antigen specific T cells

1. Detection of HER2 specific T-cell responses by cytokine flow cytometry (CFC). CFC is a highly quantitative method that will enumerate both CD4+ and CD8+ antigen specific T cells. Either 1 ml of whole blood or $1x10^6$ PBMC in 1 ml culture media (EHAA/RPMI, 10% AB serum, penicillin, streptomycin, L-glutamine) is stimulated with no antigen (PBS only), 1 μg/ml HER2 peptide mix in overlapping peptide pools, 10 μg individual HER2 CD4+ or CD8+ peptides, CMV HLA-A2 pp65 peptide, tt protein, or PHA, concentrations listed above. 2 μg/ml costimulatory molecules (CD28 and CD49d cocktail) and 10 mg/ml Brefeldin A are added to the reaction mixture and incubated for 6 hours at 37°C+5%CO_2. To remove the adherent cells, 2 mM EDTA is added and incubated at room temperature for 15 minutes. 9 mls 1X FACS lysing solution (BD Biosciences, San Jose, CA) is added and incubated at room temperature for 10 minutes to lyse the red cells and fix the PBMC. The reaction mixture is stored at -80°C freezer overnight. Cells are quickly thawed in a 37°C water bath, centrifuged and 2 mls of FACS permeabilizing solution 2 (BD Biosciences) is added. After a 10 minute incubation in the dark, the cells are washed with 10 mls wash buffer (1X PBS/0.5% BSA). Cells are thoroughly resuspended in 650 **μ**l wash buffer and 100 μl aliquoted to 6 microcentrifuge tubes. Antibodies (examples) are added as follows:

Tube #1: CD3 cychrome/CD8 FITC/IFN-gamma PE
Tube #2: CD3 cychrome/CD4 FITC/ IFN-gamma PE
Tube #3: CD3 cychrome/CD69 FITC/ IFN-gamma PE

Tube #4: CD3 cychrome/CD8 FITC/IL-4 PE
Tube #5: CD3 cychrome/CD4 FITC/IL-4 PE
Tube #6: CD3 cychrome/CD69 FITC/IL-4 PE

After a 30 minute incubation at room temperature in the dark, the cells are washed twice with wash buffer. The cells are resuspended in 300 µl 1% paraformaldehyde and then analyzed using three color flow cytometry. % responding cells are calculated counting a minimum of 50,000 events (CD8+ or CD4+ T cells). Antigen specific CD8+ or CD4+ IFNγ or IL-4 secreting cells will be reported as % responding cells for a particular population. A precursor frequency based on the total number of CD3+ cells can be calculated from this value.

2. Detection of HER2 specific T-cell responses by ELIspot. Briefly, on day 1, PBMC are plated into 96-well plates in 6-well replicates in 200 µl of RPMI-1640 containing L-glutamine, penicillin, streptomycin and 10% AB serum (T cell medium) in the presence or absence of 10 µg/ml peptide antigen or 1µg/ml TT. The cells are incubated at 37^0C+5% CO_2. On day 2 (48 hours), 2.5 x 10^5/well irradiated autologous PBMC and 10 µg/ml antigens are added. Also on day 2, nitrocellulose-backed 96-well plates (NC-plate) are coated with 10 µg/ml anti-IFN-gamma antibody in PBS at 50 µl/well. On day 3 the NC-plate is washed 3X with PBS and blocked for 2 hours with PBS containing 2% bovine serum albumin followed by 3 washes with PBS. On day 3, the cells are gently re-suspended, pooled, centrifuged, and the media is replaced. The cells are transferred into the NC-plate in a volume of 100 µl/well in T-cell medium. The NC-plate is incubated at 37ºC for a further 20-24 hours followed by washing 3X using PBS containing 0.05% Tween-20. The plate is then incubated for 2.5 hours at room temperature in 50 µl/well PBS containing 5µg/ml biotinylated anti-IFN-gamma antibody, washed 3X with PBS, and further incubated with 100 µl/well streptavidin-alkaline phosphatase at a dilution of 1:1000 in PBS for 2 hours at room temperature. After washing 3X in PBS, the plate is incubated with 100 µl/well AP-colorimetric substrate for 20-30 minutes, rinsed with cool tap water, and allowed to completely dry. Resultant spots are then enumerated using a dissecting microscope. Precursor frequencies are calculated by subtracting the mean number of spots obtained from the no antigen control wells from the mean number obtained in the experimental wells. Statistical analysis is performed using the Student's t test (Microsoft Excel 97). A positive response is defined as a precursor frequency that was both significantly ($p<0.05$) greater than the mean of control no antigen wells and detectable (i.e. >1:100,000).

3. Detection of HLA-A2 restricted HER2 specific responses with MHC tetramers. 200 µl of whole blood is mixed with 10 µl HLA-A201 CMV pp65PE/anti-CD8 FITC (Beckman Coulter, San Diego, CA) or HER2 peptide specific MHC tetramer (Beckman Coulter, San Diego, CA) and incubated at room temperature for 30 minutes in the dark. 1 ml of OptiLyse C (Beckman Coulter) is added and incubated for 10 minutes to lyse the red cells. 1 ml of 1X PBS is added and incubated for another 5 minutes. 2 mls 1X PBS is added and samples centrifuged. Cell pellet is resuspended in 500 µl 1% paraformaldehyde. The labeled cells are analyzed by flow cytometry and at least 500,000 events gated on lymphocytes are collected.

C. Immunologic detection of antigen specific antibodies

1. Quantitative ELISA for the detection of HER2 protein specific antibody responses. 96 well Immulon 4 plates (Baxter SP, Redmond, WA: Dynex Technologies) are incubated overnight at 4°C with a polyclonal affinity purified antibody to HER2 (kindly supplied by Dr. D. Ring) at a concentration of 1µg antibody per ml. Wells set up as a standard curve are incubated with purified human IgG (Sigma BioSciences, St. Louis, MO) in serial dilutions ranging from 0.03µg/well to 0.0002µg/well. After incubation, all wells are blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co.), 100µl/well for 4 hours at room temperature. The plate is washed with PBS/0.1% Tween and lysates of SKBR3, a human breast cancer cell line which overexpresses the HER2 protein, are added in alternating rows with PBS/1% BSA and incubated overnight at 4°C. After washing, the plate is incubated with human sera at the following dilutions: 1:25, 1:50, 1:100, 1:200. The sera is diluted in PBS/1% BSA/1% FBS/25 µg/ml mouse IgG/0.01% NaN_3 and then serially into PBS/1% BSA. 5µl of diluted sera is added per well and incubated 1 hour at room temperature. Goat anti-human IgG (gamma) horseradish peroxidase (HRP) (Zymed, So. San Francisco, CA) is added to the wells at a 1:50,000 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature (Amersham Co., Arlington Heights, IL). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) developing reagent is added. Color reaction is read at an optical density of 650 nm until the standard curve well containing 0.04 µg reaches 0.3 OD. The reaction is stopped with 1N HCl and the optical density read at 450nm. The OD of each serum dilution was calculated as the OD of the HER2 coated wells minus the OD of the PBS/1% BSA coated wells. A normal human serum is run on each plate as a negative control. Values are calculated in µg/ml based on the standard

curve. The final value expressed is the mean and standard deviation of quadruplicate dilutions. All positive responses will be evaluated by Western Blot. A population of 150 volunteer blood donors is run to establish the baseline of the assay. Random normal blood donors (n=10) are run every 100 plates, or quarterly, whichever comes first, and the standard curve and antibody values compared to mean and standard deviations of the experimental runs. Validation runs will be tabulated and discussed by the IMC and a quality assurance report filed with the data manager.

2. Quantitative ELISA for the detection of HER2 antibody avidity: 96 well Immulon 4 plates (Baxter SP, Redmond, WA: Dynex Technologies) are incubated overnight at 4°C with a polyclonal affinity purified antibody to HER2 (kindly supplied by Dr. D. Ring) at a concentration of 1µg antibody per ml. Wells set up as a standard curve are incubated with purified human IgG (Sigma BioSciences, St. Louis, MO) in serial dilutions ranging from 0.03µg/well to 0.0002µg/well. After incubation, all wells are blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co.), 10µl/well for 4 hours at room temperature. The plate is washed with PBS/0.1% Tween and lysates of SKBR3, a human breast cancer cell line which overexpresses the HER2 protein, are added in alternating rows with PBS/1% BSA and incubated overnight at 4°C. After washing, the plate is incubated with human sera at the following dilutions: 1:25, 1:50, 1:100, 1:200. The sera is diluted in PBS/1% BSA/1% FBS/25 µg/ml mouse IgG/0.01% NaN_3 and then serially into PBS/1%BSA. 5µl of diluted sera is added per well in duplicate and incubated 4 hours at room temperature. The plate is washed and incubated with 200 µl/well 8M Urea for 3 minutes. The plate is washed and goat anti-human IgG (gamma) horseradish peroxidase (HRP) (Zymed, So. San Francisco, CA) is added to the wells at a 1:50,000 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature (Amersham Co., Arlington Heights, IL). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) developing reagent is added. Color reaction is read at an optical density of 650 nm until the standard curve well containing 0.04 µg reaches 0.3 OD. The reaction is stopped with 1N HCl and the optical density read at 450nm. The OD of each serum dilution was calculated as the OD of the HER2 coated wells minus the OD of the PBS/1% BSA coated wells. Avidity Index is expressed as a percentage, where absorbance reading after urea wash is divided with absorbance reading without urea wash multiplied by 100.

3. Quantitative ELISA for the detection of tetanus toxoid antibodies and antibody avidity: Patients have been immunized with a DT at enrollment. 96 well Immulon 4 plates (Dynatech Laboratories) are incubated with tt protein (Lederle Labs) at a concentration of 1:1000 diluted in carbonate buffer alternating with rows of PBS/1% BSA. Wells set up as a standard curve are incubated with purified human IgG (Sigma BioSciences, St. Louis, MO) in serial dilutions ranging from 0.0217 µg/ml to 2.78 µg/ml overnight at 4°C. After incubation, all wells were blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co.), 100 µl/well for 1 hour at room temperature. The protein-coated plate is incubated with experimental sera diluted 1:400, 1:800, 1:1600 and 1:3200 in duplicate for 2 hours at room temperature. One set of duplicate is treated with 8M Urea for 5 minutes. Goat anti-human IgG (gamma) horseradish peroxidase (HRP) (Zymed, So. San Francisco, CA) are added to the wells at a 1:5,000 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature (Amersham Co., Arlington Heights, IL). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) developing reagent is added. Color reaction is read at an optical density of 650nm until the standard curve well containing 0.174 µg/ml reaches 0.3 OD. More dilute sera will be used if values are at the top or off of the standard curve. The reaction is stopped with 1N HCl and the optical density read at 450nm. The OD of each serum dilution is calculated as the OD of the protein coated wells minus the OD of the PBS/1% BSA coated wells. Values are calculated in µg/ml based on the standard curve. To calculate the % avidity index, the absorbance reading after urea wash is divided with absorbance reading without urea wash and multiplied by 100. Random normal blood donors (n=10) are run every 100 plates, or quarterly, whichever comes first, and the standard curve and antibody values compared to mean and standard deviations of the experimental runs. Validation runs will be tabulated and discussed by the IMC and a quality assurance report filed with the data manager.

4. Quantitative ELISA for the detection of CMV antibodies and antibody avidity: 96 well Immulon 4 plates (Dynatech Laboratories) are incubated with CMV lysate (Virusys) at a concentration of 7.5 µg/ml diluted in carbonate alternating with rows of PBS/1% BSA. Wells set up as a standard curve are incubated with purified

human IgG (Sigma BioSciences, St. Louis, MO) in serial dilutions ranging from 2.9 to 0.023 µg/ml. After overnight at 4°C, the plate is washed and all wells were blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co.), 100 µl/well for 1 hour at room temperature. The protein-coated plate is incubated with 100 µl sera diluted 1:800, 1:1600, 1:3200 and 1:6400 tested in duplicate for 2 hours at room temperature. One set of duplicate is treated with 8M Urea for 5 minutes. Goat anti-human IgG (gamma) horseradish peroxidase (HRP) (Zymed, So. San Francisco, CA) are added to the wells at a 1:5,000 dilution in PBS/1% BSA and incubated for 45 minutes at room temperature (Amersham Co., Arlington Heights, IL). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) developing reagent is added. Color reaction is read at an optical density of 650nm until the standard curve well containing 0.3 µg/ml reaches 0.3 OD. More dilute sera will be used if values are at the top or off of the standard curve. The reaction is stopped with 1N HCl and the optical density read at 450nm. Values are calculated in µg/ml based on the standard curve. The values are reported using the 1:800 dilution (no urea treatment) with the assay cut-off value of 200 µg/ml. Results between 100 to 200 µg/ml will need to be confirmed with western blot. To calculate the % avidity index, the absorbance reading after urea wash is divided with absorbance reading without urea wash and multiplied by 100. Random normal blood donors (n=10) are run every 100 plates, or quarterly, whichever comes first, and the standard curve and antibody values compared to mean and standard deviations of the experimental runs. Validation runs will be tabulated and discussed by the IMC and a quality assurance report filed with the data manager.

Appendix C

Data and Safety Monitoring Plan (DSMP)

Phase I-II Study of Combination Immunotherapy for the Generation of HER-2/neu (HER2) Specific Cytotoxic T Cells (CTL) *in vivo*

A. Purpose

To ensure that the Tumor Vaccine Group clinical research staff (Table 1) follow NIH/NCI/CTEP guidelines with respect to (1) accurate assessment and timely reporting of adverse drug reactions associated with investigational drugs (2) adherence to protocol and (3) accurate reporting of data. The DSMP will be attached to all protocols as an appendix and be approved by Human Subjects Division Institutional Review Board (IRB) at the University of Washington, Seattle, WA as part of the IRB protocol approval process.

The Principal Investigator is responsible for every aspect of the design, conduct and final analysis of the protocol. Regulations defining the responsibilities for assessment and reporting of all adverse events (AE), serious AE and unexpected AE are defined by the Code of Federal Regulations: 21 CFR 312.32 and Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0 published June 10, 2003 by the Cancer Therapy Evaluation Program (CTEP), a division of the NCI/NIH. A matrix of reporting requirements and schedules is in the CTEP web-site at http://ctep.infi.nih/gov.

This clinical study will rely upon the monitoring of the trial by the Principal investigator in conjunction with a Research Nurse, a Statistician, and an Independent Study Monitor assigned by the Research Trial Office of the University of Washington/Fred Hutchinson Cancer Research Center Cancer Consortium. If an adverse event occurs the Principal Investigator will be notified along with the IRB and appropriate federal agencies: FDA, NCI/CTEP and the Clinical Data Update System and/or the Adverse Event Expedited Reporting System, (AdEERS).

B. Objectives

1. To ensure that the Principal Investigator and clinical research staff follow federal and institutional regulatory guidelines with respect to timely reporting of adverse reactions associated with investigational drugs.
2. To provide instructions for completion of adverse events reporting forms as follows: a) MED WATCH FDA form 3500A, or b) Adverse Reaction (ADR) Form for Investigational Agents, or c) the Adverse Event Expedited Report for 24 hour phone/fax reporting of adverse events.
3. To define classification of adverse drug reactions as expected or unexpected.
4. To define classification of adverse drug reactions as serious or non-serious.
5. To ensure compliance and accuracy of documentation of adverse drug reactions reportable to the FDA/NIH/CTEP.
6. To ensure compliance and accuracy of reporting and documentation of adverse drug reactions reportable to the NCI by the Clinical Data Update System (CDUS, for NCI-funded trials).

C. Adverse Event Reporting Policy and Procedures

1. Evaluation of adverse events (Table 2): Patients are monitored for the development of end organ damage by assessing adverse events with serum chemistries, liver function studies, complete blood counts, urine analysis and physical exams performed every month until 30 days after the final vaccine administration. All adverse events for all systems are graded on a scale of 1-5 and attribution is assigned, using the NCI Common Terminology Criteria. If an adverse event occurs during more than one cycle of treatment, then only the most severe adverse event is reported. All information pertaining to toxicity is recorded in the source documents.
2. Definitions of adverse events:

a. Adverse Event - any unfavorable and unintended sign (including abnormal laboratory finding), symptom or disease temporally associated with the use of a medical treatment or procedure regardless of whether it is considered related to the medical treatment or procedure.

b. Expected Adverse Event - an event that may be reasonably anticipated to occur as a result of the study procedure.

c. Unexpected Adverse Event - any adverse event that is unanticipated. An event that might have been anticipated but is more serious than expected or occurs more frequently than expected, would be considered an unexpected adverse event.

d. Serious Adverse Event - grade 4 or 5 toxicity or any adverse event occurring that results in any of the following outcomes: death, a life threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization due to the adverse event, a persistent or significant disability/incapacity or a congenital anomaly/birth defect.

e. Life-threatening Adverse Event - the patient was at substantial risk of dying at the time of the adverse event or it is suspected that the use or continued use of the product would have resulted in the patient's death.

3. Scale of scoring adverse events
   a. Grade 1 = mild adverse event
   b. Grade 2 = moderate adverse event
   c. Grade 3 = severe adverse event
   d. Grade 4 = life-threatening or disabling adverse event
   e. Grade 5 = death related to adverse event.
4. Attribution of adverse event
   a. 5 (definite): the adverse event is clearly related to the investigational agent
   b. 4 (probable): the adverse event is likely related to the investigational agent
   c. 3 (possible): the adverse event may be related to the investigational agent
   d. 2 (unlikely): the adverse event is doubtfully related to the investigational agent
   e. 1 (unrelated): the adverse event is clearly NOT related to the investigational agent.
5. Adverse event reporting is outlined below by regulatory agency (Table 1):

University of Washington Human Subjects Division - expected adverse events are routinely reported as part of the annual renewal. Unexpected or more serious than expected adverse events are reportable by phone within 24 hours and followed in writing with a completed Human Subjects Adverse Effect Report

FDA (for trials using an Investigational New Drug (IND) - any unexpected, fatal or immediately life-threatening reaction must be reported to the FDA (Center for Biologics) by telephone or facsimile transmission (using Medwatch Form 3500A) as soon as possible but no later than seven calendar days after initial receipt of the information concerning the event. All unexpected, serious adverse events are reported in writing (using Medwatch Form 3500A) to the FDA (Center for Biologics) within 15 calendar days after initial receipt of the information concerning the event. Yearly written progress reports to the FDA describe all expected or non-serious unexpected adverse events.

NCI (for NCI-sponsored trials only) - adverse reactions that are expected and grade 1-3 or unexpected and grade 1 are reportable in a routine report. Adverse reactions that are unexpected and grade 2 are reportable in writing, using the Adverse Event Expedited Report (AdEERS) within 10 days to the NCI for NCI-sponsored trials, via the Clinical Trials Evaluation Program (CTEP). Adverse reactions that are expected and grade 4 or 5, or unexpected and grade 3, 4 or 5 are reportable by phone/fax, using the Adverse Event Expedited Report (AdEERS) within 24 hours. Temporary or permanent suspension of an NCI funded clinical trial is to be reported to the NCI grant program director responsible for the grant.

6. Procedure for reporting adverse events:

a. Identify the classification/attribution of the adverse event as defined above using the Modified Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v. 3.0).

b. After appropriate medical intervention has been instituted, the Principal Investigator or his/her designee will be notified within 24 hours.

c. File appropriate reports immediately by phone/fax with appropriate agencies, as described above.

d. Notify the patient's primary physician or referring physician within a medically appropriate timeframe, depending on the classification of the adverse event.

e. Submit written reports to appropriate agencies.

f. Document the adverse event in the patient's chart, using a progress note to describe the event and treatment, if appropriate.

g. File copies of all forms/correspondence relating to the adverse event in the patient's chart.

D. Clinical Trials Monitoring Operational Procedures (Table 3)

1. Clinical Data Documentation

a. Monthly monitoring:

All patients actively enrolled in the study are seen monthly. Clinical labs are evaluated each month for the development of toxicity (adverse events) related to the vaccine for up to 30 days following the last vaccine. Results are recorded on a clinical lab flow sheet that shows lab values over the course of the study. This document becomes part of the patient chart at the end of the study. Any abnormal lab values are faxed to the patient's physician and followed with a letter. The Research M.D. or the P.I. sees each study participant monthly and the following evaluations completed: toxicity evaluation, physical assessment and adverse events summary (these evaluations are part of the source document that is filled out at each visit). Grade 1 and 2 non-serious and expected adverse events will be reviewed with the P.I. at a weekly clinical trials review meeting. All other adverse events will be reported to the Principal Investigator at the time they become known and reported on further as outlined above in Section C.

Each patient chart is audited for completeness one month after enrollment. Each chart is again audited for completeness within one month of the final follow-up visit (30 days after the final vaccination). This audit will be conducted by the Research nurse every 3 months and the results will be presented to the Principal Investigator at weekly clinical meetings. The Research Nurse's chart audit record form is located in the front of each patient chart. Charts are audited for completeness, legibility, and accuracy. All source documents must be filled out completely. The Research Nurse will review all charts for subject recruitment and retention, protocol adherence, follow-up, data quality, and participant risk versus benefit. Additionally, all correspondence with primary physicians and patients is maintained in the patient chart. Copies of email communications and telephone logs of verbal communications are maintained.

b. Biannual study audit:

An Independent Study Monitor will be assigned by the Research Trials Office of the FHCRC/University of Washington's Division of Oncology and an independent study audit will be performed two times a year (approximately every 6 months). This audit will be conducted on all patients who have been enrolled on study at the time of the audit. Charts will be formally audited for completeness, accuracy and compliance to protocol. 10% of all computer data entry will also be reviewed.

2. Data validity/integrity

Systems to insure data integrity have been put into place to provide multiple checks to data entry. Subject eligibility is reviewed by the Research Nurse at screening, by the Research M.D. at enrollment, by the P.I. at a weekly meeting, quarterly by the Research Nurse chart audit, and biannually by the Independent Study Monitor. Source documents are initially reviewed by the Research Nurse monthly and quarterly for completeness and reviewed by the Independent Study Monitor biannually. All clinical laboratory monitoring data is reviewed in real time by the Research M.D. and any abnormalities are assessed. Any abnormalities are reported to the P.I. at weekly meetings. All subject's records are kept in the research file and reviewed at quarterly and biannual

audits. All toxicity scoring is reviewed with P.I. at weekly meetings and end of study toxicity scoring is done by the research nurse, reviewed by the P.I. and audited by the Independent Study Monitor and at biannual meetings as described above. All research data is checked by the P.I. and reviewed with the technologist or staff scientist who generated the data. Data is taken from the subject's source documents and entered into a data base which links data to subject by a Unique Patient Identifier and is accessible by password code only. Data entry is made by the Study Coordinator and is verified at end of study by the Research Nurse and the P.I. by reviewing all source documents and reconciling them to the study database. 10% of all data entry is reviewed by the Independent Study Monitor. At the time of study reports or publications print outs of all data from the data base are reviewed by the P.I.

Table 1. *Protocol 118 Clinical Trials Personnel*

Name	Role
Mary L. (Nora) Disis, M.D.	Principal Investigator
Lupe Salazar, M.D.	Research M.D.
Devon Webster, MD	Senior Fellow
Doreen Higgins, BSN	Research Nurse
Katherine Guthrie, Ph.D.	Statistician
Corazon de la Rosa, BSMT	Research Lab Manager
Independent Study Monitor for Bi-annual Audits	Assigned by Research Trials Office FHCRC/University of Washington/ Division of Oncology
Jennifer S. Childs, MPH	Research Coordinator
Patricia Fintak, MA	Research Coordinator

Table 2. *Adverse Event Reporting*

Regulatory Agency	**Expected Non-serious (Grade 1-2)**	**Expected Serious (Grade 3-5)**	**Unexpected Non-Serious**	**Unexpected Serious/Fatal**
UW-IRB	Annual renewal	24 hours	Annual renewal	24 hours
FDA	Yearly progress report	15 days	Yearly progress report	ASAP within 7 days-Medwatch Form 3500
NCI/CTEP	Routine report	24 hours by phone/fax	(Grade2) In writing $\leq$ 10 days	24 hours by phone/fax-ADR Form
Study Monitor	Routine report	24 hours by	Routine report	24 hours by

		phone/fax		phone/fax

NOTE: The death of any patient on a clinical trial is considered Serious Adverse Event, regardless of attribution and is required to be reported to the IRB, FDA and study sponsor.

Table 3. *Operational Procedures for Protocol 118*

Study Event	Specific Action	Performed By
Screen	Telephone screening of interested subjects	Study Coordinator/Research Nurse
	Screening checklist	Study Coordinator/Research Nurse
Eligibility	Initial review w/interested subject	Study Coordinator/Research Nurse
	Obtain source documents to determine eligibility	Study Coordinator/Research Nurse
	2º review of eligibility	MD/PI
	Weekly meeting w/PI to report study status/evaluate eligibility	Study Coordinator/Research Nurse MD
	Quarterly evaluation of subject eligibility	PI MD Research Nurse Study Coordinator
	Biannual review	Independent Study Monitor
Consent	Obtain Informed Consent	MD/PI
Monthly Visits	Blood draw	Research Nurse
	Physical assessment/toxicity evaluation	MD/PI
	Vaccinate	Research Nurse
	Monitor for immediate reactions	Research Nurse
Source Document		Filled out by Study Coordinator, Research Nurse, MD or P.I.
	Demographic Data Entry	Study Coordinator
	Monthly review of source documents for completeness	Research Nurse
	Quarterly Chart Review	Research Nurse
	Bi-annual audit for data-safety monitoring	Independent study monitor
Toxicity Monitoring	Monthly monitoring of adverse events with patient at each visit	MD
	Review of non serious and grade 2 expected adverse events weekly	P.I.
	Quarterly review of adverse events	Research Nurse/PI
	Biannual review of documentation of adverse events	Independent Study Monitor
Report of serious (grade 3, 4 or 5) or unexpected grade 2 adverse event	See sections B and C of DSMP, page 1-4	Research Nurse and P.I.
Immunologic Monitoring-Research data	Performed at specific timepoints for each subject	Research Lab Manager & Technologist
	Review of immunologic data	PI & MD
	Computer entry of immunologic data	Study Coordinator
Study review with P.I.	Weekly meeting to report study status/evaluate eligibility, subject recruitment and retention, protocol adherence, and adverse event evaluations (all grade 1 and	MD Research Nurse Study Coordinator

Study Event	Specific Action	Performed By
	expected grade 2). Daily on an as needed basis for all unexpected grade 2, or grade 3-5 adverse events.	
Twice yearly chart audits	Bi-annual evaluation of subject recruitment and retention, protocol adherence, adverse events and participant risk versus benefit. Validation of computer data entry from the source document.	Independent Study Monitor
Assurance of Data Accuracy and Integrity	Validation of computer data entry for clinical data Validation of data entry for immunologic monitoring-research data	Research Nurse Independent Study Monitor P.I.
Annual reports to: IRB FDA NCI		 Study Coordinator P.I. P.I

Appendix D

Supplemental HER2 CTL Peptide-Based Booster Vaccine Study

1. INTRODUCTION

Vaccinating women with HER2 overexpressed breast cancer against HER2-specific antigens has been shown to improve both overall survival in metastatic breast cancer (MBC) and disease free survival (DFS) in women with locally advanced non metastatic disease.[1,2] Furthermore, vaccination has been shown to both stimulate an immune response against the vaccine antigen as well epitope spreading to other antigens within the protein (intermolecular spreading) and other tumor-related proteins (intramolecular spreading).[1,2] However, immunity to the vaccines may wane as patients are greater than 6 months to a year from vaccination. This loss of immunity may explain progressive disease in patients with previous immune control. To improve prolonged immune response, booster vaccinations have been evaluated to re-stimulate HER2-specific immunity after completion of a primary vaccination series. Specifically, using GMCSF and the E75 vaccine, a single booster vaccination greater than 6 months after completing the vaccination series was demonstrated to be safe and improved immune response.[3] We therefore seek to evaluate the immunogenicity of a series of 2 booster vaccinations given 6 months apart in patients who have received at least one vaccination with the HER2 cytotoxic T lymphocyte (CTL) peptide-based vaccine and have no evidence of disease, stable disease, or slowly progressive disease.

Twenty-two HLA-A2 HER2 overexpressing stage IV patients were enrolled in the NCT00194714 trial of six HER2 CTL peptide-based vaccines along with concurrent trastuzumab. Twenty-one patients received vaccinations: Fourteen of 21 patients received all six immunizations, five of the patients completed at least three immunizations, and two of the patients only completed two immunizations. The vaccine series was well tolerated and had an acceptable safety profile with 99% grade 1 or 2 toxicities, four grade 3 toxicities which included injection site reaction, infection, ulceration and fainting. There was one unrelated grade 4 event of a stroke. Furthermore, the vaccine series was immunogenic with 90% of the patients developing new or increased HER2-specific IFN-g immunity as well as epitope spreading to other tumor associated proteins. The immunized patients also had disease response with a median progression free survival (PFS) of 17.7 months at 3 years (with 33% of patients showing no evidence of disease progression) and median overall survival of 86% at 4 years. The immunity induced by the vaccine was followed for up to a year after vaccination in 11 patients. Forty-six percent of patients maintained the same level of immunity, 36% had increased immunity, and 18% had lowered immunity after a year.[1] In long term follow up, 10 patients are currently living 10 years after completion of the initial vaccine series despite having $HER2^{+}$ MBC. As an example, one of the patients had 7 years of DFS on single agent trastuzumab after the vaccine series and continues to have considerable disease control despite slowly progressive disease while remaining on single agent trastuzumab. These patients' long disease stabilization beyond expected average DFS of 12 months with trastuzumab alone suggests that the immunity acquired from vaccination may have contributed to disease stabilization.[4,5] We therefore want to evaluate if providing additional boosters will possibly increase the anti-HER2 immunity of these previously vaccinated patients. Additionally, we will monitor for safety of the approach.

Although most cancer vaccine studies have focused on the generation of measurable immunity during active immunization, few studies have evaluated the persistence of immunity or the generation of immunologic memory. The development of immunologic memory has been shown to be essential. Indeed, it is the development of immunologic memory to the vaccinating antigen that would be the key to the success of a tumor vaccine to protect against relapse. In this booster study, we will evaluate the persistence of HER2 peptide-based specific immunity and also the presence and magnitude of a memory T-cell response after "booster" immunization with a HER2 peptide-based vaccine in previously immunized patients.

2. BACKGROUND

Immunologic memory, a hallmark of the protective immune response after immunization, is the ability to make a faster, stronger, and more effective, immune response to an antigen that has been encountered previously.[6]

Memory T-cells are clones of antigen-specific lymphocytes that are able to be maintained in homeostasis for a long time and reactivate when stimulated by a second encounter with an antigen.[6] The goal of vaccines, whether against infectious or cancer targets, is to develop these memory cells to allow for continued immune surveillance. Antigen-specific immunity can wane over time and booster vaccines can be used to reactivate the T-cells, increase memory, and trigger a more robust immune response. In infectious disease, with each antigen challenge (either an infection or booster vaccination) there is a larger increase in effector $CD8^+$ T-cells and a higher baseline memory T-cell response.[7] Therefore booster cancer vaccines would be expected to also give a second antigen challenge increasing both anti-tumor immune response and specific T-cell memory. Most breast cancer vaccine trials have evaluated the immunity that is developed immediately after primary vaccination.[8,9] However, long term follow up from these vaccine trials suggest that this immunity wanes and booster vaccinations may reactivate the anti-HER2 immune response and the memory T-cell population.

A. HER2 directed immunotherapy is safe when administered concurrently with HER2 CTL peptide based vaccination. As discussed above, patients enrolled to the original HER2 vaccine study were receiving concurrent trastuzumab and the combination immunotherapy was well tolerated with no significant cardiac toxicity observed. Over the past 2 years, the first-line treatment of $HER2^+$ MBC has been changing and now also includes other HER2 targeted agents. In 06/2012, the FDA approved the combination of pertuzumab and trastuzumab with chemotherapy for the treatment of $HER2^+$ MBC. This was based on data from the phase III CLEOPATRA study, which showed improved PFS with the combination of docetaxel and pertuzumab/trastuzumab versus docetaxel + placebo/trastuzumab.[10] Moreover, extensive cardiac surveillance conducted in CLEOPATRA did not show increased cardiotoxicity (increased rate of left-ventricular systolic dysfunction) in patients receiving concomitant pertuzumab and trastuzumab when compared to concomitant placebo and trastuzumab. As such, it is possible that patients enrolled in the original HER2 peptide vaccine study and who are now interested in the booster vaccine study may be receiving maintenance pertuzumab and trastuzumab. Given the acceptable toxicity data of the combined HER2 targeted therapy, we do not anticipate any increased cardiac toxicity if pertuzumab and trastuzumab are given concurrently with HER2 peptide booster vaccination. Therefore concurrent use of either trastuzumab or pertuzmab/trastuzumab during booster vaccination will be allowed.

B. T-cell populations essential for immune efficacy after vaccination. T-cells go through three stages in response to antigen, acute expansion in the lymph node when exposed to antigen, contraction after an acute inflammatory response where the antigen-specific T-cell populations decrease, and then development of memory T-cells in case of any further exposure to antigen. Naïve T-cells localized in the lymph node are either $CD4^+$ or $CD8^+$ T-cells and, on association with antigen presented in association with MHC class I or II receptors, are able to clonally expand into effector T-cells (either $CD4^+$ helper T-cells or $CD8^+$ cytotoxic T-cells).[6] How these T-cells clonally expand is controlled by the cytokine milieu, immune environment, and secondary signals from the antigen presenting cell.[11,12] After antigen clearance, the number of antigen specific $CD4^+$ and $CD8^+$ T-cells rapidly decline believed to be due to decreasing pro-inflammatory cytokines and decreased antigen stimulation.[6] After contraction of the antigen-specific T-cell populations, a small population of memory T-cells remain and these T-cells are called central memory T-cells which can persist for decades despite the average lifespan of single memory cells being days to weeks, suggesting that these T-cells are self-renewing and can persist without antigen present.[7] The factors that determine if a cell becomes an effector or a memory T-cell remain unclear. Central memory cells home to lymphoid tissue where they proliferate vigorously on re-encountering antigen and produce cytotoxic function slowly. Effector memory cells on the other hand proliferate less well, but produce cytokines rapidly and enter non-lymphoid tissue providing immediate protection with re-activation from memory.[6] With a second exposure to antigen, either through booster vaccination or a second infection, the secondary memory response produces higher numbers of memory T-cells.[7] Therefore providing booster vaccines should reactivate an acute HER2-specific anti-tumor immune response against disease that is present and provide further anti-tumor epitope spreading as well as increase the HER2 specific memory T-cell population against further tumor development.

C. Rationale for booster vaccination in patients immunized with HER2 CTL peptide. Booster vaccinations have been examined previously in cancer vaccines and a single booster vaccination has the

potential to mount an immune response as great if not greater than initial vaccination.[13] The booster vaccination can also widen the spectrum of $CD4^+$ and $CD8^+$ T-cells against protein specific epitopes. A booster vaccine in $HER2^+$ breast cancer patients has previously been shown to be safe and immunogenic using the HER2-specific E75 vaccine along with GMCSF adjuvant. The peptide-based HER2 vaccine E75 with GMCSF adjuvant was given as a single booster in 53 patients who had previously received the E75 vaccine by various schedules in previous clinical trials but had not received vaccination for over 6 months. The investigators found that residual $CD8^+$ T-cell immunity existed in 94.4% of patients at 6 months after vaccination but at greater than 6 months after vaccination only 48 of patients had residual immunity ($p=0.002$). Furthermore, the median residual immunity remaining was very low at 0.70% (range 0 to 3.49%). The booster vaccine was well tolerated with only grade 1 and 2 toxicity primarily local injection site reactions and 85% of the patients without E75 specific immunity had achieved increased immunity after the booster vaccination.[3] We propose to evaluate HER2 specific immunity and improved memory T-cell response generated using two boosters vaccinations of the HER2 peptide vaccine. Our group has completed a trial using 2 boosters with a DNA vaccine IGFBP2 in 8 ovarian cancer patients and these boosters were found to be safe and their immunity is currently being evaluated. We therefore seek to evaluate a similar booster series with the HER2 peptide vaccine.

D. Immune and metabolic activity measured by fluorodeoxyglucose positron emission tomography (FDG-PET) imaging. Changes in SUV quantitated by FDG-PET are a functional measure that is usually associated with disease progression, particularly when evaluating breast cancer bone metastases. In a study of 28 breast cancer patients with bone dominant disease, higher tumor FDG SUVs predicted a shorter time to disease progression ($p<0.006$).[14] However there is an emerging role for PET imaging to evaluate patients for immunologic side effects caused by monoclonal checkpoint inhibitor therapy and for measuring immune flare after vaccination.[15,16] FLT-PET imaging of KLH antigen loaded dendritic cell (DC) vaccines in 3 patients with metastatic melanoma to the lymph node showed that only antigen loaded dendritic cells increased PET avidity in draining lymph nodes and remained positive up to 3 weeks after vaccination (Aarntzen PNAS 2011). In another study, FDG-PET scans done within 7 days after receiving the 6th vaccine of a DC vaccination series showed increased metabolic responses of index lesions (even though new lesions appeared simultaneously) and improved survival, indicative of an overall antitumor effect of DC vaccines.[17] The authors concluded that it is possible that infiltration of inflammatory cells might lead to pseudo-progression in SUV; and thus, PET scans may provide a measure to evaluate the activity of immunotherapies over time. In our experience in a patient with HER2-specific adoptive T-cells, a temporal increase in tumor FDG uptake at sites of metastases over a 48 hour period that resolved by 1 month follow up was associated with significant disease stabilization of 18 months. This suggests that acute increases in tumor metabolism as measured by FDG-PET may identify inflammation induced by activated T-cells and could potentially indicate a defined level of inflammation needed to mediate disease regression in HER2 adoptive T-cell studies (Stanton *submitted*). Clinical symptoms of tumor flare, such as pain at metastatic sites, after immune modulation have been associated with beneficial clinical responses. After adoptive T-cell therapy for nasopharyngeal carcinoma or breast cancer pain at sites of disease and increase in tumor size temporally related to T-cell infusion was correlated with clinical response in specific lesions.[18,19] Therefore, we will compare baseline FDG-PET imaging prior to the second booster to FDG-PET imaging at 48 hours after the second booster vaccine to evaluate if there is any evidence of acute increase in tumor metabolism suggestive of activated T-cell infiltration and increased inflammation.

3. OBJECTIVES

A. Primary

1. Evaluate whether HER2 peptide-based booster vaccines can enhance HER2-specific immunity.
2. Evaluate whether HER2 peptide-based booster vaccines induce increase in memory T-cell populations in patients' peripheral blood.
3. Evaluate whether HER2 peptide-based booster vaccinations demonstrate acute inflammatory response in lymph nodes draining the vaccine site, and in tumor metastasis, by FDG-PET.

B. Secondary

1. Evaluate the safety of HER2 peptide-based booster vaccines in patients who previously received the HER2 peptide-based vaccines.

4. PATIENT SELECTION

A. Inclusion criteria

1. Patients must have received at least 1 vaccination of the HER2 CTL peptide-based vaccine.
2. Patients must have Stage IV breast cancer with either no evidence of disease, disease that is stable, or slowly progressive disease.
3. Patients taking HER2-targeted monoclonal antibody therapy may remain on the treatment.
   a. HER2-targeted monoclonal antibody therapy is defined as either trastuzumab monotherapy, or trastuzumab and pertuzumab combination therapy administered per standard of care.
4. Patients must be at least 28 days post cytotoxic chemotherapy prior to first vaccine
5. Patients on bisphosphonates and/or endocrine therapy are eligible and may remain on therapy.
6. Patients must be at least 28 days post immunosuppressants prior to first vaccine.
7. Patients must have an ECOG Performance Status Score ≤ 1 (Appendix A of original protocol).
8. Non-menopausal female subjects who are having sex that can lead to pregnancy must agree to contraception for the remainder of their childbearing years.
9. Laboratory values must be as follows and performed within 60 days of enrollment:
   a. WBC $\geq 1500/mm^3$
   b. Hgb ≥ 8 g/dl
   c. Platelet count $\geq 75{,}000/mm^3$
   d. Serum creatinine ≤ 2.0 mg/dl or creatinine clearance > 60 ml/min
   e. Total bilirubin ≤ 2.5 mg/dl
   f. SGOT ≤ 2.0 times the upper limit of normal
10. Patients must have recovered from major infections and/or surgical procedures.
11. Patients receiving a HER2 targeted monoclonal antibody must have adequate cardiac function, as demonstrated by normal left ventricular ejection fraction (LVEF) $\geq$ the lower limit of normal for the facility on MUGA scan or echocardiogram (ECHO) within 6 months of enrollment as per standard of care.

B. Exclusion criteria

1. Patients cannot be simultaneously enrolled on other treatment studies.
2. Any contraindication to receiving GMCSF based vaccine products.
3. Patients with any of the following cardiac conditions:
   a. Symptomatic restrictive cardiomyopathy
   b. Unstable angina within 4 months prior to enrollment
   c. New York Heart Association functional class III-IV heart failure on active treatment
   d. Symptomatic pericardial effusion
4. Patients with any clinically significant autoimmune disease uncontrolled with treatment.

5. EXPERIMENTAL DESIGN

A. Study design

This is a supplemental booster vaccine to Protocol 118 (IR# 6304): Phase I-II Study of Combination Immunotherapy for the Generation of HER-2/neu (HER2) Specific Cytotoxic T-Cells (CTL) in vivo.

B. Sample size

Up to 10 patients can be enrolled. This sample size is not derived based on statistical considerations, but rather on the number of potentially eligible patients that we feel can be accrued.

C. Booster vaccines

Patients will receive one HER2 CTL peptide-based vaccine every six months for two vaccines.

D. FDG-PET scans

Patients will undergo FDG-PET prior to their second booster and approximately 48 hours post the second booster vaccine to assess for inflammatory changes in lymph nodes draining the vaccine site and any metastatic sites.

E. Outcome measures

Primary

1. Evaluation of whether the HER2 peptide-based boosters enhance HER2-specific immunity through IFN-g ELISPOT will be determined 6 months after first and second booster vaccines. Increased HER2-specific immunity will be defined by generating a HER2 protein specific IFN-g precursor augmented over 2 times baseline.
2. Evaluation of whether there is increased memory T-cell populations in the peripheral blood by flow cytometry will be evaluated 48 hours after each of the two HER2 peptide-based booster vaccinations. The peripheral memory T-cell populations will be evaluated on PBMC using a flow cytometry panel developed in our immune monitoring laboratory. We will be evaluating CD45R0, CD62, CCR7, CD3, CD4, and CD8 antibodies for evaluation of memory T-cells.
3. Evaluation of HER2-specific memory through development of a delayed type hypersensitivity (DTH) response at the vaccination site will be determined 48 hours after each booster vaccine. A positive response will be defined as ≥ 10 mm^2 induration at the vaccination site.
4. Evaluation of immune modulation by FDG-PET will be defined as acute inflammatory response evidenced by acute increase in FDG avidity in the tumor and/or draining lymph nodes 48 hours after the 2nd booster vaccination as compared to baseline FDG-PET prior to the second vaccine.

Secondary

1. Safety and systemic toxicity will be determined by chemical and clinical parameters evaluated at various time points as described in Section 6. Toxicity grading will be evaluated per the CTEP CTCAE v3.0 and monitoring of adverse events will be done per FDA and NCI guidelines. CTCAE v.3.0 criteria was used to evaluate the original data.

6. PLAN OF TREATMENT

The following section outlines the schedule for evaluation and treatment of patients during the study.

A. First Visit-Booster Vaccination #1

1. Sign consent form for the "Supplemental HER2 CTL Peptide-Based Booster Vaccine Study".
2. Verification that patient meets eligibility criteria.
3. Medical history and complete physical examination.
   a. Clinical evaluation, including vital signs and weight, symptom assessment and ECOG scoring.
4. CBC (complete blood count with differential and platelet count), serum chemistries (electrolytes, AST, ALT, alkaline phosphatase, bilirubin, creatinine and blood urea nitrogen (BUN)).
5. Obtain up to 110 mls peripheral blood for baseline research studies.
6. Patients of child bearing potential must have a negative urine pregnancy test.
7. Vaccination will be administered i.d. (intradermal) at the site of previous primary vaccine series.
8. Post immunization monitoring: The patients will be observed for at least 60 minutes post immunization. Vital signs will be done at least 60 minutes post vaccination.

B. 48 hours after Booster Vaccination #1

1. Delayed type hypersensitivity (DTH) will be evaluated at the injection site approximately 48 hours (+24 hours) after the booster vaccine and the DTH response (i.e. induration and erythema) measured in mm^2.
2. Peripheral blood draw to evaluate HER2 specific immunity and T-cell memory (100 mls blood).

C. Booster Vaccination #2 (Six months after Booster Vaccination #1)

1. Complete physical examination.
   a. Clinical evaluation, including vital signs and weight, symptom assessment and ECOG scoring.
2. Patients of child bearing potential must have a negative urine pregnancy test.
3. CBC (complete blood count with differential and platelet count), serum chemistries (electrolytes, AST, ALT, alkaline phosphatase, bilirubin, creatinine and blood urea nitrogen (BUN)).
4. Obtain up to 110 mls peripheral blood for research studies, prior to second booster vaccination.
5. A FDG-PET scan will be performed prior to the second booster vaccination.
6. Vaccination will be administered i.d. at the site of previous vaccination.
7. Post immunization monitoring: The patients will be observed for at least 60 minutes post immunization. Vital signs will be done at least 60 minutes post vaccination.

D. 48 hours after Booster Vaccination #2

1. Delayed type hypersensitivity (DTH) will be evaluated at the injection site approximately 48 hours (+24 hours) after the booster vaccine and the DTH response (i.e. induration and erythema) measured in mm^2.
2. Peripheral blood draw to evaluate HER2 specific immunity and T-cell memory (100 mls blood).
3. A repeat FDG-PET scan will be performed to evaluate inflammatory changes in draining lymph nodes.

E. Six months after Booster Vaccination #2

1. Peripheral blood draws to evaluate HER2-specific immunity (by ELISPOT) and T-cell memory (210 mls blood).

F. Long Term Follow-up

A request for records will be sent to the patient's primary oncologist twice yearly from the last booster vaccine for a total of 5 years.

This information will be put in the study chart which is kept in a locked filing cabinet in a secured building. Study charts are kept for 30 years after the close of the study per University of Washington policy. Information requested will include:

a. Most recent laboratory evaluation (CBC, CMP)

b. Patient's disease free and overall survival status

c. Recent clinical notes which includes history, imaging reports, and physical exam notes

Notes will be reviewed by study team for vaccine toxicity and followed up with the patient's primary physician if indicated.

We will look for possible autoimmune symptoms (e.g. unexplained rash, dry eyes, unexplained diarrhea) within the above collected records.

7. EVALUATION AND MANAGEMENT OF TOXICITY

Patients must be off all immunosuppressive treatments such as chemotherapy or systemic steroid therapy a minimum of 28 days prior to each booster vaccination. Evaluation for toxicity will include a physical examination and laboratory evaluation as described in Section 5 (Plan of Treatment.).

8. STATISTICS

This study is descriptive and the statistics will be based on changes from baseline at the various time points as described in Section 5.

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SCHEDULE OF EVENTS

Visit Time Point	Procedures
Booster 1	• Sign consent • Medical history and complete physical examination • Vitals signs-including weight • ECOG scoring • Pregnancy urine dip – if applicable • Clinical labs: complete blood counts, serum chemistries • Research blood: approximately 110 mls • Symptom assessment • Booster vaccine #1 • Post-immunization monitoring
Forty-eight hours after booster #1	• DTH measurement of vaccine site • Research blood: approximately 100 mls
Booster 2: Six months post first booster vaccine	• FDG-PET scan prior to vaccination • Complete physical examination • ECOG scoring • Pregnancy urine dip – if applicable • Vitals signs-including weight • Clinical labs: complete blood counts, serum chemistries • Research blood prior to vaccination: approximately 110 mls • Symptom/toxicity assessment • Booster vaccine #2 • Post-immunization monitoring
Forty-eight hours after booster #2	• DTH evaluation • Research blood: approximately 100 mls • FDG PET Scan
Six months after booster #2	• Research blood: approximately 210 mls
Long-Term Follow-Up for 5 years	• Every 6 months for 5 years • Medical documentation request of your oncologist
• During the consent process, and at subsequent visits, patients will be instructed to hydrate sufficiently prior to visits requiring large volume blood draws.	