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13. ABSTRACT (Maximum 200 Words)

Breast cancer is the most common non-skin cancer in women and the American Cancer Society estimates that there will be 215,990 new cases of invasive breast cancer and 40,110 deaths from MBC in the United States in 2004. Thus, patients with MBC who fail conventional therapies are candidates for clinical trials using novel therapeutic approaches, including immunotherapy. Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor cytotoxic T lymphocytes against tumor-associated antigens and bacterial DNA oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) further augment the immune priming functions of DCs. We hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. Our 3 specific aims are 1) to study the mechanism of antitumor immunity mediated by the vaccination of TS/A mammary tumor-bearing BALB/c mice with CpG DNA-stimulated DCs primed *in vitro* with necrotic TS/A cells, 2) to determine optimal conditions for CpG DNA stimulation and tumor priming of human DCs, and 3) to design a Phase I/II clinical trial of DC + CpG DNA therapy for breast cancer.

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I. INTRODUCTION

Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor immunity against tumor-associated antigens by cytotoxic T lymphocytes, and bacterial DNA oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) can further amplify the immunostimulatory capacity of DCs. In this proposal we hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. In Aim 1 of this proposal, we test this hypothesis in a pre-clinical model of breast cancer using an established mammary tumor (TS/A) in syngeneic BALB/c mice. DCs are primed by necrotic tumor cells *in vitro* and then injected subcutaneously with CpG DNA in TS/A tumor-bearing mice. In Aim 2, as a prelude to a future clinical trial, we determine the optimal conditions for tumor priming and CpG DNA stimulation of human DCs. In this aim, we will develop optimal loading conditions of human DCs with tumor lysate derived from the MCF-7 human breast cancer cell line. In Aim 3, based on the pre-clinical data generated in Aims 1 and 2, we plan to undertake a Phase I/II clinical trial of immunization with autologous tumor-primed DCs and dose-escalated CpG DNA in patients with metastatic breast cancer.

II. BODY

Aim 1. To Study The Mechanism Of Antitumor Immunity Mediated By The Vaccination Of TS/A Tumor-Bearing Mice With CpG DNA-Stimulated DCs Primed In Vitro With Necrotic TS/A Cells.

In order to establish a clinical rationale for cancer therapy using tumor lysate-loaded DCs in combination with CpG DNA, 4 groups of 5 BALB/c mice were inoculated in their right flanks with $5x10^4$ syngeneic TS/A mammary tumor cells and tumors were allowed to grow until they were palpable (~1 mm^2). DC precursors were harvested from the bone marrow of BALB/c mice and cultured for 7 days in complete medium containing 1000 u/ml of both mGM-CSF and mIL-4. After 7 days, DCs were cultured overnight with freeze/thaw-generated lysate from TS/A tumors at a 5:1 tumor:DC ratio. Mice were then vaccinated subcutaneously twice, 7 days apart, in their left flanks with either (a) PBS, (b) TS/A lysate-loaded DCs alone ($5x10^5$ DCs), (c) CpG DNA alone (15 nmoles - injection 1; 10 nmoles - injection 2) (5'-TCCATGACGTTCCTGATG CT-3') or (d) DCs + CpG DNA. As shown in Figure 1(a), the combination of DCs + CpG DNA was more effective in treating the established TS/A mammary tumor than either PBS, DCs, or CpG DNA alone. Also, as shown in Figure 1(b), all mice treated with DCs + CpG DNA were alive and tumor-free compared to only 60% of mice treated with CpG DNA alone. There is therefore a strong rationale for using tumor lysate-loaded DCs in combination with CpG DNA for cancer therapy.

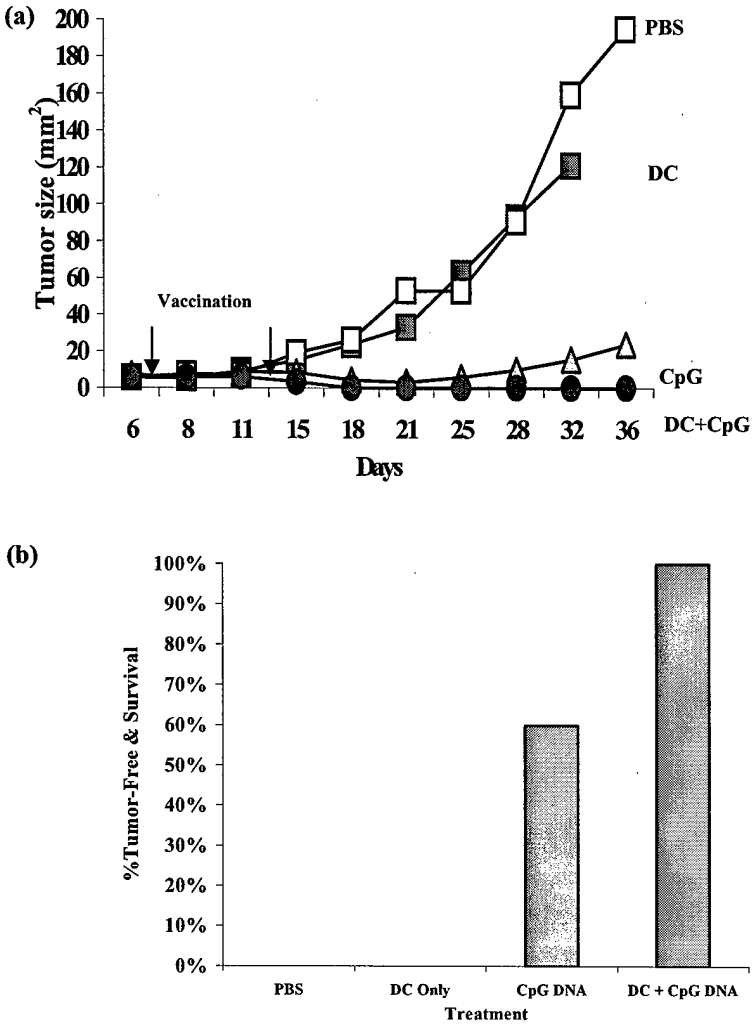


Fig. 1. (a) Tumor size and (b) % tumor-free and survival of TS/A tumor-bearing BALB/c mice treated with either PBS, TS/A lysate-loaded DCs alone, CpG DNA alone, or TS/A lysate-loaded DCs + CpG DNA.

Aim 2. To Determine Optimal Conditions For CpG DNA Stimulation And Tumor Priming Of Human DCs.

Many DC-based human clinical therapy protocols utilize many cytokines (e.g. hGM-CSF and hIL-4) and maturation factors (e.g. LPS, IL-1, TNF-α, PGE2, poly I:C) to direct peripheral blood monocytes to differentiate into monocyte-derived DC (mDC). This process is cumbersome, expensive and generally requires 7 to 8 days of *in-vitro* culture to generate functional DCs. The endpoint of this grant proposal is to develop a simpler and more efficient DC clinical protocol to treat patients with metastatic breast cancer where CpG DNA will be used as the single, primary stimulus without using any other maturation factors to drive human peripheral blood DC precursors into mature DCs.

A subset of human DCs responsive to CpG DNA has been characterized by Krug *et al* (1). These plasmacytoid DC (pDC, CD123+) express the Toll-like receptor-9 (TLR9) which is involved in the recognition of CpG motifs. The blood dendritic cell antigen-4 (BDCA-4) is a unique pDC marker that allows for the isolation of these pDCs by elution from a BDCA-4 selection column. The authors demonstrated that a CpG DNA (5'-TCGTCGTTTTGTCGTTTT GTCGTT-3') supported the survival, activation (CD80, CD86, CD40, MHC class II), chemokine

production (IL-8, IP-10) and maturation (CD83) of BDCA-4 selected pDC which were grown in culture medium containing hGM-CSF and hIL-3. In another study, Gursel *et al* (2) used another CpG DNA (5'-GTGCATCGATGCAGGGGGG-3') to stimulate human peripheral blood monocytes to mature into functionally active DC over 2-4 days. The transition from monocyte to DC was characterized by the up-regulation of CD83, CD86, CD80, CD40 and the down-regulation of CD14 in serum- and cytokine-free medium (XVIVO) containing only CpG DNA as the maturation factor. The differentiation of these monocytic DC (mDC) precursors to functional DCs was mediated by pDC present at low frequency in the peripheral blood sample, which responded to the CpG DNA by secreting IFN-alpha which, in turn, induced the maturation of the mDCs. The 2 major advantages of this latter approach compared to the former is that, first, no column selection procedure is required to isolate DCs, thereby significantly increasing the yield of mature DCs and, second, no cytokines (GM-CSF or IL-4 or IL-3) are required in the growth medium to sustain DC growth and maturation. We were therefore interested in doing a head-to-head comparison of the ability of these 2 CpG DNAs to stimulate human DCs.

OBJECTIVES

The objectives of this task were to:

1. Test the 2 above-mentioned CpG DNAs on both pDC and mDC and compare their effect on DC maturation (e.g. upregulation of MHC Class II, CD40, CD86, etc).
2. Test MCF-7 tumor lysate uptake by DCs after CpG stimulation and determine whether DC maturation markers are altered by tumor lysate loading.
3. Use autologous DC-primed T cells in an *in-vitro* cytotoxicity assay against MCF-7.

MATERIALS AND METHODS

CpG DNAs. 1: 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (used for pDC maturation or as indicated); 2: 5'-GGTGCATCGATGCAGGGGGG-3' (used for mDC maturation or as indicated).

Generation of pDC. Peripheral blood mononuclear cells (PBMC) were isolated form health human donors by Ficoll gradient separation. PDC were selected from PBMC by anti-BDCA-4 conjugated magnetic beads. pDC eluted from separation column were cultured in the presence of hGM-CSF and hIL-3 for 1 day (Fig. 2).

Generation of mDC. PBMC were separated by Percoll gradients into lymphocyte and monocyte sections. Monocytes were cultured in XVIVO® medium in the presence of CpG DNA but without any other cytokines or maturation factors. The rationale for the use of unselected mDCs is that these aliquots contain both pDC and mDCs. The addition of CpG DNA to the growth medium stimulates the pDCs to produce IFN-α which, in turn, stimulate the maturation of the mDCs in the culture (Fig. 2).

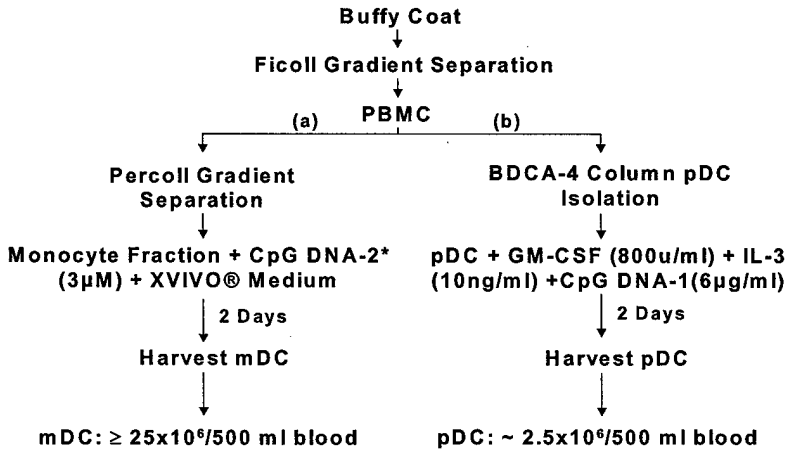


Fig. 2. Preparation of pDC and mDC from human peripheral blood

DC Surface Markers. Markers were detected through fluorescent-conjugated antibody to CD80, CD86, CD83, CD40, class II and etc by flow cytometer.

Tumor Antigen Loading. The human breast cancer cell line (MCF-7) lysate was prepared through repeated freeze and thaw cycles and was added overnight to the DC preparation in a 1:1 ratio of tumor cells to DCs. The next day, the loaded DCs were washed and labeled for FACS analysis.

Tumor Labeling. To monitor tumor uptake by DC, tumor cells were stained with DiI before loading onto DC.

Cytotoxicity Assays. Monocytes isolated from PBMC were grown for 1 day in XVIVO medium containing CpG DNA. The breast cancer cell line MCF-7 was lysed by repeated freezing and thawing. Tumor lysate was added to DC at the ratio DC: Tumor = 1 :1 for 1 day. After harvesting the tumor-pulsed DCs, they were used to prime autologous T cells (PBMC) at the ratio T:DC = 10:1. Il-2 (20 u/ml) was added to the medium on Day 5. T cells were restimulated every week with the CpG-treated DC. CTL were harvested after 3 stimulations and used as effectors against MCF-7 in an LDH cytotoxicity assay (Promega).

RESULTS

1. Test CpG DNA On Both pDC And mDC And Compare Their Responses By DC Markers.

We observed that both CpG DNAs were equally effective in upregulating both pDC- and mDC-associated cell-surface markers such as MHC class II, CD40, CD80 and CD83. Maximum upregulation of these cell-surface markers was usually seen after 48 hours of DC culture (Figs. 3a and 3b).

a) CpG DNA-1 Upregulates pDC Marker Expression

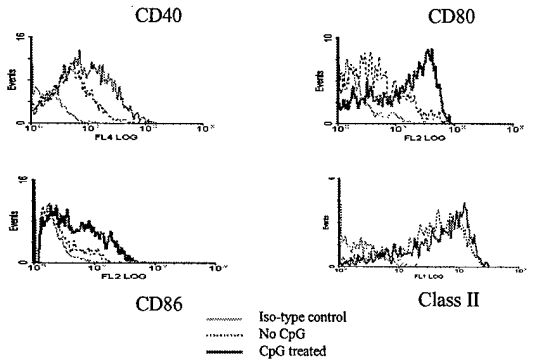
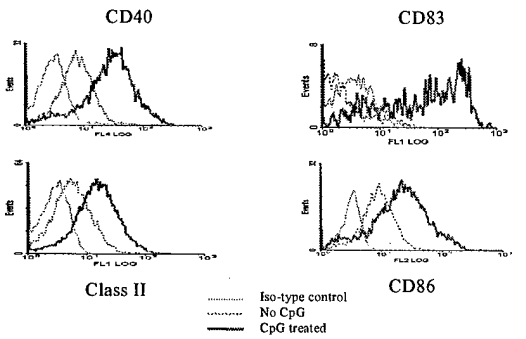


Fig 3. Upregulation of Maturation Markers by CpG DNA in a) pDC and b) mDC

b) CpG DNA-2 Upregulates mDC Marker Expression



Since CpG DNA-1 is commercially available and CpG DNA-2 is not, and since CpG DNA-1 has been tested for its ability to induce the maturation of pDC but not mDC in bulk peripheral blood, we tested whether CpG DNA-1 could induce the maturation of mDC. Thus, peripheral blood monocytes were grown in XVIVO medium containing 3 μM of CpG DNA-1. As shown in Fig. 5, this CpG DNA was equally effective in stimulating the maturation of both pDCs and mDCs.

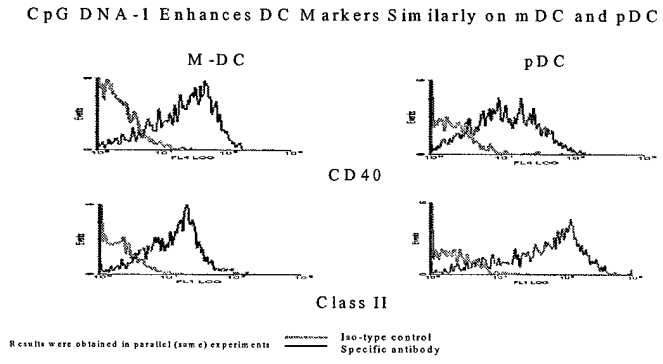


Fig. 4. CpG DNA-1 Is Equally Effective In Inducing The Maturation Of pDCs and mDCs

Therefore, we elected to use CpG DNA-1 in all of our subsequent experiments, as detailed below.

2. Test MCF-7 Tumor Lysate Uptake By DCs After CpG Stimulation And Determine Whether DC Maturation Markers Are Altered By Tumor Lysate Loading.

We observed that mDCs were efficient in taking up lysate from the MCF-7 human breast cancer cell line and that cell-surface DC marker expression was not reduced after tumor lysate loading (Fig. 6).

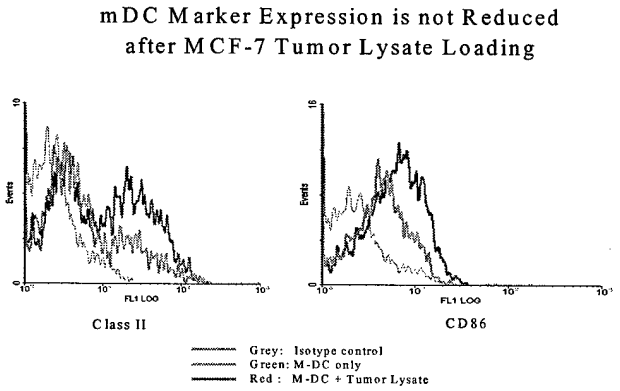


Fig. 5. mDC Marker Expression is not Reduced after MCF-7 Tumor Lysate Loading

3. Use Autologous DC-Primed T Cells In An In-Vitro Cytotoxicity Assay Against MCF-7.

When autologous CTLs were primed by mDCs loaded with MCF-7 tumor lysate, we observed a dose-dependent killing of MCF-7 (Fig. 6)

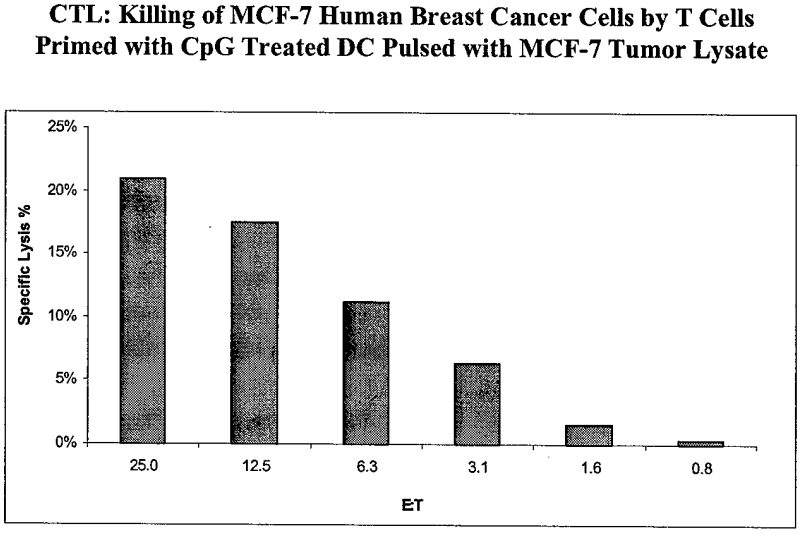


Fig. 6. Killing of MCF-7 Human Breast Cancer Cells by T Cells Primed with CpG DNA Treated DC Pulsed with MCF-7 Tumor Lysate

III. KEY RESEARCH ACCOMPLISHMENTS

Thus, we conclude that:

1. TS/A tumor lysate-loaded DCs and CpG DNA are an effective therapy for an established TS/A tumor in BALB/c mice.
2. We can generate, within 48 hours, a high number of functional DCs from bulk PBMCs using the commercially-available CpG DNA-1 as the sole DC maturation stimulus. A significant cost-saving is derived from the fact that expensive exogenous cytokines are not required for DC growth and there is no requirement for a column-selection methodology to isolate the activated DCs.
3. Human DCs stimulated by CpG DNA-1 and loaded with lysate from the MCF-7 human breast cancer cell line prime an effective cellular response against MCF-7. This is an important finding because it demonstrates that DCs that have been "matured" by CpG DNA still have the capacity to take up tumor antigen for processing and presentation to cytotoxic effector cells.

Therefore, based on the pre-clinical data detailed above, we have designed a Phase I/II clinical trial of DC + CpG DNA for women with metastatic breast cancer, as detailed below in Aim 3. As well, we are fortunate to have the support of Coley Pharmaceuticals which will provide us with clinical-grade CpG 7909 for the clinical trial (please see Letter of Support at the end of this report).

Aim 3. To Undertake A Phase I/II Clinical Trial Of Immunization With Autologous Tumor-Primed DCs And Dose-Escalated CpG DNA In Patients With Metastatic Breast Cancer.

a. Research Design And Methods

a.1 Rationale and Hypothesis

We hypothesize that CpG DNA-stimulated DCs will prime a potent anti-tumor immune response *in vivo* and we will test this hypothesis in a Phase I/II clinical trial of immunization with autologous tumor lysate-primed DCs and dose-escalated CpG DNA in patients with MBC. The rationale for this clinical trial derives from our pre-clinical data showing that (a) tumor-lysate loaded DCs and CpG DNA are effective in mediating the regression of an established murine mammary tumor *in vivo*, and (b) CpG DNA-1-stimulated human DCs can prime an anti-tumor cellular response against the human mammary carcinoma cell MCF-7.

NB: Since the trade designation for CpG DNA-1 by Coley Pharmaceuticals is CpG 7909, CpG DNA-1 will hereby be designated as CpG 7909 for the purposes of this clinical trial.

a.2 Outcome Measures

In the Phase I portion of the trial, we will (a) study the safety of and establish a starting dose for CpG 7909 in combination with the autologous DC vaccine, and (b) characterize the immunologic

response to autologous tumor induced in patients treated with the DC + CpG 7909 vaccine. In the Phase II portion of the trial, we will (a) measure the clinical tumor response to the DC + CpG 7909 vaccine and (b) characterize the immunologic response to autologous tumor induced in patients treated with the DC + CpG 7909 vaccine.

A. Clinical Endpoints

Phase I Trial. In Phase I, 5 groups of patients with measurable MBC will receive 2 sets of intradermal (i.d.) vaccinations given 7 days apart consisting of at least 10^6 tumor-loaded DCs admixed with an escalating dose (16, 32, 48, 64 and 80 mg) of CpG 7909. This dosing range of CpG 7909 has been previously tested in patients with cancer and found to be safe, with no grade III/IV toxicities (NCI Common Toxicity Criteria, v.3, http:// www.fda.gov/cder/cancer/ toxicityframe.htm) (A. Krieg, Coley Pharmaceuticals, personal communication). The safety of the CpG 7909 dosing tiers in combination with autologous DCs will be evaluated by treating 2 patients at each of the lower tiers and observing patients for toxicity for 2 weeks before treating a new patient at the next higher dose. Six patients will be treated at the maximum dose of CpG 7909 to more fully evaluate the toxicity profile at that level. If a grade III/IV toxicity is observed, the dose-escalation scheme will immediately switch to the conventional Phase I design for finding the MTD (3 to 6 patients per tier). Immunologic monitoring as detailed in Section d.2.2 below will also be undertaken.

Phase II Trial. The Phase II trial will commence at the starting dose determined in Phase I. The Phase II portion of the trial will be carried out as detailed in *Statistics* below. The endpoints of this phase of the clinical trial will be to (a) measure tumor response to the DC + CpG 7909 vaccine, and (b) define the immunologic response to autologous tumor induced in patients who have received the DC + CpG 7909 vaccine, as detailed in Section d.2.2 below. Objective evidence of tumor regression will be measured using the RECIST criteria (http://www3.cancer.gov/ dip/RECIST.htm).

B. Immunologic Endpoints

ELISPOT Assay. Peripheral blood (60 ml) will be drawn one week before the first vaccination and 7 weeks after the second vaccination, the latter to coincide with the planned radiological assessment for clinical response. Peripheral blood lymphocytes (PBL) (20 x 10^6) will be incubated in tissue culture tubes in 4 ml RPMI-1640, 10% heat-inactivated human AB serum, antibiotics, and 50 IU/ml rhIL-2. After 6 days, T cells will be evaluated in ELISPOT assays. Pre- and post-vaccination PBL (at least 10^7) will be separated into CD4+ and CD8+ fractions by selection on immunobeads. The enriched fractions will be counted and tested by flow cytometry to determine the percent of CD8+ and CD4+ T cells in each. The enriched fractions will be tested in 20h ELISPOT assays performed in triplicate wells, each containing 50,000 cells/well. The ratio of T cells to DC will be 10:1. The assays will be formatted as follows:

- ✧ CD8+T cells alone
- ✧ CD4+T cells alone
- ✧ CD8+T cells + irradiated tumor alone
- ✧ CD4+T cells + irradiated tumor alone

- ✧ CD8+T cells + unprimed DC
- ✧ CD4+T cells + unprimed DC
- ✧ CD8+T cells + tumor-loaded DC
- ✧ CD4+T cells + tumor-loaded DC
- ✧ CD8+T cells + Hepatitis B sAg
- ✧ CD4+T cells + Hepatitis B sAg
- ✧ CD8+T cells + DC + Hepatitis B sAg
- ✧ CD4+T cells + DC + Hepatitis B sAg

After incubation, the plates will be developed, and the numbers of spots in experimental and control wells will be counted, using a Zeiss image analysis system. A one-tailed permutation test at $\alpha = .05$ will be conducted to test the difference between experimental and controls wells. If the test is rejected, the ELISPOT assay results will be expressed as the difference between the mean number of spots in the experimental wells - mean number of spots in the control wells, or as the frequency of IFN-γ-secreting cells in total cells plated per well. If the permutation test is not rejected, the mean frequency of INF-γ-secreting cells in the experimental wells will be set to zero. The final results will be expressed as the frequency of CD4+ and CD8+ T cells responding to cryptic tumor antigens in the population tested. The expectation is that patients responding to the DC vaccination will demonstrate a significant difference in the frequency of responding CD4+ and CD8+ T cells before and after DC vaccination.

d.3 Statistics

Mr. Bill Gooding, a Biostatistician at the University of Pittsburgh Cancer Institute, will provide his ongoing input into this clinical trial. He is a Co-Investigator with salary support.

This is a combined Phase I/II clinical trial to find a starting dose of CpG 7909 and to evaluate the efficacy of the combination of CpG 7909 and autologous dendritic cells at the selected dose of CpG 7909. In the Phase I portion, the dose of CpG 7909 will be escalated in five tiers to 80 mg. The safety of the first 4 dose tiers will be evaluated by treating 2 patients at each tier and observing patients for toxicity for 2 weeks before treating a new patient at the next higher dose. At the highest dose, 80mg, 6 patients will be treated even if no grade III/IV toxicities are observed in order to more fully evaluate the toxicity profile at that level. If a grade III/IV toxicity (NCI Common Toxicity Criteria) is observed at any dose tier, the dose escalation scheme will immediately switch to the conventional Phase I design for finding MTD (3 to 6 patients per tier with de-escalation). If grade III/IV toxicities are observed, the MTD will be defined as the highest dose with fewer than 1/3 of patients experiencing a grade III/IV toxicity. If no grade III/IV toxicities are observed, 80 mg CpG 7909 will be chosen as the starting dose for the phase II portion of the study without declaring an MTD.

The Phase II trial will then commence at the starting dose determined in Phase I. The sample size will be based upon the two-stage design of Simon (32), which allows a trial to be stopped at the end of the first of two stages if the therapy under study shows a poor response rate. Twelve patients will be required for the first phase (including the last 6 patients on Phase I) and 35 patients will be needed for the complete trial provided the trial is not stopped at the end of Stage I due to poor response. The sample size has been chosen as the sample size with the smallest expected total sample size among designs with the following characteristics: The rate of clinical response that is considered too low to warrant further study is 10%; the minimum

response rate that would signify that the therapy would merit further study is 30%; type I and type II error rates were both set to 10%. The therapy will be rejected as a candidate for further study if a) there are fewer than 2 clinical responses among the 12 patients treated on the first stage – in which case the trial will be stopped, or b) the second stage is completed with 5 or fewer responses among the 35 patients treated on both stages.

Data analysis on both the phase I and phase II portions of the study will characterize immunologic response. The immunologic measurement to be evaluated includes pre and post-vaccination T cell reactivity as measured by the ELISPOT assay obtained from PBMC samples at baseline and 7 weeks post-vaccine. Estimates of changes in T cell reactivity and confidence intervals will be calculated. Vaccine-mediated effects will be examined via either a signed rank test for continuous data or McNemar's test for binary data. Since this is a vaccine trial with a variable number of tumor-pulsed dendritic cells available for the vaccine, immunogenic and clinical responses will be compared to the final number of delivered DCs in the vaccine as well as any phenotypic or functional characteristics of the vaccine.

d4. Potential Pitfalls

As detailed above, potential pitfalls for this clinical trial include an insufficient overall clinical response and/or excess toxicity. In both instances, strict stopping rules have been incorporated into this clinical trial for patient safety and benefit. If there is an insufficient overall clinical response, the clinical trial will be stopped as detailed above. If excess toxicity is encountered in spite of these adjustments, the trial will be stopped.

b. Human Subjects

b.1 Clinical Protocol

b.1.1 Patient Selection

- Patients must have biopsy-proven MBC.
- Patients must have sufficient tumor (at least 10^9 cells, or a 1 cm^3 nodule) to harvest for the DC vaccine preparation.
- Patients must have radiologically-measurable residual MBC after resection of the tumor nodule harvested for the DC vaccine.
- Patients must have reactivity to at least 1 of 3 PCI skin tests (see below).
- Patients must have fully recovered from surgery, and must not have received any chemotherapy, radiotherapy, hormonal therapy, or immunotherapy within 4 weeks preceding vaccination.
- Patients with treated and stable brain metastases are eligible.
- Patients requiring therapy with steroids are not eligible.
- Patients must have an expected survival of at least three months.
- Patients must have an ECOG Performance Status (PS) of ≤ 1 (Karnofsky PS ≥ 80).
- Patients must have the following initial and subsequent pretreatment laboratory parameters:

  Granulocytes: $\geq 1{,}500/mm^3$
  Lymphocytes: $\geq 700/mm^3$

Platelets: $\geq 100{,}000/mm^3$
Serum Creatinine: ≤ 2.0 mg/100 ml
Serum Bilirubin: ≤ 2.0 mg/100 ml

- Patients must be able to give written informed consent.
- Patients must not be pregnant or lactating.

b.1.2 PCI Skin Test

- Following evaluation and meeting the eligibility criteria noted above, the multitest PCI skin test with antigens for cellular hypersensitivity will be applied in initial patient screening (≤ 4 weeks prior to 1st DC vaccination).
- Each test contains a disposable applicator which is preloaded with 1 of 3 antigens for i.d. injection, including tetanus, tuberculin, Candida, and a normal saline solution control.
- Induration will be measured at 24 and 48 hours by a Study Investigator or an assigned representative (Nurse, PA, NP) and must measure ≥ 2 mm.
- Patients must have reactivity to at least 1 of the 3 antigens to be eligible for entry into the clinical trial.

b.1.3 Treatment Plan

All patients must start their treatment within 4 weeks of a positive PCI skin test.

Tumor Cell Harvest

- Tumor cells may will be collected either from solid metastatic deposits or from malignant effusions.
- Tumor cells will be delivered to the IMCPL for DC vaccine preparation, as detailed below.

DC Vaccine Preparation

- All patients will undergo a single leukapheresis, processing two-and-a-half times the patient's blood volume to obtain cells for the generation of DCs. The product will then be delivered to the IMCPL for processing.
- DC precursors will be cultured for 2 days in serum-free XVIVO® medium containing 6 µg/ml CpG 7909 to generate functional DCs.
- Autologous tumor cells obtained from patients will undergo 5 freeze/thaw cycles to induce cell lysis.
- DCs will be then be pulsed overnight with the tumor lysate (5:1 ratio of tumor cells to DC) or with hepatitis B surface antigen (HBSA) @ 37°C and washed next day 3 times with sterile saline. Since patients will be injected at 8 different sites as described below, 7/8 of the total DCs will be pulsed with tumor lysate, and 1/8 DCs will be pulsed with the recall hepatitis B surface antigen.
- The lysate-pulsed DCs will then be tested for sterility, purity (by flow cytometry), endotoxin and mycoplasma.
- The DC vaccine will then be split into 3 aliquots:
  - ⅓ of the DC vaccine will be used for the 1st DC vaccination (Day 1).

- ⅓ of the DC vaccine will be frozen, and then thawed and cultured for 1 day prior to the 2nd DC vaccination (Day 8).
- ⅓ of the DC vaccine will be frozen and used for the *in-vitro* assays detailed below.

DC Vaccine Administration

- For each scheduled day of vaccination, DCs will be counted, equally distributed among 8 syringes (i.e., 7 syringes containing the tumor-loaded DCs and 1 syringe containing the HBSA-pulsed DCs) and mixed with the scheduled dose of CpG 7909.
- Patients will be administered the DC + CpG 7909 vaccine i.d. on Days 1 and 8 at 8 different sites using the right and left anterior chest, proximal arms, right and left lower abdomen and proximal thighs for each vaccination.
- It is expected that a total of at least 10^6 DCs will be administered with each day of vaccination.
- Phase-I Dosing of CpG 7909. In the Phase I portion of this trial, we plan to dose-escalate CpG 7909 in 5 dose tiers (see below). Since patients will receive 2 vaccinations 7 days apart in each dose tier, a total of 16 i.d. injections will be administered to each patient (i.e., 8 injections on Day 1 and 8 injections on Day 8). Therefore, the total scheduled dose of CpG 7909 for each dose tier will be equally distributed among 16 syringes (i.e., each syringe will receive 1/16 of the total scheduled dose of CpG 7909 for a given tier). With respect to dose selection for each tier, patients enrolled into clinical trials using CpG 7909 as a single agent have been treated to a dose of 40 mg per week without major toxicities (defined as Grade III-IV toxicities by Common Toxicity Criteria) (A. Krieg, Coley Pharmaceuticals, personal communication). Since we plan to treat 5 cohorts of patients in the Phase I portion of this trial, we will escalate CpG 7909 in 5 dose tiers comprising 16 injections per tier (i.e., 8 injections on Day 1 and 8 injections on Day 8). Since our maximal planned dose of CpG 7909 will not surpass the recommended maximal dose of 40 mg per week (or a total of 80 mg for Days 1 and 8), the dose-escalation schema of CpG 7909 to be administered in tiers 1-5 will be 16, 32, 48, 64 and 80 mg, respectively, and will be apportioned as detailed in the table below.

Tier	Day-1 (mg/syringe)	Day-1 Total (mg/syringe x 8 syringes)	Day-8 (mg/syringe)	Day-8 Total (mg/syringe x 8 syringes)	Total Dose (mg) (Days 1 and 8)
1	1	8	1	8	16
2	2	16	2	16	32
3	3	24	3	24	48
4	4	32	4	32	64
5	5	40	5	40	80

- Phase II Dosing of CpG 7909. The starting dose of CpG 7909 for the Phase II portion of the trial will be the MTD determined in Phase I. Similarly to the Phase I trial, patients will receive 2 vaccinations 7 days apart, for a total of 16 i.d. injections (i.e., 8 injections on Day 1 and 8 injections on Day 8). Therefore, the Phase II dose of CpG 7909 will be equally distributed among the 16 syringes (i.e., each syringe will receive 1/16 of the total scheduled dose of CpG 7909) for i.d. administration with DCs.

- All patients will be treated in the NIH-funded General Clinical Research Center (GCRC) of the UPCI.

Additional Vaccinations

- If a patient has either a partial response or stable disease by RECIST criteria, the patient will be eligible for another 2 sets of DC + CpG DNA vaccination, as detailed above in *DC Vaccine Administration*, using the same dose of CpG DNA as given in the previous 2 sets of vaccination. However, additional vaccination will require a repeat leukapheresis to generate DCs and repeat harvesting of tumor cells.
- If a patient has a complete response, no further vaccinations will be administered and the patient will be followed up at 3-month intervals as detailed below in *Long-Term Follow-Up*.
- If a patient has progressive disease, no further vaccinations will be administered.

b.1.4 Treatment Evaluations

Pre-Treatment

Following evaluation and meeting the eligibility criteria detailed above, all patients will undergo the following investigations ≤ 4 weeks prior to vaccination:

- History & physical examination
- Bloodwork:
  - ✧ CBC, differential, platelets
  - ✧ Electrolytes, BUN, creatinine
  - ✧ AST, ALT, alkaline phosphatase, total bilirubin, albumin, Ca++
  - ✧ Anti-HIV-1/2, HBsAg, anti-HCV, anti-HTLV-I/II, anti-HBc, HIV-1 p24 antigen, anti-CMV, and syphilis prior to leukapheresis
  - ✧ 60 ml blood draw for immune monitoring (see Section d.2.2) (≤ 1 week prior to 1st vaccination)
- Radiology:
  - ✧ CT of the chest and abdomen
  - ✧ Total body bone scan
  - ✧ MRI or CT scan of the brain

During Treatment

At 3 weeks after the 2nd (i.e., Day-8) vaccination, patients will undergo:

- History & physical examination
- Bloodwork:
  - ✧ CBC, differential, platelets
  - ✧ Electrolytes, BUN, creatinine
  - ✧ AST, ALT, alkaline phosphatase, total bilirubin, albumin, Ca++

Post-Treatment

At 7 weeks after the 2nd (i.e., Day-8) vaccination, patients will undergo:

- History & physical examination
- Bloodwork:
  - ✧ CBC, differential, platelets
  - ✧ Electrolytes, BUN, creatinine
  - ✧ AST, ALT, alkaline phosphatase, total bilirubin, albumin, Ca++
  - ✧ 60 ml blood draw for immune monitoring (see section d.2.2)
- Radiology:
  - ✧ CT of the chest and abdomen
  - ✧ Total body bone scan (if previously positive)
  - ✧ MRI or CT scan of the brain (if previously positive)

Long-Term Follow-Up

For patients who have either responded to therapy or have stable disease and are not receiving any further therapy, follow-up will be every 3 months in the outpatient clinic with:

- History & physical examination
- Bloodwork:
  - ✧ CBC, differential, platelets
  - ✧ Electrolytes, BUN, creatinine
  - ✧ AST, ALT, alkaline phosphatase, total bilirubin, albumin, Ca++
- Radiology:
  - ✧ CT of the chest and abdomen
  - ✧ Total body bone scan (if previously positive)
  - ✧ MRI or CT scan of the brain (if previously positive)

Schedule Of Evaluations / Study Calendar

Parameter	*Pretreatment (≤4 weeks from 1st vaccination)*	*Treatment (1st and 2nd vaccinations)*	*Post-Treatment (7 Weeks after 2nd vaccination)*	*Long-Term (Every 3 months)*
PCI Skin Test	X			
History	X	X	X	X
Physical Examination	X	X	X	X
Weight	X	X	X	X
Vital Signs	X	X	X	X
Performance Status (ECOG)	X	X	X	X
CBC, differential, platelets	X	X	X	X
Electrolytes (Na, K, Cl, CO_2), BUN, creatinine	X	X	X	X
AST, ALT, alkaline phosphatase, bilirubin, albumin, Ca++	X	X	X	X

Anti-HIV-1/2, HBsAg, anti-HCV, anti-HTLV-I/II, anti-HBc, HIV-1 p24 antigen, anti-CMV and syphilis	X			
Serum HCG	X			
Peripheral Blood for Immune Monitoring	X (≤1 week prior to 1st vaccination)		X	
Chest CT Scan	X		X	X
Abdominal CT Scan	X		X	X
Brain CT/MRI Scan	X		X (if initially positive)	X (if initiall positive)
Total Body Bone Scan	X		X (if initially positive)	X (if initiall positive)

b.1.5 Research Sample Collection, Transport, Delivery

- Blood for immune monitoring (Section d.2.2) will be drawn from a peripheral vein into 6 green-top tubes (60 ml) no more than 1 week prior to, and 7 weeks after completion of the DC + CpG 7909 vaccination.
- The blood will be drawn either in the GCRC prior to the 1st DC vaccination, or in the outpatient clinic of either the Hillman Cancer Center of the UPCI or the Breast Cancer Program Clinic of Magee Women's Hospital ≤1 week prior to 1st vaccination and delivered to the IMCPL.

b.1.6 Setting For Study

- Patients will receive the DC + CpG 7909 vaccine in the GCRC of the University of Pittsburgh.
- Outpatient follow-ups will be either at the Hillman Cancer Centre of the University of Pittsburgh Cancer Institute or the Breast Cancer Clinic at Magee Womens Hospital of the University of Pittsburgh Medical Center.

b.1.7 Adverse Events

Definitions

- A serious adverse event (experience) or reaction is any untoward medical occurrence that, at any dose, results in death, is life-threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability / incapacity, or is a congenital anomaly / birth defect.
- The definition of serious adverse event (experience) also includes important medical event. Medical and scientific judgment should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the other outcomes listed in the

definition above. These should also usually be considered serious. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

- The definition of "related" is that there is a reasonable possibility that the drug caused the adverse experience.

Treatment-Related Toxicities

- Grade 2 hypersensitivity reactions are reportable only if symptoms recur after rechallenge.
- Grade 4 expected myelosuppression need not be reported but should be documented.
- Any death from any cause while a patient is receiving treatment on this protocol or up to 30 days after the last dose of protocol treatment, or any death which occurs more than 30days after protocol treatment has ended which is felt to be treatment related, must be reported.

Non Treatment-Related Toxicities

- Toxicities which fall within the definitions listed above must be reported as an ADR/second primary regardless if they are felt to be treatment related or not.
- Toxicities unrelated to treatment that do not fall within the definitions above, must simply be clearly documented.

Criteria For Removal Of Patients From Study

- Patients will be removed from the study under the following circumstances:
  - ✧ Disease progression.
  - ✧ Extraordinary medical circumstances: If at any time the constraints of this protocol are detrimental to the patient's health, the protocol treatment will be discontinued.
  - ✧ Patient withdraws consent.
  - ✧ Patient in whom treatment is delayed for $\geq$ 3 weeks.
- Patients who are removed from study treatment are followed until death.

b.1.8 Estimated Patient Accrual And Trial Duration

- Phase I: 14 patients / 12 months.
- Phase II: 21 patients / 12 months.

b.2. Protection of Human Subjects

b.2.1 Risks to the Subjects

Human Subjects Involvement and Characteristics. Up to 14 patients will be required for the first phase of the trial (including the last 6 patients on Phase I) and approximately 35 patients will be

needed for the complete trial, as described in Section d.3 ("Statistics"). Inclusion and exclusion criteria for enrollment into the clinical trial are detailed in Section e.1.1 ("Human Subjects – Patient Selection").

Sources of Material. DC precursors will be collected from peripheral blood by leukapheresis and tumor material will be collected and processed in the IMCPL of the Hillman Cancer Center, as described in Section e.1.3 ("Treatment Plan").

Potential Risks. The most common and serious side effects for this study are listed in Section e.1.9 ("What Are The Possible Risks, Side Effects, And Discomforts Of This Research Study?"). In addition, there is always the risk of very uncommon or previously unknown side effects occurring, including death.

b.2.2 Adequacy of Protection Against Risks

Recruitment and Informed Consent. Subjects will be recruited from the outpatient clinic of the Magee Womens Hospital Comprehensive Breast Cancer Program of the University of the Pittsburgh Medical Center, as well as from referrals from oncologists who are members of the University of Pittsburgh Medical Center (UPMC) Cancer Centers Network, or from oncologists outside the UPMC network. The participating oncologists will identify patients who may meet the eligibility criteria for the study. If patients express an interest in learning more about the proposed study, they will sign a HIPAA consent form allowing them to be approached by the study coordinator. The study coordinator will then discuss the study with the patients and will confirm eligibility with the principal investigator or co-investigator. The study coordinator and the principal investigator or co-investigator will then explain the study to patients and answer any questions posed by individuals who are interested and eligible. These individuals will then be asked to sign the informed consent form.

Protection Against Risk. As a safety measure, all patients will be treated in the outpatient facility of the GCRC of the Hillman Cancer Center, University of Pittsburgh Cancer Institute, where close monitoring of patients by skilled nursing staff and physicians experienced with the administration of DC-based vaccines is ensured. In the event of severe toxicities, patients will be admitted across the street to the oncology inpatient ward of Shadyside Hopital, University of Pittsburgh Medical Center, for expert care. With respect to patient confidentiality, patient records will at all times be treated as confidential documents, and data collection will adhere to the strictest concern for patient privacy and confidentiality. Every effort will be undertaken to minimize the risk to confidentiality of data and to anonymity of subjects. All subjects will be assigned a unique code number under which all data will be stored. All information will be backed up on a nightly basis. Security of data will be upheld through the use of password protection and restricted access to users. Consent forms and a list of the match between subject names and code names will be retained in a locked file cabinet. All staff will be required to sign a confidentiality agreement and to complete the following online education modules sponsored by the Research Conduct and Compliance Office of the University of Pittsburgh: 1) Research Integrity; 2) Human Subjects Research; and 3) HIPAA Researchers Privacy Requirements, prior to contact with any subject or any data. Data safety and monitoring will be undertaken as detailed is Section e.6 below ("Data Safety and Monitoring Plan").

b.2.3 Potential Benefits of the Proposed Research to the Subjects and Others

Subjects may not directly benefit from participating in this study and the DC vaccine that they receive may be harmful to them. As with any investigational study, there may be adverse events or side-effects that are currently unknown, and it is possible that certain of these unknown risks could be permanent, serious, or life-threatening. Nevertheless, based on the preliminary data presented in this proposal, there is strong evidence to suggest that DC-based vaccines are successful in the treatment of patients with advanced cancer and it is possible that the addition of CpG DNA to our DC vaccine will improve the clinical outcome of patients with metastatic breast cancer.

b.2.4 Importance of the Knowledge to be Gained

While there are already many published reports of DC-based vaccines for the therapy of patients with cancer, the innovative significance of this proposal lies in the fact that, for the first time, a DC-based clinical trial will be undertaken using CpG DNA to enhance the immune-priming function of DCs. This immunotherapeutic approach will (a) allow us to ascertain how the generation of an anti-tumor immune response as measured in peripheral blood correlates with clinical response, and (b) provide a scientific basis for the future development of novel DC- and CpG DNA-based therapies of breast cancer, whose evolution will be primed by the results obtained in this clinical trial.

b.3. Inclusion of Women

There will be no gender restriction in this clinical trial. Because of unknown risks to the fetus with DC vaccination and/or CpG DNA, women of childbearing potential will be carefully counseled prior to starting therapy.

b.4. Inclusion Of Minorities

Patients enrolled in the clinical trial detailed in this proposal will be individuals who have metastatic breast cancer. Patients will be selected to include both genders as well as major efforts to include minority populations. The population of patients that we draw from our region is ~12% minority (see table below). A concerted effort will be made to engage those patients who are limited in their access to patient care.

	American Indian or Alaskan Native	Asian or Pacific Islander	African American	Hispanic	White, not of Hispanic origin	Other or Unknown	Total
Female	0	0	3	1	31	-	35
Male	0	0	0	0	0	-	0
Total	0	0	3	1	31	-	35

b.5. Inclusion Of Children

Although breast cancer is rare in patients <21 years old, children will not be specifically excluded from this clinical trial.

b. 6. Data And Safety Monitoring Plan

All Phase I and II clinical trials are discussed and reviewed at a weekly IRB approved meeting every Monday at 8:30 a.m. where clinicians, nurses, protocol coordinators, statisticians, scientists and technicians review all the patients enrolled in Phase I and II clinical trials. Patients are reviewed as to toxicity response, any untoward or adverse events, laboratory correlates, pharmacokinetic and pharmacodynamic parameters at each dose level. Toxicity is reported to the group, which is chaired by Dr. Merrill Egorin. Decisions to continue treating subjects at the next dose level and/or if the trial accrual should continue are also discussed during these meetings. Any modifications necessary to ensure patient safety are discussed and modifications will be submitted to the IRB. All serious adverse events will be reported to the IRB according to the established guidelines. Serious adverse events will also be reported to the sponsor and/or other regulatory agency as per their requirements. Toxicity and safety data is further evaluated monthly at the Protocol Review Committee of the University of Pittsburgh Cancer Institute (UPCI). All study data reviewed and discussed during these meetings will be kept confidential. The decisions and analysis of safety and toxicity issues are made by this panel by consensus. All data which is stored in a secured database in UPCI, will be submitted to the NABTC and entered on a secured computerized Protocol Data Management System (PDMS) at the NABTC Data Management Center. Only those individuals directly involved with this study will have access to this secured information. The NABTC Data Management Center Protocol Manager will perform the ongoing protocol compliance with the support of the study investigators. All toxicities encountered during the study will be evaluated on an ongoing basis according to the NCI Common Toxicity Criteria version 2.0 and recorded prior to each course of therapy. In addition, all toxicities will be reported to the IRB along with any Data Safety meeting minutes in the annual renewal report. Life-threatening toxicities will be reported immediately to the NABTC Study Chairman, the University of Pittsburgh's IRB, and the FDA according to established guidelines. Investigators will receive telephone notification of life-threatening revisions with follow-up by fax and e-mail from the NABTC. Life-threatening protocol revision will be implemented immediately and will be reported to the institutional IRB. The NABTC Study Chairman will be the final arbiter of response or toxicity should a difference of opinion exist. Any modifications necessary to ensure patient safety are discussed and modifications will be submitted to the IRB in compliance with the IRB's policy for the reporting of adverse events as outlined in Chapter 3.0, sections 3.4 and 3.5 of the IRB Reference Manual.

III. REPORTABLE OUTCOMES

Two papers will be written to describe the data generated in Aims 1 and 2 after planned mechanistic studies are completed. Furthermore, a third paper will be generated encompassing the data derived from the planned clinical trial

IV. CONCLUSION

Based on our data, we conclude that CpG DNA-stimulated DCs prime a potent anti-tumor immune response.

V. REFERENCES

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VI. LETTER OF SUPPORT

Please find appended a letter of support from Coley Pharmaceuticals who will be supplying CpG 7909 for this clinical trial.

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April 8, 2004

Joseph Baar, MD, PhD
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Dear Dr. Baar:

Thank you for submitting your proposal for an external collaboration with Coley Pharmaceutical Group. The Coley external collaborations committee met on March 11, 2004 to review your proposal.

We are pleased to inform you that your proposal (*A Phase I/II Clinical Trial of Immunization with Autologous Tumor-Primed Dendritic Cells and CpG DNA in Patients With Metastatic Breast Cancer)* was approved. Ross Pettit, Senior Director, Global Clinical Development, will be in contact with you soon to discuss the process for moving ahead with the collaboration. We are excited about your research and look forward to working with you.

Regards,

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