FEASIBILITY, SAFETY, AND EFFICACY EVALUATION OF ALPHA-TYPE-1 DENDRITIC CELL (DC)-BASED VACCINES LOADED WITH ALLOGENEIC PROSTATE CELL LINE IN COMBINATION WITH ANDROGEN ABLATION IN PATIENTS WITH PSA PROGRESSION AFTER LOCAL THERAPY FOR PROSTATE CANCER

UPCI Protocol #:	06-070
BB-IND:	13,061
Version date:	06/02/2017
Phase:	2
Agents:	Alpha-type-1 Dendritic Cell Vaccine

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NCT#00970203

ABBREVIATIONS

αDC1	alpha-type-1-polarized DC (monocyte-derived DC generated in the presence of GM-CSF and IL-4 and matured under the influence of IL-1β/TNFα/p:IC/IFNγ/IFNα β□□
Ag	antigen
CTL	cytotoxic T lymphocyte
DC	dendritic cells
GMP	good manufacturing practice
i.d.	intradermal
IL-	interleukin-
i.l.	intralymphatic
IFN	interferon
IMCPL	Immunomonitoring and Cell Products Laboratory (cGMP facility of the UPCI, specialized in preparation of cell-based vaccines)
IRB	Institutional Review Board
NK	natural killer
PAP	prostatic acid phosphatase
PCa	prostate cancer
PGE2	prostaglandin E2
pI:C	polyinositic: polycystydylic acid
PRC	Protocol Review Committee
PSA	prostate specific antigen
PSA-DT	PSA doubling time
PSMA	prostate specific membrane antigen
s.c.	subcutaneous
Th	T helper
TNF	tumor necrosis factor
UPCI	University of Pittsburgh Cancer Institute

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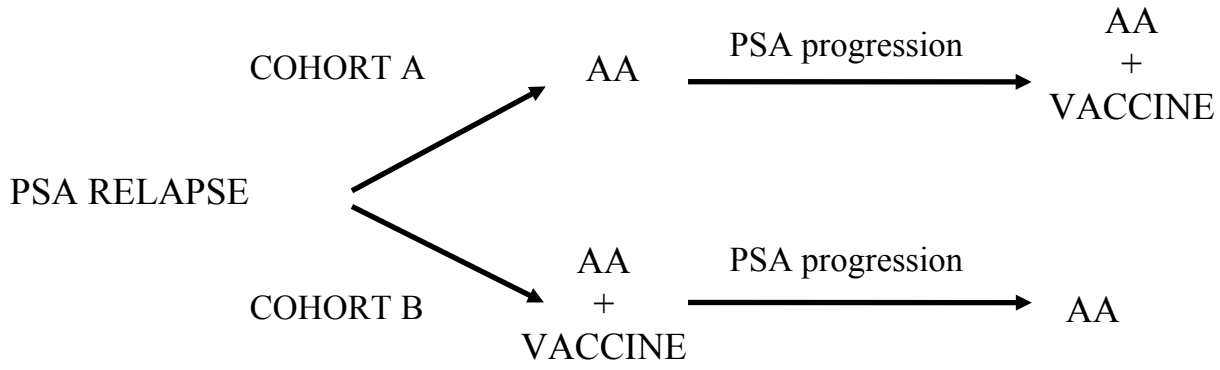
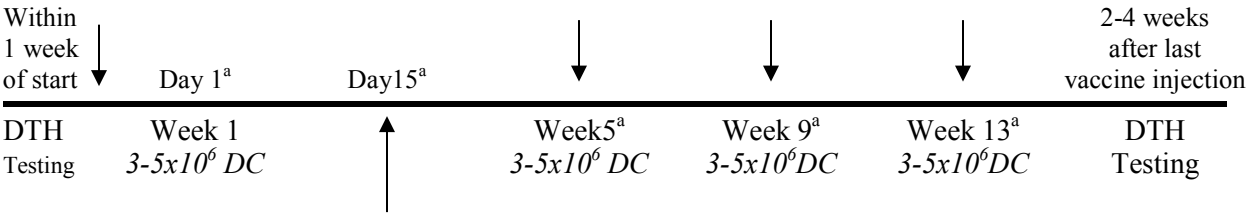
SUMMARY

This study will evaluate the feasibility, safety, and efficacy of intradermal vaccination of prostate cancer patients with alpha-type-1-polarized dendritic cells (αDC1) loaded with apoptotic allogeneic tumor (LNCap). The study will target men with recurrent prostate cancer, who failed local therapy, have no measurable metastasis, but have a rising PSA with a doubling time of less than 10 months. The selection of this study group enables us to evaluate **time to PSA progression**, a highly relevant, **clinical primary endpoint of efficacy in this two arm study.** In order to facilitate infiltration of vaccination-induced T cells into tumor site(s) and to reduce tumor-specific tolerance, **subjects will receive the vaccine in combination with limited androgen ablation (AA)** with a LHRH analogue for 3 months. Subjects will be randomly assigned to one of two cohorts. In cohort A subjects will be first treated with limited AA alone for 3 months, and at the time of PSA relapse (PSA ≥ 1 ng/dL) will receive the DC vaccine in conjunction with AA. In cohort B, the sequence of treatment will be reversed. Efficacy will be estimated as the within-subject difference in time to PSA relapse following the combination treatment as compared to the AA alone, thus, each subject will serve as his own control. All subjects will commence the αDC1-based vaccination 2 weeks prior to treatment with the LHRH analogue. Each subject will receive 1 *intradermal (i.d.)* dose of the vaccine at weeks 1, 5, 9, and 13 for a total of 4 doses. Additional courses of vaccination may be administered to subjects without evidence of disease progression every 3 months (±1 month) for up to 12 months depending on the number of doses originally produced and available after the 4 intended protocol doses. All doses of the vaccine will be administered intradermally *(i.d.)*.

STUDY DESIGN



VACCINE SCHEMA



***Lupron 22.5 mg** i.m. **or Zoladex 10.8 mg** s.c.*
The LHRH analogue will be administered
2 weeks after the 1st dose of the vaccine.

Study cohorts:

- **Cohort A:**
  LHRH analogue or limited androgen ablation (AA) followed by Vaccine + AA at time of PSA relapse

- **Cohort B:**
  Vaccine + AA followed by AA at time of PSA relapse

[a] Vaccine injections may be delivered on a ± 2 workday schedule.

1 OBJECTIVES

Dendritic Cell (DC)-based vaccination is a new treatment option for cancer patients. While the previous DC-based vaccination trials have shown the safety of this approach and its ability to induce objective clinical responses, the overall efficacy of DC-based vaccines is still disappointing (Rosenberg et al., 2004). Our preliminary in vitro data indicate that type-1-polarized DC (αDC1) are 40-fold more potent than the current "gold standard" of clinically-applied DC (sDC) in their ability to induce tumor-specific CTLs (see Appendix I for the Summary of the Preclinical Data and the Protocol and Appendix II for our original publication describing CDC1 (Mailliard et al., 2004)). Thus this trial will enroll subjects with prostate cancer who have failed local therapy and present with PSA-only disease. At the time of study entry, subjects must have a PSA doubling time of less than 10 months and an absolute PSA level of > 2.0 ng/mL. This is a two-arm study, whereby subjects will either be randomized to limited androgen ablation (AA) for 3 months followed by the DC vaccine + AA at the time of PSA relapse (PSA > 1 ng/dL) or the converse sequence of treatment. Thus all subjects will be treated with both AA as well as AA + vaccine, with each subject, in essence, serving as his own control, in estimating the time to PSA recovery following limited AA. The αDC1 vaccine will be administered every 4 weeks x 4 and then, if vaccine is available from the original production, every 3 months (±1 month) in subjects who continue to respond.

1.1 Primary Objectives

- Feasibility objective: the ability to successfully generate and administer the alpha-DC1 vaccine.
- Safety objective: assess the tolerability and toxicity of the alpha-DC1 vaccine.
- Efficacy objective: evaluate the effect of the alpha-DC1 vaccine on time to PSA progression compared to AA alone. PSA progression is defined as a rise in the PSA value to ≥ 1.0 ng/mL.

1.2 Secondary Objectives

- To determine the change in PSA velocity prior to and following the proposed treatment.
- To evaluate (in all subjects) the vaccination-induced DTH responses to LNCap, the cell line vaccine, and to compare this with vaccination-induced responses to tumor-untreated antigen (KLH).
- To evaluate the vaccination-induced changes of Th1/Th2 profiles of the responses to PAP and PSMA.
- To evaluate the CTL responses in blood to the whole LNCap cells (in all subjects) and (in all subjects who are HLA-A2 positive) the CTL responses to HLA-A2.1 restricted peptides derived from PAP and PSMA.
- To comprehensively evaluate the $CD4^+$ and $CD8^+$ T cell responses (fine specificity and Th1/Th2/T_{reg} cytokine profile) to the previously-identified and novel immunogenic epitopes of PAP and PSMA, using the EPIMAX system.

2 BACKGROUND AND RATIONALE

2.1 Prostate Cancer

In 2007, approximately 225,000 new patients will be diagnosed with prostate cancer (PCa) in the US, and about 31,000 will succumb to the disease (most from metastatic disease) (Jemal et al., 2005). Standard treatment of organ-confined PCa includes surgery or radiation therapy, and is effective in the short term, but up to one third of patients relapse. Current systemic therapy (androgen ablation and chemotherapy) for PCa is limited, primarily palliative, and is associated with significant morbidity (Vogelzang, 1997). Most patients with advanced disease are treated with hormonal therapy and/or chemotherapy, and go on to develop resistance to both these modalities (DiPaola, 1999; Tannock et al., 2004). Prostate-specific vaccine therapy, if effective and if initiated early, i.e., at the time of initial relapse, offers the prospect of prolonging survival with minimal morbidity. Thus, we have chosen to focus on men with PCa, with a PSA relapse after either surgery and/or radiation therapy (Catalona and Smith, 1998; Dillioglugil et al., 1997; Roach et al., 1999). This population of patients has less tumor burden, less molecular mechanisms of drug resistance, and may be more likely to respond to immune approaches to therapy (Blades et al., 1995; Sanda et al., 1995). Men with PSA-only disease also represent the largest sub-population of patients with PCa, for whom there is no standard treatment. Furthermore, they represent a heterogeneous group, with widely varying PSA-doubling times (Bubley et al., 1999; Pound et al., 1999).

2.2 PSA Progression after Local Therapy

Patients with PSA progression after local therapy represent an ideal population for the study of novel immune approaches. Current therapy for patients with PSA progression after local therapy is unclear, and a subset of patients can be selected with greater likelihood of progression to metastatic disease, supporting the need for novel approaches. Based on recent literature, men with a PSA-doubling time (PSA-DT) of less than 10 months are at the highest risk of rapid progression and are often offered androgen ablation therapy, either on an intermittent or a continuous schedule (Gulley et al., 2005; Pound et al., 1999). However, studies have not been completed to determine a benefit of early androgen ablation therapy in patients with PSA progression after local therapy (Gulley et al., 2005). Prior studies of adjuvant androgen ablation therapy have demonstrated a benefit in only select populations of patients (Hanks et al., 2003; Lawton et al., 2001; Messing et al., 1999). Two studies demonstrated a survival benefit of adjuvant androgen ablation in concert with definitive radiation therapy in patients with locally advanced prostate cancer (Hanks et al., 2003; Lawton et al., 2001). Another study demonstrated a survival benefit to androgen ablation therapy for stage D1 disease after surgery (Messing et al., 1999).

Predictive factors for a high risk of PSA-recurrence after local therapy have also been identified. Thus Pound et al. demonstrated that time to biochemical progression, Gleason score, and PSA

doubling time were predictive of the probability and time to the development of metastatic disease (Pound et al., 1999). Recently, D'Amico et al. also demonstrated the predictive value of PSA-DT, both pre-operatively as well as after definitive local therapy (D'Amico et al., 2004). These data support using Gleason score and PSA-DT as entry criteria to select and stratify patients at high risk of disease recurrence for novel approaches to systemic therapy.

Our target population for treatment will be men with prostate cancer, who have relapsed following surgery and/or radiation, and now have a rising PSA with a PSA doubling time (PSA-DT) of less than 10 months. This patient population is ideal for an immunologic intervention given that: i) this is the largest sub-population of men with prostate cancer in the U.S.; ii) there is no standard treatment for this sub-group; and iii) this sub-group has minimal systemic disease burden. We will carefully monitor immunological responses to vaccination at different time points, but the primary endpoint of vaccine efficacy will be time to PSA progression and stabilization of PSA levels. Several recent vaccine studies have validated failure of PSA progression as a good surrogate endpoint for lack of progression in clinical trials in prostate cancer (Bubley et al., 1999; Kelly et al., 1993; Scher et al., 2004).

2.3 Dendritic Cells in Tumor Immunology

Several lines of evidence derived from both murine studies and human clinical trials suggest that cancer can be susceptible to immune-based therapies. Studies using dendritic cells (DCs), (Steinman, 1991; Steinman and Cohn, 1973) to stimulate tumor-specific immune responses have been particularly encouraging (Banchereau and Steinman, 1998; Nestle et al., 1998; Schuler and Steinman, 1997; Steinman, 1991). DCs are the most potent antigen presenting cells (APCs), capable of efficiently internalizing and presenting antigen in the context of co-stimulatory signals and cytokines essential to the induction of effective long-lasting T-cell mediated immunity. Animal models have demonstrated that DCs, pulsed ex vivo with defined tumor antigens or material derived from tumor cells, can induce protective tumor-specific immune responses and are capable of mediating the regression of established disease (Zitvogel et al., 1996). Human clinical trials, including our own, demonstrate that DCs pulsed with defined tumor-relevant antigenic peptides, or, alternatively, with tumor cell lysates or apoptotic bodies, can induce tumor-specific-specific immune responses and even occasional complete tumor regression in late stage cancer patients (Banchereau et al., 2001; Nestle et al., 1998).

Several competing approaches to DC therapy are currently being evaluated in clinical trials. In this context, two emerging issues appear to be critical for the development of "optimal" DC-based immunization strategies. First, defining a strategy for the delivery of tumor antigens that facilitates efficient DC presentation of a broad range of class I- and class II-restricted epitopes appears to be critical to the induction of effective antigen-specific T-cell immunity. Second, recent results suggest that in order to induce tumor regression and promote long-term disease-free status, DC-based strategies need to drive the in vivo expansion and maintenance of

Th1/Tc1-type T-cells (both $CD8^+$ and $CD4^+$) effector function. Human DCs may be readily obtained in large numbers from peripheral blood by short-term in vitro culture in media containing interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Banchereau et al., 2001; Fong and Engleman, 2000; Fukao, 2002; Langenkamp et al., 2001; Lopez and Hart, 2002; Scher et al., 2004).

2.4 Type-1 Polarized DC

(αDC1: see Appendix I for the summary of preclinical data and Appendix II for our published description of αDC1s)

Two functions of dendritic cells are believed to be important for the ability of DCs to induce Th1 cells and CTLs, (the types of immune cells most desirable in cancer immunotherapy): high co-stimulatory activity and high production levels of anticancer cytokines, especially IL-12 (Romani et al., 2001; Schuler-Thurner et al., 2002; Trinchieri, 2003; Vieira et al., 2000). Clinical trials to date have relied on the use of either fully-matured DCs exhibiting high stimulatory function, but low IL-12 secretion, or immature DCs that display low stimulator/high IL-12 secretion functions. We have recently developed a novel culture method to generate mature DCs that are both highly stimulatory and produce exceedingly high levels of IL-12 (Vieira et al., 2000; Vieira et al., 1998). Such DC will be referred to as type-1 polarized DC or αDC1. We have recently succeeded in adopting our original αDC1 protocols based on fetal bovine serum-supplemented cultures, to allow αDC1 generation in serum free media, allowing for the facilitated application of αDC1s in clinical trials of cancer immunotherapy. Our in vitro observations show that such type-1-polarized DCs (αDC1s) induce up to **40-fold higher** frequencies of tumor-specific CTLs during in vitro sensitizations when compared to conventional mature DCs. Furthermore, these same αDC1s are highly effective inducers of tumor-specific Th1-type $CD4^+$ T cell responses.

Beyond merely exhibiting the unique combination of high immunostimulatory function and high production capacity for cytokines, αDC1s exhibit a stable phenotype that is resistant to tumor-associated immunosuppressive factors, including IL-10 and PGE_2 (Kalinski et al., 1997; Kalinski et al., 1999b; Vieira et al., 1998): these suppressive factors are overexpressed in many advanced cancers, including prostate cancer (Attiga et al., 2000; Chen and Hughes-Fulford, 2000; Chen et al., 1997; Gabrilovich and Pisarev, 2003; Gerosa et al., 2002; Goto et al., 1999; Kalinski et al., 1997; Kalinski et al., 1999b; Liu et al., 2002). αDC1s can produce IL-12p70 upon interaction with $CD4^+$ T cells that are unable to produce IFNγ or other IL-12 co-inducing factors. This suggests the possibility of using αDC1s to boost the clinical efficacy of cancer vaccines, despite the presumed immuno-suppressive environment of immunocompromised (Th2-, Th3- or Tr1-dominated) cancer patients (Vieira et al., 2000).

The objective of DC-based cancer immunotherapy is to induce tumor-specific cytotoxic T cells (CTLs), IFN-γ-producing T helper type-1 cells (Th1 cells), and to boost the antitumor activity of

natural killer (NK) cells. The past developments in the area of DC-based cancer vaccination focused on the selection of most appropriate cancer-specific antigens (delivery of *"signal 1"*) and generating the DCs that are fully mature and express high levels of co-stimulatory molecules (needed for the delivery of *"signal 2"),* the signals believed to be essential for the development of immune responses of high specificity and magnitude. In contrast, our own work concentrates on the development of the vaccination strategies allowing us to induce the right type of immunity (delivery of **"signal 3"**) (Kalinski et al., 1997; Kalinski et al., 1999a; Kalinski et al., 1999b; Mailliard et al., 2003; Mailliard et al., 2004; Vieira et al., 2000). Our goal is to selectively stimulate the responses of CTLs, Th1 cells and NK cells, without enhancing the ineffective, and often counterproductive, Th2- and B cell-dominated type-2 responses, known to spontaneously arise in patients with advanced cancer (Tatsumi et al., 2002).

Based on the **novel paradigm of polarization of dendritic cells** (Kalinski et al., 1999a), we have recently developed the first clinically-applicable protocol to generate type-1 polarized DC (DC1 or αDC1). Such DC1s are the first clinically-applicable type of **DCs that combine all 3 properties deemed important for their *in vivo* activity** as inducers of anticancer immunity. These are: 1) fully-mature status; 2) high migratory responsiveness to lymph node-produced chemokines; and 3) high IL-12p70-producing function. Such a combination was missing in all previously-applied protocols for preparing DC, where final DC maturation (needed to generate DC with high expression of co-stimulatory molecules and the ability to localize in the T cell areas of the lymph nodes) was associated with irreversible loss of their ability to produce the key Th1-, CTL-, and NK cell-activating cytokine with anti-cancer properties, IL-12 (Kalinski et al., 1999b; Langenkamp et al., 2001). DC1s show a selectively enhanced ability to induce type-1 anti-cancer responses, when compared to the current "gold standard" of clinically-used DCs. Our studies in the melanoma model have shown that peptide-pulsed DC1s induce up to **40-fold higher numbers of tumor-specific CTLs**, during *in vitro* sensitization of peripheral blood T cells from the patients (see ***Appendix I for the summary of preclinical data and Appendix II for our published description of αDC1s***). Importantly for clinical applications of DC-based vaccines, we could also show that type-1 DC polarization makes **DC resistant to prostaglandin E_2** (PGE_2), an immunosuppressive factor produced by prostate cancer (Attiga et al., 2000; Boutemmine et al., 2002; Filella et al., 2000; Liu et al., 2002; Tjandrawinata et al., 1997). An additional consideration is the ability of DC1 to **cross-present apoptotic tumor**, an important factor allowing us to use as a source of tumor-relevant antigens, *whole tumor cells, rather than defined peptide epitopes*, broadening the spectrum of tumor-relevant targets for immunization and eliminating the need to restrict the study to patients with a defined MHC type (usually HLA-A2). This last property of DC1s also facilitates the use of either autologous tumor or allogeneic prostate cancer cell lines as a source of antigenic material (Hrouda et al., 2000; Nouri-Shirazi et al., 2000; Pandha et al., 2004; Todryk et al., 2004). Allogeneic cell lines offer the advantage of standardized preparation of the vaccine, and a potential possibility to further boost the

immunogenic properties of vaccine-carrying DCs by promoting their interaction *in vivo* with high numbers of allo-specific $CD8^+$ T cells and NK cells in tumor-bearing hosts (Mailliard et al., 2002; Mailliard et al., 2003; Nouri-Shirazi et al., 2000).

Our concurrent studies, performed in collaboration with the groups of Drs. David Bartlett and John Kirkwood, resulted in the design and FDA approval and recent implementation of αDC1-based clinical trials in melanoma (UPCI 03-118; **BB-IND 11,754**) and colorectal cancer (UPCI 05-063; **BB-IND 13,234**). **Clinical activity of vaccination was observed in 2 of the initial 4 melanoma subjects** who completed the treatment (1PR 12M+ in a subject with Stage IIIb disease; 1 SD 11M+ in a subject with stage IV lung disease). The two first subjects with resected metastatic CRC are undergoing follow-up for time to recurrence and immunologic evaluations. No side effects were observed in either of these settings. In addition, our collaborators from the Departments of Dermatology (Drs. L. Geskin and L. Falo) and Neurosurgery (Dr. Hideho Okada) **used this platform to vaccinate subjects with advanced cutaneous T cell lymphoma (CTCL) and recurrent glioma**, observing lack of serious side effects and the **occurrence of clinical responses in both of these settings.**

2.5 Dendritic cell (DC) Immunotherapy for Prostate Cancer

Prostate-specific immunotherapy and/or induction of autoimmune prostatitis are two related immunological approaches, which may offer the prospect of an effective treatment for prostate cancer (Fong et al., 1997; Liu et al., 1997). The recent identification of dendritic cells (DC) as powerful professional antigen-presenting cells capable of inducing primary T cell responses in vivo and in vitro has generated widespread interest in the application of DC to tumor immunology (Fong and Engleman, 2000; Schuler and Steinman, 1997; Steinman, 1991). DC also have the capacity to break peripheral tolerance and induce CTL responses to "self" antigens (Banchereau and Steinman, 1998). Prostate tumor cells express a variety of tissue-specific antigens, including prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), and prostate-specific membrane antigen (PSMA), all of which have been considered as potential targets for T cell immunotherapy, DC vaccination, or induction of therapeutic autoimmune prostatitis. PSA peptides containing HLA A*0201-binding motifs can induce CTL responses that lyse peptide-loaded target cells and prostate tumor cells (Correale et al., 1997; Correale et al., 1998; Xue et al., 1997). Similarly, DC pulsed with HLA A*0201-binding PAP peptides are able to induce CTL responses against A*0201-positive prostate tumor cells (Machlenkin et al., 2005; Peshwa et al., 1998). These encouraging laboratory results have formed the basis of clinical trials for DC-based immunotherapy targeted against PSA, PAP, and PSMA (Burch et al., 2004; Fong et al., 2001; Heiser et al., 2002; Murphy et al., 2000). In a recent Phase I trial of PSA mRNA-pulsed DC administered to subjects with metastatic prostate cancer, PSA-specific T cell responses were detected in all subjects, and vaccination was associated with a significant decrease in PSA in six of seven subjects (Heiser et al., 2002). No dose-limiting toxicity or other

adverse effects were noted.

Working on the principle that xenogeneic antigens are more immunogenic than self antigens DC loaded with mouse PAP have been tested in a Phase I clinical trial in subjects with metastatic prostate cancer. All subjects developed T cell responses to the mouse PAP, and eleven of twenty-one also developed T cell responses to the homologous human PAP (Fong et al., 2001). Both Phase I and Phase II trials have been conducted with DC loaded with a fusion protein consisting of PAP linked to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Burch et al., 2004; Small et al., 2000). The treatment was well tolerated, and 38% of subjects mounted immune responses to the PAP-GM-CSF fusion protein. Although, immunologic tolerance to the aforementioned proteins could be circumvented, the clinical benefit of these vaccines' remains to be determined. Patients with PCa have been reported to have multiple immune defects: both in antigen presenting cells, as well as in tumor-reactive T cells (Kennedy-Smith et al., 2002). Functional defects in circulating T cells, have also been noted in patients with PCa, i.e., reduced expression of the T cell zeta-chain (associated with signaling). Interestingly, Meidenbauer et al. reported that 50% of the patients treated with PSA-based vaccination therapy, had normal recovery of the T cell zeta chain (Meidenbauer et al., 2000). Additionally, defects in the antigen processing machinery have also been described in PCa (Sanda et al., 1995). Furthermore, our own data also speak to the presence of PCa-associated immunosuppression: in particular, a) defects in dendropoiesis, and b) the propensity for DC to undergo apoptosis (Shurin et al., 2001; Tourkova et al., 2004). Thus generation of DCs resistant to immunosuppression and/or apoptosis (as proposed), may be of benefit (Kalinski et al., 1999a).

2.6 Allogeneic Tumor Cells as a Source of Antigen

(See Appendix III for the data on DC-mediated cross-presentation of LNCap-associated epitopes to CTLs)

Although there are a number of prostate-specific proteins amenable to immune targeting, the immunogenicity of these proteins is not well understood, and a priori, there is no perfect antigen. Given the theoretical appeal of targeting more than one cancer-associated protein, the in vivo efficacy of whole-tumor cell vaccines was initially demonstrated in preclinical animal studies (Suckow et al., 2005; Todryk et al., 2004; Vieweg et al., 1994). Several of these studies observed immunologic memory capable of protective immunity as well as curative effects against established subcutaneous (s.c.) tumors using Dunning rat prostatic carcinoma cells (MAT-LyLu) (Vieweg et al., 1994). Autologous whole cell vaccines for human prostate cancer were initially investigated by Simons et al (Simons et al., 1999). Surgically harvested prostate tumor cells were irradiated and engineered to secrete GM-CSF via a replication-defective retrovirus. A small Phase I study (n=11) demonstrated interesting immunologic response and confirmed the safety of this approach. The numerous technical difficulties involved in the preparation of autologous cells, however, represented a significant limitation. Thus the focus of research was shifted to

allogeneic vaccines, which are readily available from established prostate cancer cell lines. This type of vaccine may be manufactured on a larger scale for distribution. In addition, patients do not need to be HLA-matched for these vaccines as antigens can be presented by cross priming. A GM-CSF secreting vaccine, GVAX (Cell Genesys, South San Francisco, CA), is an admix of prostate cancer cell lines PC-3 and LNCaP, and is currently in two large Phase III clinical trials in hormone-refractory prostate cancer. The impetus for these studies was promising survival data from smaller Phase II studies (Michael et al., 2005; Simons et al., 1999). Another Phase II trial utilizing a different allogeneic whole cell, Onyvax-P (Onyvax Ltd, London, UK) consisting of irradiated prostate cancer cell lines OnyCap23, LNCaP, and P4E6, demonstrated improved median time to disease progression in patients with AIPC (Eaton et al., 2002).

In this trial we also plan to use an allogenic cell line: LNCaP, as a source of tumor antigens to be loaded on each subject's autologous αDC1s. We have verified that this cell line is a relevant source of DC-cros-presented CTL epitopes of PCa (Nouri Shirazi et al 2004: ***see Protocol Appendix III***). In addition to circumventing the perceived problems with obtaining autologous tumor material in the current group of patients, the selection of allogeneic cell line has been supported on our data showing that human resting $CD8^+$ T cells (such as allo-specific T cells promote the ability of DCs to induce Th1 and CTL responses (Mailliard et al 2002). Thus LNCap will act in our vaccine both as a source of both PCa-specific antigens and additional tumor-unrelated "heterologous" (helper) antigens (alloantigens).

2.7 Feasibility of Generating αDC1-based Vaccines for Prostate Cancer Patients

(Also see Appendix I for the summary of preclinical results)

We have successfully verified that αDC1 can be generated from the monocytes of prostate cancer patients. The current study will verify the ability to generate αDC-1 on a large scale, store them, and subsequently use them for vaccination. Compared to standard DCs, αDC1s from the same patients produce 10-100 times more IL-12p70, the known Th1-, CTL-, and NK cell-activating factor (***see Appendix I and II***). In accordance with the resistance of polarized DCs to tumor-related suppressive factors, such as PGE_2 (Vieira et al 2002), we observed that high IL-12-producing mature $CD83^+/CCR7^+$ αDC1 are not negatively affected by the presence of apoptotic bodies from UV-irradiated LNCap cells during DC maturation (***see Appendix III***). The expansion, testing, and banking of the LNCaP master cell line that will be used as a source of prostate cancer antigens in the αDC1s vaccines has already been arranged with Bio_Reliance (***see Appendix IV***). The final documentation and the results of all tests of such master cell line will be delivered to the FDA prior to the accrual of any subjects to the current protocol. With regard to other vaccine components, KLH, a "heterologous $CD4^+$ helper antigen", has been approved for the use in a similar DC-based melanoma trial (UPCI 03-118; **BB-IND 11,754**) and is available in sufficient amount for the currently proposed trial.

All vaccines will be prepared by the experienced staff of the IMCPL (the cGMP-compliant facility of the UPCI), according to the procedures covered by the **FDA master file (BB-MF 12,244) of the IMCPL.**

2.8 Prostate Cancer-Associated Antigens or Tumor-Associated Antigens (TAAs)

Over the past 10 years, numerous TAAs (PSA, PAP, PSMA, etc) and their derivative HLA-A2-presented peptides have been identified in prostate cancer. These antigens can be used in the form of antigenic proteins and/or peptides. In addition, these and additional TAA, unique to individual tumors are present in whole apoptotic tumor cells and can be cross-presented by DC (Nouri-Shirazi et al., 2000) (***see Appendix III***). Our vaccine will consist of αDC1 loaded with allogeneic apoptotic tumor that is expected to contain all of these different classes of antigens. Thus vaccination-induced immune responses against allogeneic whole tumor are likely to be directed against both TAAs as well as allogeneic molecules. To validate that our αDC1-based vaccines are effectively presenting such tumor related epitopes *in vivo*, in vaccinated subjects, an important secondary endpoint in this study will be testing immune responses against HLA-A2 restricted peptides derived from prostate TAAs (*see Table 1 below*), in any subjects who are HLA-A2 positive (expectedly 40-50%).

TABLE 1. Prostate Cancer HLA-A2-restricted TAAs Used as Readouts (Secondary Efficacy Endpoints)

Antigen	Epitopes	Sequence	Reference
PAP	*PAP-3*	NH2-**ILLWQPIPV**-COOH	(Harada et al., 2004; Machlenkin et al., 2005; Peshwa et al., 1998)
PAP	*PAP-5*	NH2-**ALDVYNGLL**-COOH	(Harada et al., 2004; Machlenkin et al., 2005; Peshwa et al., 1998)
PSA	PSA_{147}	NH2-**KLQCVDLHV**-COOH	(Alexander et al., 1998; Chakraborty et al., 2003; Correale et al., 1997)
PSMA	$PSMA_{27}$	NH2-**VLAGGFFLL**-COOH	(Harada et al., 2004; Kobayashi et al., 2003)
STEAP	STEAP-3	NH2-**LLLGTIHAL**-COOH	(Machlenkin et al., 2005)

KLH as a "heterologous" helper antigen:

In general, most TAAs are considered to be "weak" antigens, inducing low-frequency, low-avidity $CD4^+$ T cell responses as a consequence of being "self" antigens that are subject to tolerance programming. In addition, recent reports have demonstrated an undesirable bias of tumor-specific $CD4^+$ ("helper") T cells towards the TH2 phenotype (Tatsumi et al., 2002), that is likely to be ineffective in providing helper signals for CTL generation. To circumvent the problem that Th1-type T cells responsive to TAAs are limiting in subjects receiving our vaccines, similar to the strategy adopted in our melanoma protocol UPCI 03-118 (BB-IND 11,754), we propose to include "heterologous", tumor-unrelated helper antigen KLH in our

vaccine formulation. In order to avoid preferential induction of KLH-specific responses and to avoid the risk of the strong DTH reactions to the booster doses of the vaccines, this protein will be included only in the first dose of the vaccine and will not be included in the vaccines used as booster doses.

2.9 Experience with DC-based Vaccines and Cancer Vaccine Trials at UPCI

Dendritic cell based vaccines have been investigated extensively in a number of centers and to date, have not been reported to be associated with limiting toxicities or adverse side effects. UPCI has a long standing interest, a significant track record, and a robust infrastructure to support vaccine trials.

3 RATIONALE FOR TRIAL AND TARGET PATIENT POPULATION

Over the past decade, owing to aggressive PSA screening, patients with prostate cancer (PCa) are being diagnosed earlier and undergo both definitive treatment as well as androgen ablation (for those who relapse after surgery or radiation) early in the course of their disease. Consequently, the fastest growing subsets of patients with PCa include: i) men with a rising PSA following local therapy, i.e., irradiation and/or prostatectomy; and ii) men with a rising PSA following failure of hormonal therapy. These men with PSA only disease are virtually certain to develop clinically manifest prostate cancer, though the interval from detection of PSA increase to clinical disease may be very variable. In the hormone-naïve serologic failures, the mean time to development of metastasis may vary from 4 to 7 years (Dillioglugil et al., 1997; Pound et al., 1999). Androgen deprivation is the standard of care for men with advanced prostate cancer but the optimal timing for such therapy is unclear. There is no evidence that continuous androgen deprivation for men with PSA elevation following local therapy improves quality or quantity of survival – and androgen deprivation is not without consequences: osteoporosis, diminished libido, loss of muscle mass, increase body fat and hot flashes. Hence alternatives to androgen deprivation are urgently needed – especially for this patient population. Likewise, there is no accepted standard of treatment for the non-metastatic androgen independent population, where the time to development of metastasis may vary from 9 to 24 months. Hence these two sub-populations of men with PCa, are ideal for testing novel therapies.

With respect to immunotherapy, since it is known that androgen ablation promotes an influx of T cells into the prostate (Mercader et al., 2001), in the proposed trial we plan on immunizing men in concert with limited (3 months) androgen ablation. We plan on introducing androgen ablation 2 weeks after immunization, in order to focus the effector phase of prostate cancer-specific immune response on the existing prostate cancer sites. We anticipate that such timing of both treatments will allow the circulating inflammatory-type cancer-specific T cells (Th1 cells and CTLs) induced by the first dose of the αDC1-based vaccine, to enter the existing tumor sites damaged by the hormone therapy and to eliminate the surviving cancer cells. The second dose of the vaccine, administered 2 weeks after the hormone therapy and the 2 remaining vaccine doses,

are anticipated to re-enforce such an effect, cumulatively prolonging the time to disease progression, compared to what is typically observed after limited androgen ablation: PSA recurrence, as testosterone levels recover in 3 to 6 months. Thus, we hypothesize that the combination of the αDC1-based vaccine and limited androgen ablation, will significantly delay time to PSA recurrence, despite a normalization of testosterone levels. Finally, we also anticipate that the combination treatment proposed above, will further enhance the activity of vaccination by counteracting tumor-related, prostate-specific immune tolerance, that has been demonstrated in experimental murine models of prostate cancer (Drake et al., 2005).

In order to allow the vaccine-constituting αDC1s to act as a medium of prolonged feedback interactions between the expanding populations of $CD4^+$ and $CD8^+$ T cells, the first priming dose of the αDC1-based vaccine will be administered intradermally over a period of four days, similar to our melanoma protocol UPCI 03-118 (*see previous sections)*. We expect that such prolonged period of the presence of LN-immigrating DCs is particularly important during the priming cycle of vaccination, when the frequencies of functional tumor-specific CD4+ and CD8+ T cells are the lowest. Since we expect that the first cycle of vaccination will successfully increase the pools of functional $CD4^+$ and $CD8^+$ T cells, we expect that such prolonged period of vaccine delivery will be less important in case of the booster doses of vaccination. Therefore, for the feasibility reason, we plan to perform all booster doses as single injections.

For the same reason (low-frequency of tumor specific T cells during the first priming cycle of vaccination), during this cycle alone, we also propose to use KLH as a "heterologous" helper antigen, given that most prostate tissue-derived antigens are considered to be "weak" antigens, inducing low-frequency, low-avidity $CD4^+$ T cell responses as a consequence of being "self" antigens that are subject to tolerance programming. In addition, recent reports have demonstrated an undesirable bias of tumor-specific $CD4^+$ ("helper") T cells towards the Th2 phenotype (Tatsumi et al., 2002), that is likely to be ineffective in providing helper signals for CTL generation. To circumvent the problem that Th1-type T cells responsive to self are limiting in subjects receiving our vaccines, we propose to include "heterologous", tumor-unrelated helper antigen, KLH (Nestle et al., 1998) in our formulation.

Thus, our goal is to develop protocols to induce autoimmune prostatitis as a novel immunotherapy for prostate cancer, through dendritic cell-induced stimulation of $CD8^+$ cytotoxic T cell (CTL) responses against prostate autoantigens. This is based on the premise that a vigorous autoimmune response against prostate-specific proteins, capable of destroying normal prostate tissue, will also destroy malignant prostate tissue, provided the malignant tissue expresses these proteins. Since the prostate gland is routinely removed or ablated as a part of the treatment for early prostate cancer, any remaining/recurring prostatic tissue could be destroyed immunologically. Three prostate tissue specific antigens are currently being evaluated in clinical trials: prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and prostate specific

membrane antigen (PSMA). However the immunogenicity of these proteins is not well understood, and a priori, there is no perfect prostate-specific immunologic target. Hence our focus is on the allogenic tumor cell line - LNCaP. Additionally, we will also incorporate limited (3 months) androgen deprivation (with LHRH analogues) in the treatment protocol to enhance the immune-potentiating effects of this manipulation, as described above (Drake et al., 2005; Mercader et al., 2001).

Our target population for treatment will be men with prostate cancer, who have relapsed following surgery and/or radiation, and now have a rising PSA with a PSA doubling time (PSA-DT) of less than 10 months. This patient population is **ideal for an immunologic intervention** given that: i) this is the largest sub-population of men with prostate cancer in the U.S.; ii) there is no standard treatment for this sub-group; and iii) this sub-group has minimal systemic disease burden (Havranek et al., 2002; Kaufman et al., 2004). We will carefully monitor immunological responses to vaccination at different time points, but the primary endpoint of vaccine efficacy will be stabilization of PSA levels or failure of the PSA to progress. Several recent studies have validated failure of PSA progression as a good surrogate endpoint for lack of progression in clinical trials in prostate cancer (Scher et al., 2004).

This is a 2 group crossover trial in which subjects are randomly assigned to one of two treatment arms:

Cohort A: 3 months of androgen ablation (AA) to be followed at PSA progression by 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine (DC1).

Cohort B: 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine followed at PSA progression by 3 months of AA.

In this crossover trial each subject will serve as their own control. Following either therapy the time to PSA progression, defined as the time between treatment and the first instance of PSA increase to 1 ng/mL, will be estimated from monthly PSA monitoring. The endpoint is the difference between the time to PSA progression for the combination of AA + DC1 compared to AA alone.

4 SUBJECT SELECTION

4.1 Eligibility Criteria

- Patients with histologically proven prostate cancer and tumors limited to the prostate (including seminal vesicle involvement, provided all visible disease was surgically removed) who have completed local therapy and have an elevated PSA after surgery or rising PSA after radiation therapy, as defined below.
- Age 18 years or older
- Histologically confirmed diagnosis of prostate cancer.
- Previous treatment with definitive surgery or radiation therapy or both.

- No evidence of metastatic disease on physical exam, CT/MRI/CXR (see Section 7.1 for radiologic imaging), and bone scan within 4 weeks prior to randomization.
- Prior neoadjuvant/adjuvant hormonal, androgen deprovation therapy, or chemotherapy is allowed if it was last used > 12 months prior to first vaccination.
- No therapy modulating testosterone levels (such as leuteinizing-hormone releasing-hormone agonists/antagonists and antiandrogens) is permitted within 12 months prior to first vaccination. Agents such as 5α-reductase inhibitors, ketoconazole, megestrol acetate, systemic steroids (replacement doses of steroids are allowed), PC-SPES, and Saw Palmetto are not permitted at any time during the period that the PSA values are being collected.
- Hormone-sensitive prostate cancer as evident by a serum total testosterone level > 150 ng/dL or > 6 nmol/L at the time of enrollment within 4 weeks prior to randomization.
- All patients must have evidence of biochemical progression as determined by a reference PSA value followed by 1 confirmatory rising PSA value, higher than the previous value, obtained at least 2 weeks apart. All of these PSA values must be obtained at the same reference lab, and all must be done within 6 months prior to enrollment.
- The most recent of the PSA values must be ≥ 2.0 ng/mL. This measurement must be obtained within 1 month prior to enrollment.
- The PSA doubling time (PSA-DT) must be less than 12 months.
- ECOG performance status 0 or 1.
- Patients must have normal organ and marrow function as defined below:
    - Absolute neutrophil count > 1,500/μL
    - Platelets > 100,000/μL
    - Total bilirubin 1.5 x upper limit of normal (ULN)
    - SGOT (AST) and SGPT (ALT) < 2.5 x institutional ULN
    - Creatinine 1.5 x ULN
- The effects of dendritic cell vaccines on the developing human fetus are unknown. For this reason men must agree to use adequate contraception (hormonal or barrier method of birth control) prior to study entry and for the duration of study participation.

4.2 Exclusion Criteria

- Patients must not be receiving other investigational agents or concurrent anticancer therapy.
- No uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements.
- Patients must not have active eczema, atopic dermatitis, or other exfoliative skin conditions (e.g., burns, impetigo, varicella zoster, severe acne, contact dermatitis, psoriasis, herpes or other open rashes or wounds).
- Presence of an active acute or chronic infection, including urinary tract infection, HIV or viral hepatitis. HIV patients are excluded based on immunosuppression which may render

them unable to respond to the vaccine; patients with chronic hepatitis are excluded because of concern that hepatitis could be exacerbated by the injections. If clinically indicate, HIV/viral hepatitis testing will be performed to confirm status.

- Patients with a history of auto-immune disease such as, but not restricted to, inflammatory bowel disease, systemic lupus erythematosus, ankylosing spondylitis, scleroderma, or multiple sclerosis. Patients receiving replacement thyroid hormone would be eligible.
- No concurrent use of systemic steroids, except for local (topical, nasal, or inhaled) steroid use. Adrenal replacement doses of corticosteroids are allowed.
- Subjects with concurrent additional malignancy (with exception of non-melanoma skin cancers and superficial bladder cancer or malignancy within last 3 years).

4.3 Gender/Ethnicity

Study entry is open to male patients of all ethnic backgrounds. The racial and ethnic characteristics of the proposed subject population will reflect the demographics of Pittsburgh and the surrounding area and/or the patient population of the UPMC Health Systems. We shall attempt to recruit subjects in respective proportions to these demographics. No exclusion criteria will be based on race, ethnicity, gender, or socioeconomic status. In recent Phase I/II trials at UPCI, approximately 11% of subjects were non-caucasian. Given the limited size of the patient population to be accrued on this trial, there will be limited power to determine differences in efficacy between different racial groups. However, we will describe any apparent differences in efficacy or toxicity.

4.4 Subject Recruitment

Approximately 12-16 subjects are expected to enter the study. All of whom will be accrued through the University of Pittsburgh Cancer Institute.

For the proposed trial, all the subjects will be accrued at UPCI Hillman Cancer Center. There is an extensive clinical practice in urologic cancers between the Medical Oncology offices and the Urology offices at UPCI/UPMC. In addition, UPCI has vast community links and a large clinical investigations program through its economically aligned network of over 60 medical and radiation oncologists who practice in community-based sites throughout western PA and adjacent areas of Ohio and West Virginia. In the UPMC/UPCI Network registry for Genitourinary Organs, 2004-2005, 1117 new cases of prostate cancer were diagnosed across the system hospitals. Across the entire state of Pennsylvania, 10,300 new cases of prostate cancer are expected to be diagnosed this year. At current rates, we expect one out of every 6 will be seen in the UPMC/UPCI network. Since most of these patients are referred to UPCI for novel clinical investigations, we expect rapid accrual to the above protocol. In the last 2 years, over 125 subjects/year have been accrued to clinical trials in the UPCI prostate cancer program.

Potential subjects will be approached by clinicians who are directly involved in their care. ”Cold-

calling" will not be used to recruit subjects and "Finder's fees" for referring a potential subject for participation in a research study are prohibited. Physicians and other health care professionals in the area are aware of active studies at UPCI by means of various publications including the World Wide Web. Such publications and Web listings are not advertisements for specific studies. Rather, they are public listings of trials available. Subjects will *not* receive any monetary compensation for participation.

5 STUDY DESIGN AND METHODS

This is a 2 group crossover trial in which subjects are randomly assigned to one of two treatment arms:

Cohort A: 3 months of androgen ablation (AA) to be followed at PSA progression by 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine (DC1).

Cohort B: 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine followed at PSA progression by 3 months of AA.

In this crossover trial each subject will serve as their own control. Following either therapy the time to PSA progression is defined as the time between treatment and the first instance of PSA increase to 1 ng/mL. The endpoint is the difference between the time to PSA progression for the combination of AA + DC1 compared to AA alone. A **total of 12 evaluable subjects** (6 subjects/arm) will be enrolled on the trial. Subjects who do not complete both courses (AA and AA+DCV) will be replaced. This schema will also help us better estimate the time to PSA recovery following 3 months of limited androgen ablation in our cohort of subjects.

All subjects in Cohort B will commence αDC1-based vaccination 2 weeks prior to treatment with the LHRH analogue. Each subject will receive 1 intradermal (*i.d.*) dose of the vaccine at weeks 1, 5, 9, and 13 for a total of 4 doses. The first dose will be considered the 'priming' dose and injections on weeks 5, 9, and 13 are 'booster' doses. Depending upon cells/volume the injections may utilize 1-2 syringes being administered on a single day. Additional courses of vaccination, if available after the 4 intended protocol doses, can be administered to any subject without evidence of disease progression, every 3 months (±1 month) for up to 12 months. The schema for the vaccine can be found on page 6.

The LHRH analogue (Lupron 22.5 mg or Zoladex 10.8 mg) will be administered 2 weeks after the 1st dose of the DC vaccine.

We assume that 3 months of AA alone will be followed by PSA progression at a median of 6 months with 90% of PSA recovery times between 5 and 7 months. We suspect that a second course of AA will delay time to testosterone recovery by approximately 10% and that testosterone recovery and PSA failure will likely correlate very closely. Therefore we anticipate that a second course of AA will delay time to PSA relapse by approximately 10%. The purpose of randomizing to one of two treatment sequences is to permit an unbiased estimate of

the improvement due to DC1 treatment effect in the presence of any 2nd course variability. In this study, on treatment PSA-relapse is defined as a PSA ≥ 1.0. We consider the clinically significant objective to be a within-subject delay in PSA progression time due to the addition of DC1 by 30% or to 7.8 months.

Our estimates of time to testosterone and PSA recovery are based on a recent study that we participated in (Figg et al, Lancet Oncology [***in press***]; Figg et al, ASCO Proceedings, 2008, Abstract #5015)**.**

6 TREATMENT PLAN

Subjects will undergo a thorough pre-study evaluation (see below), and then undergo leukapheresis to generate the DC-based vaccine. Each subject will receive 1 dose of *intradermal (i.d.)* αDC1-based vaccine at weeks 1, 5, 9, and 13 for a total of 4 doses.

6.1 Leukapheresis

Leukapheresis is a minimal risk procedure. All selected subjects will undergo a single 90-120 minute-long limited leukapheresis before vaccination. The product is delivered immediately to the University of Pittsburgh Cancer Institute Immunologic Monitoring and Cellular Products Laboratory from the UPCI Outpatient Clinic pheresis area. Leukapheresed product will be immediately processed as described in the laboratory SOP. The product will be cryopreserved as described in the Chemistry, Manufacturing and Control section of the IND application. If cytopenia (WBC < 2000/mm^3, or platelets < 40,000/mm^3) develops during, or as a result of, leukapheresis, the procedure will be postponed until recovery. Samples from each cell product will be obtained for hemoglobin, hematocrit, total WBC and differential, and platelet count.

6.2 DC Vaccine Administration

Each dose will consist of a single allogeneic tumor cell vaccine administered intradermally using 1-2 1-mL syringe(s) and needle appropriate for intradermal injections and will hold a total of 0.5 mL of the vaccine preparation. The allogeneic tumor dendritic cell vaccine will be injected in the vicinity of the major nodal basins of the thigh or arm. The nodal basin must not have been dissected.

- Week 1: Both thighs - 1.5 to 2.5 x 10^6* cells on each side (target: 3-5 x 10^6 cells total)
- Week 5: Both arms - 1.5 to 2.5 x 10^6* cells on each side (target: 3-5 x 10^6 cells total)
- Week 9: Both thighs - 1.5 to 2.5 x 10^6* cells on each side (target: 3-5 x 10^6 cells total)
- Week 13: Both arms - 1.5 to 2.5 x 10^6* cells on each side (target: 3-5 x 10^6 cells total)

* If the cell count is lower, the total number of cells will be divided equally between the 2 sites. However, at the Investigators discretion, the vaccine may have to be repeated if the cell count is less than< 1.5 x 10^6/per site.

In the event of a prior node dissection, the priming dose will be administered in the arms.

The vaccine will be administrated by the Investigator/Sub-investigator or a member of the advanced practice staff.

Subjects may undergo additional vaccinations, every 3 months (± 1 month) if they demonstrate lack of disease progression on week 16 (or later) and if they have vaccine remaining.

6.3 Dose Limiting Toxicities

This study will utilize the NCI CTCAE version 4.0 for toxicity and Adverse Event (AE) reporting (http://ctep.cancer.gov/forms/).

The following, if they occur and are felt to be attributable to the investigational treatment, are considered to be dose limiting toxicities (DLT). Any subject experiencing one of these will be taken off study and no further injections will be given:

- ≥ Grade 2 or greater bronchospasm,
- ≥ Grade 2 or greater allergic reaction or generalized urticaria,
- ≥ Grade 2 or greater autoimmune disease
- ≥ Grade 3 injection site reaction due to vaccine, implanted port, or catheter
- ≥ Grade 3 hematologic and non-hematologic toxicities
- Infection, local or systemic

Management associated with the above adverse events is outlined below. Subject accrual will cease if 2 subjects have a DLT related to vaccine administration. The observation period for DLTs will be 1 week.

6.4 Study Discontinuation Criteria

Subjects MUST be discontinued from study therapy AND withdrawn from the study for the following reasons:

- Withdrawal of informed consent (subject's decision to withdraw for any reason)
- Any clinical adverse event, laboratory abnormality or intercurrent illness which, in the opinion of the Investigator/Sub-Investigator, indicates that treatment with study therapy is not in the best interest of the subject
- Pregnancy: All WOCBP should be instructed to contact the Investigator/Sub-Investigator immediately if they suspect they might be pregnant (e.g., missed or late menstrual period) at any time during study participation.
- Investigator decides to close the clinical trial prematurely.

6.5 Supportive Care Guidelines

Toxicities of the vaccination itself are expected to be minimal. For fever, acetaminophen will be utilized (325 mg tabs, 1 or 2 by mouth every 4 hours). Fevers lasting more than 8 hours after treatment will be evaluated in terms of potential infection. For mild, local pain, oral opiates will be planned (oxycodone, 5-10 mg by mouth every 3-4 hours). Pain that is of more than mild-moderate grade will be investigated for sources other than the therapy, and managed accordingly. In case of the occurrence of the acute hypersensitivity reactions, administration of IV antihistamines, corticosteroids, and epinephrine may also be required. Any cases of infection will

be treated with antibiotics, according to established guidelines.

6.6 Duration of study therapy

In the absence of treatment delays due to adverse events, treatment will continue for at least 4 doses or until one of the following criteria applies:

- Disease progression,
- Intercurrent illness that prevents further administration of treatment,
- Unacceptable adverse event(s),
- Severe reaction to the DTH testing with the tumor cell lysate prior to vaccination,
- Subject decides to withdraw from the study, or
- General or specific changes in the subject's condition rendering the subject unacceptable for further treatment in the judgment of the investigator/sub-investigator.
- Dosing delays > 4 weeks

7 STUDY EVALUATIONS AND STUDY CALENDAR

7.1 Screening

Baseline evaluations are to be conducted within 14 days prior to the leukapheresis .. Scans and x-rays must be done within six weeks prior to the start of therapy.

- Complete physical examination (with ECOG performance status (PS), weight, height, and BSA)
- Radiologic imaging to evaluate the status of disease (all scans must be obtained within 30 days of starting therapy): Includes bone scan and CT scan of chest (or CXR), abdomen, and pelvis.
- Chemistry profile including: electrolytes, creatinine, blood urea nitrogen, glucose, SGOT, SGPT, total bilirubin, calcium, and albumin.
- Serum testosterone
- PSA levels in the blood (as a tumor marker). To be eligible, subjects must have at least two PSA levels checked at least 2 weeks apart, shown to be rising, and with the the last PSA being ≥ 2.0 ng/dL
- CBC, differential, platelet
- ANA Testing (if clinically indicated)
- Known history of HIV, HbsAg, and Anti-HCV. For subjects at high risk of HIV/viral hepatitis, HIV/viral hepatitis testing will be performed to confirm status.
- Following confirmation of eligibility, based on the above tests and procedures, the following, additional pre-treatment procedures will be performed:
    - Leukapheresis – for vaccine production and a portion will be obtained for *in vitro* assays (immune parameters of $CD4^+$ and $CD8^+$ T cell responses against tumor-associated and tumor-irrelevant control antigens).

- DTH placement within 48-72 hours before the day of first dendritic cell vaccine administration (baseline testing) to allow for it to be read on the day of and prior to the 1st vaccine injection.

7.2 Evaluation during Treatment

During dendritic cell vaccine cycles (weeks 1, 5, 9, and 13) the following will be performed:

- Complete physical examination (with ECOG performance status and weight)
- Laboratory tests including: CBC, differential, platelet, and chemistry profile each visit (see Section 7.1 and Study Calendar).
- Blood for in vitro assays will be obtained within 1 week after each cycle of vaccination.
- PSA at week 13

7.3 Post-Treatment

- Complete history and physical examination.
- For subjects with suspected disease progression on therapy, radiographic imaging as indicated.
- PSA at week 17, 21, and every 4 weeks thereafter. Confirmatory PSA should be performed 1 month post progression
- Testosterone at week 17, 25, and every 2 months thereafter
- CBC, differential, platelets at week 25 and every 3 months thereafter
- Chemistry profile (see Section 7.1) at week 25 and every 3 months thereafter
- Blood for in vitro assays (immune parameters of CD4+ and CD8+ T cell responses against tumor-associated and tumor-irrelevant control antigens) will be obtained 1 week and 6 weeks after completion of treatment. The assays will involve the evaluation of cytokine production, cytotoxicity, and changes in cell phenotype of T cell subsets.
- Leukapheresis - a second limited leukapheresis will be performed during week 19 to facilitate immune-monitoring.
- DTH testing (post-testing: 2 to 4 weeks after last vaccine, i.e., during week 15 through 17). The subjects will be asked to agree to the biopsies being taken from the sites of the DTH tests, following their readout.
- EPIMAX analysis may be used to define the magnitude and cytokine production profiles of CD4+ and CD8+ T cell responses to the overlapping peptide libraries and individual peptides covering the entire span of PAP and PSMA molecules
- Subjects with lack of disease progression at 12 months after the last treatment may be monitored for disease free survival, as well as for overall survival.

7.4 Long Term Follow-up

Patients will continue to be followed after the last vaccine injection and/or progression based upon the timing of their standard of care for their disease.

7.5 Study Calendar

Screening evaluations are to be conducted within 14 days prior to leukapheresis. Scans and x-rays must be done within 6 weeks prior to the start of therapy.

There is a window of ±1 week available for scheduling treatment and/or procedures at the discretion of the Investigator/Sub-investigator. This applies also if a course is missed or a subject's treatment and/or testing day(s) need to be rescheduled due to the subject's inability to comply with the study calendar (i.e., hospitalizations, business, vacation plans, travel from long distances for study treatment, in advance of the scheduled date to allow ready access to the result(s), reduce financial burden on the subject [i.e. non-UPMC insurance coverage] or reduce travel inconvenience, illness, transportation issues, holidays, family emergencies, etc.).

	Screening	**Baseline**	**Treatment**					**Post-treatment**						
Parameter / Week #	-4 to -1		1	3	5	9	13	14	15	16	17	19	21	25
Informed Consent	X													
History, PE, ECOG PS	X		X		X	X	X				X		X	X
Serum PSA[g] - *every 4 weeks after 12 weeks of hormones*	X						X				X		X	X
Serum Testosterone [k]	X										X			X
ANA testing (if clinically indicated)	X													
HIV/viral hepatitis testing	X[f]													
CT scan and Bone Scan [i]	X													
CBC, platelets, differential	X		X		X	X	X							X[h]
Chemistry Profile	X		X		X	X	X							X[h]
Leukapheresis (90-120 mins)[e]		X										X[d]		
Skin test (DTH)		X[b]							X[c]		X[c]			
Vaccination[a]			X		X	X	X					X[a]		
Blood (70 cc) for in vitro assays [j]			X		X	X	X							

	Screening	Baseline	Treatment					Post-treatment						
Parameter / Week #	-4 to -1		1	3	5	9	13	14	15	16	17	19	21	25
LHRH Analogue				X										

a. Subjects may undergo additional vaccinations, every 3 months (±1 month) if they demonstrate lack of disease progression on week 16 (or later) and if they have vaccine remaining.
b. DTH placement should be performed within 48-72 hours prior to the day of the first dendritic cell vaccine administration to ensure the test can be read on the day and prior to the first vaccine administration.
c. DTH testing (post-testing: within 2 – 4 weeks after last vaccine, i.e. week 15 through 17). At the discretion of the investigator/sub-investigator, subjects may be asked to agree to biopsies being taken from the sites of the DTH tests, following their readout.
d. A second limited leukapheresis will be performed at the end of the study to facilitate immune-monitoring.
e. EPIMAX analysis may be performed on blood samples obtained with the leukapheresis procedure.
f. When clinically indicated, HIV/viral hepatitis testing will be performed to confirm disease status.
g. Confirmatory PSA should be performed 1 month post progression.
h. CBC and Chemistry profile every 3 months after week 25.
i. Scans must be obtained within 6 weeks of starting therapy. For subjects with suspected disease progression on therapy, radiographic imaging as indicated during post-treatment.
j. Blood for in vitro assays will be obtained within one week after each cycle of vaccination.
k. Testosterone at week 17, 25, and every 2 months thereafter.

8 MEASUREMENT OF EFFECT

8.1 Efficacy endpoints

- The primary efficacy endpoint is to evaluate the effect of the alpha-DC1 vaccine on time to PSA progression. PSA progression is defined as a rise in the PSA value to $\geq$ 1.0 ng/mL.
- The secondary efficacy objectives include:
    - To determine the change in PSA velocity prior to and following the proposed treatment,
    - To estimate time to development of metastatic disease,
    - To evaluate the immunologic response to the vaccine in all subjects,
    - To evaluate the immune response to HLA-A2.1 restricted peptides derived from PAP and PSMA in subjects who are A2.1 positive,
    - EPIMAX analysis will be used to define the magnitude and cytokine production profiles of CD4+ and $CD8^+$ T cell responses to the overlapping peptide libraries and individual peptides covering the entire span of PAP and PSMA molecules.

8.2 Safety endpoints

The primary safety endpoint is to assess the tolerability and toxicity of the alpha-DC1 vaccine.

8.3 Evaluation of Clinical Responses

The primary clinical endpoint is the time to PSA progression. Since all subjects will be treated with androgen ablation for 3 months, the expectation is that at the outset all subjects will have undetectable PSA levels. As subjects recover their testosterone levels (in 3 to 6 months), they are expected to develop a PSA relapse (5 to 7 months).

Thus time to progression (TTP) will be defined as time from starting of androgen ablation (week 2) to time of either developing *PSA progression* (see below) or the development of metastatic disease (whichever occurs sooner). Typically PSA progression will preceed the development of metastatic disease.

Progressive Disease: All subjects are expected to have a PSA < 0.1 following androgen ablation. Thus PSA progression will be defined as a PSA rise of at least 1.0 ng/dL, measured at least twice, with the two values being at least 1 month apart. Changes in PSA below 1.0 ng/dL will be evaluated for the PSA slope and PSADT.

PSA Slope:The change in PSA will be graphically depicted and a PSA slope calculated. The change in PSA slope (and PSADT) pre-treatment, during treatment, and off- treatment will be determined to see if vaccine therapy has any disease modifying effects, over and above castration. This end-point is exploratory, but may lead to continued interest in this agent if the PSA slope (or PSADT) is decreased during treatment.

Clinical Progression: The appearance of new lesions on examination or radiographs (CT scan or

bone scan), or development of symptoms consistent with metastatic disease (i.e.bone pain).

Identification of Lesions: All measurements should be taken and recorded in metric notation. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before randomization. A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the sum LD. The sum LD is used as reference to assess progression.

Clinical Progression free survival: This is the interval from entry to study to date of first documented lesion.

8.4 Evaluation of Safety

The primary safety objective of this study is to evaluate the feasibility and safety of vaccination with type-1-polarized dendritic cells (αDC1) loaded with apoptotic allogeneic tumor and tumor-unrelated "heterologous helper antigens". Based on the past experience from the University of Pittsburgh Cancer Institute (UPCI) and other clinical centers performing DC-based vaccinations, our hypothesis is that the vaccination with DC will be well-tolerated, and not associated with serious adverse side effects.

8.5 Measurement of the Immunologic Effects

The immunologic endpoints of this study will include an in vivo assessment of DTH responses against the allogeneic tumor cell lysate, and (on additional sites) responses to the "heterologous helper antigen" KLH. In addition, we will perform the in vitro assessment of the increase in the frequency of circulating tumor-specific T-cells as determined by IFNγ ELISPOT against the allogeneic tumor cells used in the vaccine and in case of HLA-A2+ subjects against the individual TAAs relevant to prostate cancer (Table 1). All of the subjects will be tested for PMBC responses to KLH. To allow for these evaluations, 70 cc of peripheral blood will be drawn 4 times (1 x pre- and 3 times post-vaccination, see study calendar). As additional tertiary endpoints, we will perform and IFNγ and IL-5 ELISPOT, and cytotoxicity assays. EPIMAX analysis will be used to define the magnitude and cytokine production profiles of $CD4^+$ and $CD8^+$ T cell responses to the overlapping peptide libraries and individual peptides covering the entire span of PAP and PSMA molecules.

8.5.1 Immunologic Assays

- DTH against the allogeneic tumor cell lysate and (on additional sites) to the "heterologous helper antigen" KLH. Following the readout, the subjects will be asked for a separate consent for a biopsy of the DTH test site to perform the in situ and ex vivo analyses of the DTH-infiltrating T cells.
- Cytokine production in ELISPOT assay (IFNγ/IL-5), using the cells isolated from 70 cc of peripheral blood will be used to detect the frequencies of IFNγ-producing CTLs.

- In all HLA-A2 subjects we will also test the responses to all individual TAAs identified in Table 1.
- All of the subjects will be tested for the responses against the whole tumor cells, against KLH and against recombinant PAP and PSA proteins, in order to evaluate the overall increase and the anticipated vaccination-induced Th2 to Th1 shift of $CD4^+$ Th cell responses.
- EPIMAX analysis will be used to define the magnitude and cytokine production profiles of $CD4^+$ and $CD8^+$ T cell responses to PAP and PSMA molecules. The reactivity will be first tested against the pooled batches of the overlapping peptide libraries. Any "positive" pools will be subdivided into individual peptides, in order to define fine specificity of the responses. This analysis will allow us to cover all length of PSMA and PAP, in any subject, independently on HLA type, allowing us to detect immune responses to the previously-identified and potentially novel epitopes.
- We will also analyze CTL cytotoxic activity [to additionally confirm the results of ELISPOT assays],
- We will also monitor the overall numbers of $CD4^+$ and $CD8^+$ T cells, as well as the frequencies of undesirable $CD4^+/CD25^+$/FoxP3/$GITR^+$/ Treg cells. In case of success, these additional novel parameters may be then included in the overall evaluation of this clinical study as additional tertiary end-points.

8.5.2 DTH Testing

Delayed-type hypersensitivity is an in vivo assessment of inflammatory Th1-type T-lymphocyte reactivity against specific antigens. It is the only in vivo assay currently available for the measurement of cellular immunity in humans. A positive DTH response against antigen at cutaneous injection sites will be defined as an increase of 3 mm of induration post dendritic cell vaccine over pre-dendritic cell vaccine. Vehicle only will be used as a negative control in both pre- and post- dendritic cell vaccine evaluations. At each time-point, individual separate sites will be injected with the allogeneic tumor cells used in the vaccine (100 μL in lysate form) and with KLH (100 μg KLH in 200 μL 0.9% sodium chloride). Intradermal injection of saline alone will be used as the negative controls (100 μL 0.9% sodium chloride). Place 3 intradermal injections containing the following (from IMCPL lab):

1) 100 μL of allogeneic tumor cells vaccine (cell line)
2) 100 μg KLH in 200 μL 0.9% sodium chloride
3) 100 μL 0.9% sodium chloride for Negative Control

All testing will be performed on the anterior forearm separated by at least 2 cm in the vertical and horizontal axis.Tattooing of the DTH sites will be done using permanent marker to assure that the site in not injected twice. Skin tests will be read at 48-72 hours by trained clinical personnel.

8.6 Alpha-1 Polarized Dendritic Cells Loaded with Allogeneic Apoptotic Cells

Manufacture of the vaccine will take place in the IMPCL, accordingly to the cGMP-compliant procedures.

8.7 LNCaP Cell Line

The GMP grade cell line ***(See Appendix IV and the letter from our collaborator, Dr. Palucka)*** received from Baylor Institute is maintained by the UPCI IMCPL.

8.8 LHRH Analogue

We will plan on using the q 3 month depot preparation of either Goserelin Acetate 10.8 mg (Zoladex) (NSC-606864) or ELIGARD® 22.5 mg (Lupron) for once every 3 month dosing (NDA #21-379).

8.8.1 Goserelin Acetate

Chemical Structure: pyro-Glu-His-Trp-Ser-Tyr-D-Ser (But)-Leu-Arg-Pro-Azgly-NH_2

Molecular Weight: 1269 daltons (as base)

Solubility: Soluble in water, dimethylformamide, dimethylsulfoxide, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide

Pharmacological Class: LHRH agonist

Zoladex™ is supplied as a 10.8 mg solid depot formulation preloaded in a disposable syringe device mounted on a #14 gauge hypodermic needle. It should be administered subcutaneously every 12 weeks into the upper abdominal wall using sterile technique under the supervision of a physician. While a delay of a few days is permissible, every effort should be made to adhere to the q 12 week schedule.

8.8.2 ELIGARD® 22.5 mg

Active ingredient: Leuprolide acetate

Chemical name: 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide acetate

Relative Molecular Weight: 1,209

Physical Appearance: Lyophilized, white to off-white powder

Solubility: Soluble in water and in acetic acid

Pharmacological Class: Synthetic non-peptide GnRH agonist

Eligard is provided as a powder and solvent for solution/suspension for injection (prolonged release), provided as 2 sterile, pre-filled, polypropylene syringes. One syringe contains the LA powder and the other syringe contains the Atrigel® Delivery System. When reconstituted,

ELIGARD® 22.5 mg injection is a white to off-white, opaque, viscous liquid. It should be administered once every 3 months.

8.9 KLH Protein

This clinical-grade KLH (Immucothel), used as a "heterologous helper antigen", is being provided by Biosyn. It has previously been approved for use in our concurrent melanoma trial: UPCI 03-118/BB-IND 11,754, IRB# IRB0507089). KLH will be loaded only on αDC1s used in the first cycle of vaccination (first vaccine) and will be used as a one of the readouts of the ability of the vaccines to induce DTH responses (see secondary endpoints): the strategy used in our currently active melanoma protocol UPCI 03-118.

9 STATISTICAL CONSIDERATIONS

9.1 Study Design and Endpoints

This is a 2 group crossover trial in which subjects are randomly assigned to one of two treatment arms:

- 3 months of androgen ablation (AA) to be followed at PSA progression by 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine (DCV)
- 3 months of the combination of AA and alpha-type 1 dendritic cell vaccine followed at PSA progression by 3 months of AA.

The primary objectives of this novel trial are to evaluate feasibility, safety, and a preliminary estimate of efficacy. Following either treatment arm the time to PSA progression, defined as the time between treatment and the first instance of PSA increase to 1.0 ng/mL, will be estimated from monthly PSA monitoring. The efficacy endpoint is the difference between time to PSA progression for the combination of AA + DCV compared to AA alone.

9.2 Number of Subjects

For the feasibility objective, the goal is to evaluate the production of bulk vaccine, storage of vaccine, and subsequent administration. If this is successful in the first 4 subjects, we will go on to accrue a total of 12 subjects, 6 per arm, for safety testing, with 6 subjects per arm being consistent with a standard Phase I design.

9.3 Characteristics of the Design

The difference in PSA progression between AA and AA + DCV will be calculated for each individual subject. We note that if the standard deviation of this difference is 1.5 months, a one tailed signed rank test at $\alpha = .05$ will have 98% power to detect a improvement of 1.8 months, equivalent to a hypothesized 30% improvement in time to PSA recovery (6 months vs 7.8 months).

9.4 Safety Evaluation

Serious adverse advents are uncommon in cancer vaccine trials. For example, no treatment-related deaths or grade 4 toxicities have occurred among 652 subjects enrolled in Phase I studies by CTEP, NCI's Cancer Therapy Evaluation Program (Horstmann et al., 2005). Rather than a separate phase of the study to characterize vaccine safety, we will use continual Bayesian monitoring of SAEs (Berry, 2006) with a stopping rule that permits the investigator to suspend the trial for review by the Data and Safety and Monitoring Committee (DSMC). We have selected prior probabilities of SAEs to conform to our prior beliefs that the probability of a treatment-related SAE, caused by αDC1 vaccine is as low as 5%. The prior probability distribution was selected to permit at most a 10% chance that this prior probability could be double the expected prior probability. Assuming the prior probability follows a beta distribution, the stopping rule will be based on the updated posterior probability of an SAE, which also has a beta distribution. As each subject is observed for an SAE, the posterior probability of an SAE can be calculated. If at any time during the trial the probability is ≥ .50 that the treatment-related SAE rate exceeds 5%, the study will be suspended pending review by the DMSC. In order to permit continuous monitoring of serious toxicities, a look-up table has been provided (below). This table is based upon cumulative observed SAEs at any point in the trial and can be used to signal when the therapy should be suspended and the trial results reviewed by the DSMC.

TABLE 4. Number of Observed Treatment-Related SAEs and Associated Posterior Probabilities Needed to Suspend the Study

Subjects	Total Observed SAE's	Pr($\pi \geq .05$)*	B(SAE)**
1	1	.690	.05
2	1	.675	.098
3	1	.660	.143
4	1	.645	.186
5	1	.630	.226
6	1	.615	.264
7	1	.601	.302
8	1	.586	.337
9	1	.572	.370
10	1	.558	.401
11	1	.543	.431
12	1	.529	.460

π is the posterior probability of a treatment-related SAE. Pr($\pi \geq .05$) is the chance that the posterior probability of a treatment-related SAE given the observed number of subjects treated and observed SAEs, exceeds .05.

** B is the binomial probability of observing at least the number of SAEs in column 2 if the underlying rate of SAE is 5%.

This table presents the minimum number of SAEs that dictate suspension of the trial in accordance with the stopping rule. According to this table a single SAE due to the αDC1-based vaccine among any of the 10 subjects will trigger suspension and review by the DSMC.

9.5 Data Analysis

Subjects will be required to have monthly PSA monitoring. This will enable linear interpolation between PSA samples to detect the time at which PSA progression occurs following each course of therapy. The efficacy endpoint is estimated by the difference between time to PSA progression for the AA+DCV arm and the AA arm. This difference will be tested for significance with a one tailed signed rank test. Other conventional estimates of clinical efficacy will also be calculated including PSA doubling time and PSA velocity. The PSA-DT will be calculated as follows:

The logarithm of a minimum of two PSA measurements will be plotted against the time of each PSA measurement. If 3 or more measurements are available a linear regression equation will be estimated. Two sequential PSA measurements (actual or estimated by linear regression) will be denoted as PSA1 and PSA2. Denote their times of blood samples as t1 and t2, respectively. Thus the PSA doubling time is: log (2)[t2 - t1] / [log(PSA2) - log(PSA1)].

Further analysis will involve testing the immunologic response of subjects receiving the αDC1-based vaccine. The principal measure of improved immunogenicity will be the comparison of pre to post αDC1-based vaccine T cell response to prostatic acid phosphatase (PAP) as measured by the IFN-γ Elispot assay. We expect that prostate cancer subjects undergoing androgen ablation in the absence of immune stimulation will have a negative or very low Elispot count for measuring reaction to PAP. Even with a nominal count of 5 spots in the group, a sample of 7 subjects with androgen ablation alone and 7 subjects treated with androgen ablation + αDC1-based vaccine will provide 90% power to detect a difference of double the Elispot count (average of 5 to 10 spots) using a one tailed alpha level Wilcoxon test if the Elispot counts have a Poisson distribution. Additional immunologic testing will conducted by analyzing subject profiles before treatment and after each course of therapy. Immunologic endpoints include T cell reactivity by IFNγ or IL-5 Elispot, cytokine release assays for cytotoxicity and DTH tests (as measured by induration in mm). Changes over time will be tested with the Friedman Test or, if there are missing values, the Mack-Skillings procedure.

9.6 Subjects Randomization

Subjects will be randomized through the email randomization program of the Biostatistics Facility of the UPCI.

10 DATA SAFETY MONITORING PLAN

Investigator/Sub-investigators, regulatory, CRS management, clinical research coordinators, clinical research associates, data managers, and clinic staff meet monthly in disease center Data Safety Monitoring Boards (DSMB) to review and discuss study data to include, but not limited to, the following:

- serious adverse events

- subject safety issues
- recruitment issues
- accrual
- protocol deviations
- unanticipated problems
- breaches of confidentiality

All toxicities encountered during the study will be evaluated on an ongoing basis according to the NCI Common Toxicity Criteria version 4. All study treatment associated adverse events that are serious, at least possibly related and unexpected will be reported to the IRB. Any modifications necessary to ensure subject safety and decisions to continue, or close the trial to accrual are also discussed during these meetings. If any literature becomes available which changes the risk/benefit ratio or suggests that conducting the trial is no longer ethical, the IRB will be notified in the form of an Unanticipated Problem submission and the study may be terminated.

All study data reviewed and discussed during these meetings will be kept confidential. Any breach in subject confidentiality will be reported to the IRB in the form of an Unanticipated Problem submission. The summaries of these meetings are forwarded to the UPCI DSMC which also meets monthly following a designated format.

For all research protocols, there will be a commitment to comply with the IRB's policies for reporting unanticipated problems involving risk to subjects or others (including adverse events). DSMC progress reports, to include a summary of all serious adverse events and modifications, and approval will be submitted to the IRB at the time of renewal.

Protocols with subjects in long-term (survival) follow-up or protocols in data analysis only, will be reviewed twice a year rather than monthly by the disease center DSMB.

Both the UPCI DSMC as well as the individual disease center DSMB have the authority to suspend accrual or further investigate treatment on any trial based on information discussed at these meetings.

All records related to this research study will be stored in a locked environment. Only the researchers affiliated with the research study and their staff will have access to the research records.

11 ADVERSE EVENT REPORTING

11.1 Definitions

Adverse event: Any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related.

Life-threatening adverse event or *life-threatening suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "life-threatening" if, in the view of either the

investigator or sponsor, its occurrence places the patient or subject at immediate risk of death. It does not include an adverse event or suspected adverse reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse event or *serious suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

Suspected adverse reaction: Any adverse event for which there is a reasonable possibility that the drug caused the adverse event. For the purposes of IND safety reporting, "reasonable possibility" means there is evidence to suggest a causal relationship between the drug and the adverse event. Suspected adverse reaction implies a lesser degree of certainty about causality than adverse reaction, which means any adverse event caused by a drug.

Unexpected adverse event or *unexpected suspected adverse reaction:* An adverse event or suspected adverse reaction is considered "unexpected" if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or, if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan or elsewhere in the current application, as amended. For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure referred only to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure listed only cerebral vascular accidents. "Unexpected," as used in this definition, also refers to adverse events or suspected adverse reactions that are mentioned in the investigator brochure as occurring with a class of drugs or as anticipated from the pharmacological properties of the drug, but are not specifically mentioned as occurring with the particular drug under investigation.

11.2 Review of Safety Information: Sponsor Responsibilities[1]

The sponsor must promptly review all information relevant to the safety of the drug obtained or

[1] 21CFR Sec.312.50

otherwise received by the sponsor from foreign or domestic sources, including information derived from any clinical or epidemiological investigations, animal or in vitro studies, reports in the scientific literature, and unpublished scientific papers, as well as reports from foreign regulatory authorities and reports of foreign commercial marketing experience for drugs that are not marketed in the United States.

11.3 Review of Safety Information: Investigator Responsibilities[2]

An investigator shall promptly report to the sponsor of the IND application any adverse effect that may reasonably be regarded as caused by, or probably caused by, the drug. If the adverse effect is alarming, the investigator shall report the adverse effect immediately. An investigator shall provide the sponsor with an adequate report shortly after completion of the investigator's participation in the investigation.

11.4 IND safety reports

The sponsor must notify FDA and all participating investigators (i.e., all investigators to whom the sponsor is providing drug under its INDs or under any investigator's IND) in an IND safety report of potential serious risks, from clinical trials or any other source, as soon as possible, but in no case later than 15 calendar days after the sponsor determines that the information qualifies for reporting under Sections 12.4.1 to 12.4.4 below. In each IND safety report, the sponsor must identify all IND safety reports previously submitted to FDA concerning a similar suspected adverse reaction, and must analyze the significance of the suspected adverse reaction in light of previous, similar reports or any other relevant information.

11.4.1 Serious and unexpected suspected adverse reaction

The sponsor must report any suspected adverse reaction that is both serious and unexpected. The sponsor must report an adverse event as a suspected adverse reaction only if there is evidence to suggest a causal relationship between the drug and the adverse event, such as:

- A single occurrence of an event that is uncommon and known to be strongly associated with drug exposure (e.g., angioedema, hepatic injury, Stevens-Johnson Syndrome);
- One or more occurrences of an event that is not commonly associated with drug exposure, but is otherwise uncommon in the population exposed to the drug (e.g., tendon rupture);
- An aggregate analysis of specific events observed in a clinical trial (such as known consequences of the underlying disease or condition under investigation or other events that commonly occur in the study population independent of drug therapy) that indicates those events occur more frequently in the drug treatment group than in a concurrent or historical control group.

[2] 21 CFR Sec. 312.64

11.4.2 Findings from other studies

The sponsor must report any findings from epidemiological studies, pooled analysis of multiple studies, or clinical studies (other than those reported under section 12.4.1), whether or not conducted under an IND, and whether or not conducted by the sponsor, that suggest a significant risk in humans exposed to the drug. Ordinarily, such a finding would result in a safety-related change in the protocol, informed consent, investigator brochure (excluding routine updates of these documents), or other aspects of the overall conduct of the clinical investigation.

11.4.3 Findings from animal or in vitro testing

The sponsor must report any findings from animal or in vitro testing, whether or not conducted by the sponsor, that suggest a significant risk in humans exposed to the drug, such as reports of mutagenicity, teratogenicity, or carcinogenicity, or reports of significant organ toxicity at or near the expected human exposure. Ordinarily, any such findings would result in a safety-related change in the protocol, informed consent, investigator brochure (excluding routine updates of these documents), or other aspects of the overall conduct of the clinical investigation.

11.4.4 Increased rate of occurrence of serious suspected adverse reactions

The sponsor must report any clinically important increase in the rate of a serious suspected adverse reaction over that listed in the protocol or investigator brochure.

11.4.5 Submission of IND safety reports

The sponsor must submit each IND safety report in a narrative format or on FDA Form 3500A or in an electronic format that FDA can process, review, and archive. FDA will periodically issue guidance on how to provide the electronic submission (e.g., method of transmission, media, file formats, preparation and organization of files). The sponsor may submit foreign suspected adverse reactions on a Council for International Organizations of Medical Sciences (CIOMS) I Form instead of a FDA Form 3500A. Reports of overall findings or pooled analyses from published and unpublished in vitro, animal, epidemiological, or clinical studies must be submitted in a narrative format. Each notification to FDA must bear prominent identification of its contents, i.e., "IND Safety Report," and must be transmitted to the review division in the Center for Drug Evaluation and Research or in the Center for Biologics Evaluation and Research that has responsibility for review of the IND. Upon request from FDA, the sponsor must submit to FDA any additional data or information that the agency deems necessary, as soon as possible, but in no case later than 15 calendar days after receiving the request.

11.4.5.1 Unexpected fatal or life-threatening suspected adverse reaction reports

The sponsor must also notify FDA of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible but in no case later than 7 calendar days after the sponsor's initial receipt of the information.

11.4.5.2 Reporting format or frequency

FDA may require a sponsor to submit IND safety reports in a format or at a frequency different than that required under this paragraph. The sponsor may also propose and adopt a different reporting format or frequency if the change is agreed to in advance by the director of the FDA review division that has responsibility for review of the IND.

11.4.5.3 Investigations of marketed drugs

A sponsor of a clinical study of a drug marketed or approved in the United States that is conducted under an IND is required to submit IND safety reports for suspected adverse reactions that are observed in the clinical study, at domestic or foreign study sites. The sponsor must also submit safety information from the clinical study as prescribed by the post marketing safety reporting requirements (e.g., 310.305, 314.80, and 600.80 of this chapter).

11.4.5.4 Reporting study endpoints

Study endpoints (e.g., mortality or major morbidity) must be reported to FDA by the sponsor as described in the protocol and ordinarily would not be reported under paragraph (c) of this section. However, if a serious and unexpected adverse event occurs for which there is evidence suggesting a causal relationship between the drug and the event (e.g., death from anaphylaxis), the event must be reported under *Serious and unexpected suspected adverse reaction* as a serious and unexpected suspected adverse reaction even if it is a component of the study endpoint (e.g., all-cause mortality).

11.4.6 Follow-up

- The sponsor must promptly investigate all safety information it receives.
- Relevant follow-up information to an IND safety report must be submitted as soon as the information is available and must be identified as such, i.e., "Follow-up IND Safety Report."
- If the results of a sponsor's investigation show that an adverse event not initially determined to be reportable under Section 12.4 is so reportable, the sponsor must report such suspected adverse reaction in an IND safety report as soon as possible, but in no case later than 15 calendar days after the determination is made.

11.4.7 Disclaimer

A safety report or other information submitted by a sponsor under this part (and any release by FDA of that report or information) does not necessarily reflect a conclusion by the sponsor or FDA that the report or information constitutes an admission that the drug caused or contributed to an adverse event. A sponsor need not admit, and may deny, that the report or information submitted by the sponsor constitutes an admission that the drug caused or contributed to an adverse event.

11.5 Reporting adverse events to the responsible IRB

In accordance with applicable policies of the University of Pittsburgh Institutional Review Board (IRB), the Sponsor-Investigator will report, to the IRB, any observed or volunteered adverse event that is determined to be 1) *associated with the investigational drug or study treatment(s)*; 2) *serious*; and 3) *unexpected*. Adverse event reports will be submitted to the IRB in accordance with the respective IRB procedures.

Applicable adverse events will be reported to the IRB as soon as possible and, in no event, later than 10 calendar days following the sponsor-investigator's receipt of the respective information. Adverse events which are 1) *associated with the investigational drug or study treatment(s)*; 2) *fatal or life-threatening*; and 3) *unexpected* will be reported to the IRB within 24 hours of the Sponsor-Investigator's receipt of the respective information.

Follow-up information to a reported adverse event will be submitted to the IRB as soon as the relevant information is available. If the results of the Sponsor-Investigator's follow-up investigation show that an adverse event that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting; the Sponsor-Investigator will report the adverse event to the IRB as soon as possible, but in no event later than 10 calendar days, after the determination was made.

12 QUALITY CONTROL AND QUALITY ASSURANCE

Independent monitoring of the clinical study for protocol and GCP compliance will be conducted periodically (i.e., at a minimum of annually) by qualified staff of the Education and Compliance Office – Human Subject Research, Research Conduct and Compliance Office, University of Pittsburgh.

The Sponsor-Investigator and the University of Pittsburgh and UPMC will permit direct access of the study monitors and appropriate regulatory authorities to the study data and to the corresponding source data and documents to verify the accuracy of this data.

13 DATA HANDLING AND RECORD-KEEPING

The Investigator will maintain records in accordance with Good Clinical Practice guidelines.

The Investigator will retain the specified records and reports for up to 2 years after the marketing application is approved for the investigational drug; or, if a marketing application is not submitted or approved for the investigational drug, until 2 years after investigations under the IND have been discontinued and the FDA so notified.

14 ETHICS

14.1 Institutional Review Board (IRB) approval

The Investigator will obtain, from the University of Pittsburgh Institutional Review Board (IRB), prospective approval of the clinical protocol and corresponding informed consent form(s);

modifications to the clinical protocol and corresponding informed consent forms, and advertisements (i.e., directed at potential research subjects) for study recruitment.

The only circumstance in which a deviation from the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator will promptly notify the University of Pittsburgh IRB of the deviation.

The University of Pittsburgh IRB operates in compliance with FDA regulations at 21 CFR Parts 50 and 21 CFR 56, and in conformance with applicable International Conference on Harmonization (ICH) Guidelines on Good Clinical Practice (CGP).

In the event that the University of Pittsburgh IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of the Sponsor's decision to modify the previously accepted clinical protocol:

The Investigator will submit (i.e., in advance of implementing the change) a Protocol Amendment to the IND describing any change to the Phase I clinical protocol that significantly affects the safety of the subjects. For changes that do not affect critical safety assessments, the revisions to the clinical protocol will be addressed in the Annual Report to the IND.

14.2 Ethical and scientific conduct of the clinical research study

The clinical research study will be conducted in accordance with the current IRB-approved clinical protocol; ICH GCP Guidelines adopted by the FDA; and relevant policies, requirements, and regulations of the University of Pittsburgh IRB, University of Pittsburgh and UPMC, Commonwealth of Pennsylvania, and applicable federal agencies.

14.3 Subject informed consent

The Investigator will make certain that an appropriate informed consent process is in place to ensure that potential research subjects, or their authorized representatives, are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Investigator, or a sub-investigator(s) designated by the Investigator, will obtain the written, signed informed consent of each subject, or the subject's authorized representative, prior to performing any study-specific procedures on the subject. The date and time that the subject, or the subject's authorized representative, signs the informed consent form and a narrative of the issues discussed during the informed consent process will be documented in the subject's case history. The Investigator will retain the original copy of the signed informed consent form, and a copy will be provided to the subject, or to the subject's authorized representative.

The Investigator will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and

that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Investigator will obtain the informed consent of enrolled subjects for continued participation in the clinical study

15 REFERENCES

Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H. M., Kubo, R. T., Sette, A., and et al. (1994). Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. Immunity *1*, 751-761.

Alexander, R. B., Brady, F., Leffell, M. S., Tsai, V., and Celis, E. (1998). Specific T cell recognition of peptides derived from prostate-specific antigen in patients with prostate cancer. Urology *51*, 150-157.

Asai, T., Storkus, W. J., and Whiteside, T. L. (2000). Evaluation of the modified ELISPOT assay for gamma interferon production in cancer patients receiving antitumor vaccines. Clin Diagn Lab Immunol *7*, 145-154.

Attiga, F. A., Fernandez, P. M., Weeraratna, A. T., Manyak, M. J., and Patierno, S. R. (2000). Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. Cancer Res *60*, 4629-4637.

Banchereau, J., Palucka, A. K., Dhodapkar, M., Burkeholder, S., Taquet, N., Rolland, A., Taquet, S., Coquery, S., Wittkowski, K. M., Bhardwaj, N.*, et al.* (2001). Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res *61*, 6451-6458.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barratt-Boyes, S. M., Zimmer, M. I., Harshyne, L. A., Meyer, E. M., Watkins, S. C., Capuano, S., 3rd, Murphey-Corb, M., Falo, L. D., Jr., and Donnenberg, A. D. (2000). Maturation and trafficking of monocyte-derived dendritic cells in monkeys: implications for dendritic cell-based vaccines. J Immunol *164*, 2487-2495.

Berry, D. A. (2006). Bayesian clinical trials. Nat Rev Drug Discov *5*, 27-36.

Blades, R. A., Keating, P. J., McWilliam, L. J., George, N. J., and Stern, P. L. (1995). Loss of HLA class I expression in prostate cancer: implications for immunotherapy. Urology *46*, 681-686; discussion 686-687.

Boutemmine, D., Bouchard, N., Boerboom, D., Jones, H. E., Goff, A. K., Dore, M., and Sirois, J. (2002). Molecular characterization of canine prostaglandin G/H synthase-2 and regulation in prostatic adenocarcinoma cells in vitro. Endocrinology *143*, 1134-1143.

Bubley, G. J., Carducci, M., Dahut, W., Dawson, N., Daliani, D., Eisenberger, M., Figg, W. D., Freidlin, B., Halabi, S., Hudes, G.*, et al.* (1999). Eligibility and response guidelines for phase II clinical trials in androgen-independent prostate cancer: recommendations from the Prostate-Specific Antigen Working Group. J Clin Oncol *17*, 3461-3467.

Burch, P. A., Croghan, G. A., Gastineau, D. A., Jones, L. A., Kaur, J. S., Kylstra, J. W., Richardson, R. L., Valone, F. H., and Vuk-Pavlovic, S. (2004). Immunotherapy (APC8015, Provenge) targeting prostatic acid phosphatase can induce durable remission of metastatic androgen-independent prostate cancer: a Phase 2 trial. Prostate *60*, 197-204.

Catalona, W. J., and Smith, D. S. (1998). Cancer recurrence and survival rates after anatomic radical retropubic prostatectomy for prostate cancer: intermediate-term results. J Urol *160*, 2428-2434.

Chakraborty, N. G., Stevens, R. L., Mehrotra, S., Laska, E., Taxel, P., Sporn, J. R., Schauer, P., and Albertsen, P. C. (2003). Recognition of PSA-derived peptide antigens by T cells from prostate cancer patients without any prior stimulation. Cancer Immunol Immunother *52*, 497-505.

Chen, Y., and Hughes-Fulford, M. (2000). Prostaglandin E2 and the protein kinase A pathway mediate arachidonic acid induction of c-fos in human prostate cancer cells. Br J Cancer *82*, 2000-2006.

Chen, Y. M., Yang, W. K., Ting, C. C., Tsai, W. Y., Yang, D. M., Whang-Peng, J., and Perng, R. P. (1997). Cross regulation by IL-10 and IL-2/IL-12 of the helper T cells and the cytolytic activity of lymphocytes from malignant effusions of lung cancer patients. Chest *112*, 960-966.

Correale, P., Walmsley, K., Nieroda, C., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Y. (1997). In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen. J Natl Cancer Inst *89*, 293-300.

Correale, P., Walmsley, K., Zaremba, S., Zhu, M., Schlom, J., and Tsang, K. Y. (1998). Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide. J Immunol *161*, 3186-3194.

D'Amico, A. V., Chen, M. H., Roehl, K. A., and Catalona, W. J. (2004). Preoperative PSA velocity and the risk of death from prostate cancer after radical prostatectomy. N Engl J Med *351*, 125-135.

Dilliogluglil, O., Leibman, B. D., Kattan, M. W., Seale-Hawkins, C., Wheeler, T. M., and Scardino, P. T. (1997). Hazard rates for progression after radical prostatectomy for clinically localized prostate cancer. Urology *50*, 93-99.

DiPaola, R. S. (1999). Approaches to the treatment of patients with hormone-sensitive prostate cancer. Semin Oncol *26*, 24-27.

Drake, C. G., Doody, A. D., Mihalyo, M. A., Huang, C. T., Kelleher, E., Ravi, S., Hipkiss, E. L., Flies, D. B., Kennedy, E. P., Long, M.*, et al.* (2005). Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell *7*, 239-249.

Eaton, J. D., Perry, M. J., Nicholson, S., Guckian, M., Russell, N., Whelan, M., and Kirby, R. S. (2002). Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. BJU Int *89*, 19-26.

Filella, X., Alcover, J., Zarco, M. A., Beardo, P., Molina, R., and Ballesta, A. M. (2000). Analysis of type T1 and T2 cytokines in patients with prostate cancer. Prostate *44*, 271-274.

Fong, L., Brockstedt, D., Benike, C., Breen, J. K., Strang, G., Ruegg, C. L., and Engleman, E. G. (2001). Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy. J Immunol *167*, 7150-7156.

Fong, L., and Engleman, E. G. (2000). Dendritic cells in cancer immunotherapy. Annu Rev Immunol *18*, 245-273.

Fong, L., Ruegg, C. L., Brockstedt, D., Engleman, E. G., and Laus, R. (1997). Induction of tissue-specific autoimmune prostatitis with prostatic acid phosphatase immunization: implications for immunotherapy of prostate cancer. J Immunol *159*, 3113-3117.

Fukao, T. (2002). Dendritic-cell-based anticancer vaccination: has it matured? Trends Immunol *23*, 231-232.

Gabrilovich, D., and Pisarev, V. (2003). Tumor escape from immune response: mechanisms and targets of activity. Curr Drug Targets *4*, 525-536.

Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G., and Trinchieri, G. (2002). Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med *195*, 327-333.

Goto, S., Sato, M., Kaneko, R., Itoh, M., Sato, S., and Takeuchi, S. (1999). Analysis of Th1 and Th2 cytokine production by peripheral blood mononuclear cells as a parameter of immunological dysfunction in advanced cancer patients. Cancer Immunol Immunother *48*, 435-442.

Gulley, J. L., Figg, W. D., Steinberg, S. M., Carter, J., Sartor, O., Higano, C. S., Petrylak, D. P., Chatta, G., Hussain, M. H., and Dahut, W. L. (2005). A prospective analysis of the time to normalization of serum androgens following 6 months of androgen deprivation therapy in patients on a randomized phase III clinical trial using limited hormonal therapy. J Urol *173*, 1567-1571.

Hanks, G. E., Pajak, T. F., Porter, A., Grignon, D., Brereton, H., Venkatesan, V., Horwitz, E. M., Lawton, C., Rosenthal, S. A., Sandler, H. M., and Shipley, W. U. (2003). Phase III trial of long-term adjuvant androgen deprivation after neoadjuvant hormonal cytoreduction and radiotherapy in locally advanced carcinoma of the prostate: the Radiation Therapy Oncology Group Protocol 92-02. J Clin Oncol *21*, 3972-3978.

Harada, M., Matsueda, S., Yao, A., Ogata, R., Noguchi, M., and Itoh, K. (2004). Prostate-related antigen-derived new peptides having the capacity of inducing prostate cancer-reactive CTLs in HLA-A2+ prostate cancer patients. Oncol Rep *12*, 601-607.

Havranek, E. G., Whelan, M. A., Greenhalgh, R., Dalgleish, A. G., and Pandha, H. (2002). Advances in prostate cancer immunotherapy. Surg Oncol *11*, 35-45.

Heiser, A., Coleman, D., Dannull, J., Yancey, D., Maurice, M. A., Lallas, C. D., Dahm, P., Niedzwiecki, D., Gilboa, E., and Vieweg, J. (2002). Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest *109*, 409-417.

Horstmann, E., McCabe, M. S., Grochow, L., Yamamoto, S., Rubinstein, L., Budd, T., Shoemaker, D., Emanuel, E. J., and Grady, C. (2005). Risks and benefits of phase 1 oncology trials, 1991 through 2002. N Engl J Med *352*, 895-904.

Hrouda, D., Todryk, S. M., Perry, M. J., Souberbielle, B. E., Kayaga, J., Kirby, R. S., and Dalgleish, A. G. (2000). Allogeneic whole-tumour cell vaccination in the rat model of prostate cancer. BJU Int *86*, 742-748.

Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R. C., Ghafoor, A., Feuer, E. J., and Thun, M. J. (2005). Cancer statistics, 2005. CA Cancer J Clin *55*, 10-30.

Kalinski, P., Hilkens, C. M., Snijders, A., Snijdewint, F. G., and Kapsenberg, M. L. (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J Immunol *159*, 28-35.

Kalinski, P., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. (1999a). T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today *20*, 561-567.

Kalinski, P., Schuitemaker, J. H., Hilkens, C. M., Wierenga, E. A., and Kapsenberg, M. L. (1999b). Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J Immunol *162*, 3231-3236.

Kaufman, H. L., Wang, W., Manola, J., DiPaola, R. S., Ko, Y. J., Sweeney, C., Whiteside, T. L., Schlom, J., Wilding, G., and Weiner, L. M. (2004). Phase II randomized study of vaccine treatment of advanced prostate cancer (E7897): a trial of the Eastern Cooperative Oncology Group. J Clin Oncol *22*, 2122-2132.

Kelly, W. K., Scher, H. I., Mazumdar, M., Vlamis, V., Schwartz, M., and Fossa, S. D. (1993). Prostate-specific antigen as a measure of disease outcome in metastatic hormone-refractory prostate cancer. J Clin Oncol *11*, 607-615.

Kennedy-Smith, A. G., McKenzie, J. L., Owen, M. C., Davidson, P. J., Vuckovic, S., and Hart, D. N. (2002). Prostate specific antigen inhibits immune responses in vitro: a potential role in prostate cancer. J Urol *168*, 741-747.

Kobayashi, K., Noguchi, M., Itoh, K., and Harada, M. (2003). Identification of a prostate-specific membrane antigen-derived peptide capable of eliciting both cellular and humoral immune responses in HLA-A24+ prostate cancer patients. Cancer Sci *94*, 622-627.

Langenkamp, A., Messi, M., Lanzavecchia, A., and Sallusto, F. (2001). Kinetics of dendritic cell activation: impact on priming of Th1, Th2 and nonpolarized T cells. Nat Immunol *1*, 311-316.

Lawton, C. A., Winter, K., Murray, K., Machtay, M., Mesic, J. B., Hanks, G. E., Coughlin, C. T., and Pilepich, M. V. (2001). Updated results of the phase III Radiation Therapy Oncology Group (RTOG) trial 85-31 evaluating the potential benefit of androgen suppression following standard radiation therapy for unfavorable prognosis carcinoma of the prostate. Int J Radiat Oncol Biol Phys *49*, 937-946.

Liu, K. J., Chatta, G. S., Twardzik, D. R., Vedvick, T. S., True, L. D., Spies, A. G., and Cheever, M. A. (1997). Identification of rat prostatic steroid-binding protein as a target antigen of experimental autoimmune prostatitis: implications for prostate cancer therapy. J Immunol *159*, 472-480.

Liu, X. H., Kirschenbaum, A., Lu, M., Yao, S., Klausner, A., Preston, C., Holland, J. F., and Levine, A. C. (2002). Prostaglandin E(2) stimulates prostatic intraepithelial neoplasia cell growth through activation of the interleukin-6/GP130/STAT-3 signaling pathway. Biochem Biophys Res Commun *290*, 249-255.

Lopez, J. A., and Hart, D. N. (2002). Current issues in dendritic cell cancer immunotherapy. Curr Opin Mol Ther *4*, 54-63.

Machlenkin, A., Paz, A., Bar Haim, E., Goldberger, O., Finkel, E., Tirosh, B., Volovitz, I., Vadai, E., Lugassy, G., Cytron, S.*, et al.* (2005). Human CTL epitopes prostatic acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3 as candidates for prostate cancer immunotherapy. Cancer Res *65*, 6435-6442.

Mackensen, A., Krause, T., Blum, U., Uhrmeister, P., Mertelsmann, R., and Lindemann, A. (1999). Homing of intravenously and intralymphatically injected human dendritic cells generated in vitro from CD34+ hematopoietic progenitor cells. Cancer Immunol Immunother *48*, 118-122.

Mailliard, R. B., Egawa, S., Cai, Q., Kalinska, A., Bykovskaya, S. N., Lotze, M. T., Kapsenberg, M. L., Storkus, W. J., and Kalinski, P. (2002). Complementary dendritic cell-activating function of CD8+ and CD4+ T cells: helper role of CD8+ T cells in the development of T helper type 1 responses. J Exp Med *195*, 473-483.

Mailliard, R. B., Son, Y. I., Redlinger, R., Coates, P. T., Giermasz, A., Morel, P. A., Storkus, W. J., and Kalinski, P. (2003). Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J Immunol *171*, 2366-2373.

Mailliard, R. B., Wankowicz-Kalinska, A., Cai, Q., Wesa, A., Hilkens, C. M., Kapsenberg, M. L., Kirkwood, J. M., Storkus, W. J., and Kalinski, P. (2004). alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. Cancer Res *64*, 5934-5937.

Maloy, K. J., Erdmann, I., Basch, V., Sierro, S., Kramps, T. A., Zinkernagel, R. M., Oehen, S., and Kundig, T. M. (2001). Intralymphatic immunization enhances DNA vaccination. Proc Natl Acad Sci U S A *98*, 3299-3303.

Meidenbauer, N., Harris, D. T., Spitler, L. E., and Whiteside, T. L. (2000). Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. Prostate *43*, 88-100.

Mercader, M., Bodner, B. K., Moser, M. T., Kwon, P. S., Park, E. S., Manecke, R. G., Ellis, T. M., Wojcik, E. M., Yang, D., Flanigan, R. C.*, et al.* (2001). T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A *98*, 14565-14570.

Messing, E. M., Manola, J., Sarosdy, M., Wilding, G., Crawford, E. D., and Trump, D. (1999). Immediate hormonal therapy compared with observation after radical prostatectomy and pelvic lymphadenectomy in men with node-positive prostate cancer. N Engl J Med *341*, 1781-1788.

Michael, A., Ball, G., Quatan, N., Wushishi, F., Russell, N., Whelan, J., Chakraborty, P., Leader, D., Whelan, M., and Pandha, H. (2005). Delayed disease progression after allogeneic cell vaccination

in hormone-resistant prostate cancer and correlation with immunologic variables. Clin Cancer Res *11*, 4469-4478.

Murphy, G. P., Tjoa, B. A., Simmons, S. J., Rogers, M. K., Kenny, G. M., and Jarisch, J. (2000). Higher-dose and less frequent dendritic cell infusions with PSMA peptides in hormone-refractory metastatic prostate cancer patients. Prostate *43*, 59-62.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998). Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med *4*, 328-332.

Nouri-Shirazi, M., Banchereau, J., Bell, D., Burkeholder, S., Kraus, E. T., Davoust, J., and Palucka, K. A. (2000). Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses. J Immunol *165*, 3797-3803.

Pandha, H. S., John, R. J., Hutchinson, J., James, N., Whelan, M., Corbishley, C., and Dalgleish, A. G. (2004). Dendritic cell immunotherapy for urological cancers using cryopreserved allogeneic tumour lysate-pulsed cells: a phase I/II study. BJU Int *94*, 412-418.

Peshwa, M. V., Shi, J. D., Ruegg, C., Laus, R., and van Schooten, W. C. (1998). Induction of prostate tumor-specific CD8+ cytotoxic T-lymphocytes in vitro using antigen-presenting cells pulsed with prostatic acid phosphatase peptide. Prostate *36*, 129-138.

Pound, C. R., Partin, A. W., Eisenberger, M. A., Chan, D. W., Pearson, J. D., and Walsh, P. C. (1999). Natural history of progression after PSA elevation following radical prostatectomy. Jama *281*, 1591-1597.

Roach, M., 3rd, Lu, J., Pilepich, M. V., Asbell, S. O., Mohiuddin, M., Terry, R., and Grignon, D. (1999). Long-term survival after radiotherapy alone: radiation therapy oncology group prostate cancer trials. J Urol *161*, 864-868.

Romani, N., Ratzinger, G., Pfaller, K., Salvenmoser, W., Stossel, H., Koch, F., and Stoitzner, P. (2001). Migration of dendritic cells into lymphatics-the Langerhans cell example: routes, regulation, and relevance. Int Rev Cytol *207*, 237-270.

Rosenberg, S. A., Yang, J. C., and Restifo, N. P. (2004). Cancer immunotherapy: moving beyond current vaccines. Nat Med *10*, 909-915.

Sanda, M. G., Restifo, N. P., Walsh, J. C., Kawakami, Y., Nelson, W. G., Pardoll, D. M., and Simons, J. W. (1995). Molecular characterization of defective antigen processing in human prostate cancer. J Natl Cancer Inst *87*, 280-285.

Scher, H. I., Eisenberger, M., D'Amico, A. V., Halabi, S., Small, E. J., Morris, M., Kattan, M. W., Roach, M., Kantoff, P., Pienta, K. J.*, et al.* (2004). Eligibility and outcomes reporting guidelines for clinical trials for patients in the state of a rising prostate-specific antigen: recommendations from the Prostate-Specific Antigen Working Group. J Clin Oncol *22*, 537-556.

Schuler-Thurner, B., Schultz, E. S., Berger, T. G., Weinlich, G., Ebner, S., Woerl, P., Bender, A., Feuerstein, B., Fritsch, P. O., Romani, N., and Schuler, G. (2002). Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med *195*, 1279-1288.

Schuler, G., and Steinman, R. M. (1997). Dendritic cells as adjuvants for immune-mediated resistance to tumors. J Exp Med *186*, 1183-1187.

Shurin, G. V., Aalamian, M., Pirtskhalaishvili, G., Bykovskaia, S., Huland, E., Huland, H., and Shurin, M. R. (2001). Human prostate cancer blocks the generation of dendritic cells from CD34+ hematopoietic progenitors. Eur Urol *39 Suppl 4*, 37-40.

Simon R, Optimal two-stage designs for phase II clinical trials, *Controlled Clinical Trials* 10:1-10, 1989

Simons, J. W., Mikhak, B., Chang, J. F., DeMarzo, A. M., Carducci, M. A., Lim, M., Weber, C. E., Baccala, A. A., Goemann, M. A., Clift, S. M., *et al.* (1999). Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. Cancer Res *59*, 5160-5168.

Small, E. J., Fratesi, P., Reese, D. M., Strang, G., Laus, R., Peshwa, M. V., and Valone, F. H. (2000). Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. J Clin Oncol *18*, 3894-3903.

Steinman, R. M. (1991). The dendritic cell system and its role in immunogenecity. Annu Rev Immunol *9*, 271-296.

Steinman, R. M., and Cohn, Z. A. (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med *137*, 1142-1162.

Suckow, M. A., Wolter, W. R., and Pollard, M. (2005). Prevention of de novo prostate cancer by immunization with tumor-derived vaccines. Cancer Immunol Immunother *54*, 571-576.

Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., Oudard, S., Theodore, C., James, N. D., Turesson, I., *et al.* (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med *351*, 1502-1512.

Tatsumi, T., Kierstead, L. S., Ranieri, E., Gesualdo, L., Schena, F. P., Finke, J. H., Bukowski, R. M., Mueller-Berghaus, J., Kirkwood, J. M., Kwok, W. W., and Storkus, W. J. (2002). Disease-associated bias in T helper type 1 (Th1)/Th2 CD4(+) T cell responses against MAGE-6 in HLA-DRB10401(+) patients with renal cell carcinoma or melanoma. J Exp Med *196*, 619-628.

Tjandrawinata, R. R., Dahiya, R., and Hughes-Fulford, M. (1997). Induction of cyclo-oxygenase-2 mRNA by prostaglandin E2 in human prostatic carcinoma cells. Br J Cancer *75*, 1111-1118.

Todryk, S. M., Eaton, J., Birchall, L., Greenhalgh, R., Soars, D., Dalgleish, A. G., Melcher, A. A., and Pandha, H. S. (2004). Heated tumour cells of autologous and allogeneic origin elicit anti-tumour immunity. Cancer Immunol Immunother *53*, 323-330.

Tourkova, I. L., Yamabe, K., Foster, B., Chatta, G., Perez, L., Shurin, G. V., and Shurin, M. R. (2004). Murine prostate cancer inhibits both in vivo and in vitro generation of dendritic cells from bone marrow precursors. Prostate *59*, 203-213.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol *3*, 133-146.

Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L., and Kalinski, P. (2000). Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol *164*, 4507-4512.

Vieira, P. L., Kalinski, P., Wierenga, E. A., Kapsenberg, M. L., and de Jong, E. C. (1998). Glucocorticoids inhibit bioactive IL-12p70 production by in vitro-generated human dendritic cells without affecting their T cell stimulatory potential. J Immunol *161*, 5245-5251.

Vieweg, J., Rosenthal, F. M., Bannerji, R., Heston, W. D., Fair, W. R., Gansbacher, B., and Gilboa, E. (1994). Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. Cancer Res *54*, 1760-1765.

Vogelzang, N. (1997). New developments in the treatment of advanced prostate cancer. Seminars in Oncology *23 (6)*, 1-5.

Xue, B. H., Zhang, Y., Sosman, J. A., and Peace, D. J. (1997). Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen. Prostate *30*, 73-78.

Zitvogel, L., Mayordomo, J. I., Tjandrawan, T., DeLeo, A. B., Clarke, M. R., Lotze, M. T., and Storkus, W. J. (1996). Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T hepler cell 1-associated cytokines. JExpMed *183*, 87-97.

APPENDIX I: SUMMARY OF PRECLINICAL DATA

Based on a **novel paradigm of polarization of dendritic cells** [1,2], we have recently developed the first clinically-applicable protocol (serum-free conditions) to generate alpha-type-1 polarized DC (αDC1) [3]. Such αDC1s are the first clinically-applicable type of **DCs that combine all 3 properties deemed important for their *in vivo* activity** as inducers of anticancer immunity. These are: 1) fully-mature status; 2) high migratory responsiveness to lymph node-produced chemokines; and 3) high IL-12p70-producing function (*Fig 1*). Such combination was missing in all previously-applied protocols of preparing DC, where final DC maturation (needed to generate DC with high expression of costimulatory molecules and the ability to localize in the T cell areas of the lymph nodes) was associated with irreversible loss of their ability to produce the key Th1-, CTL-, and NK cell-activating cytokine with anti-cancer properties, IL-12 [4,5].

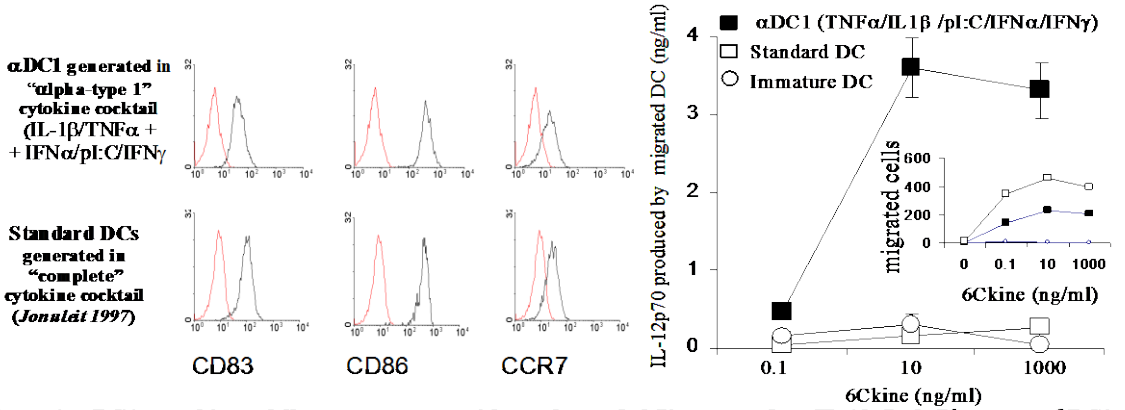


Figure 1. αDC1s combine a fully-mature status with an elevated ability to produce IL-12. Left. Phenotype of DC1. **Right.** αDC1 are stable and maintain high IL-12p70 producing capacity following migration in response to lymph-node-associated 6C-kine. αDC1; standard sDCs (IL-1β/TNFα/IL-6/PGE_2-matured DC), or immature DCs were exposed to increasing concentrations of 6C-kine in a migration chambers. Migrated DCs in the bottom chamber were stimulated with CD40L-transfected J558 cells. *The inset*: The corresponding relative numbers of DCs migrating in response to 6C-kine.

Compared to the current "gold standard" of clinically-used DCs, αDC1s show selectively enhanced ability to induce type-1 anti-cancer responses, when**.** Our studies in the melanoma model have shown that peptide-pulsed DC1s induce up to **40-fold higher numbers of tumor-specific CTLs**, during *in vitro* sensitization of peripheral blood T cells from the patients, and induce strong antitumor activity of NK cells (***Ref 3 and Fig. 2***).

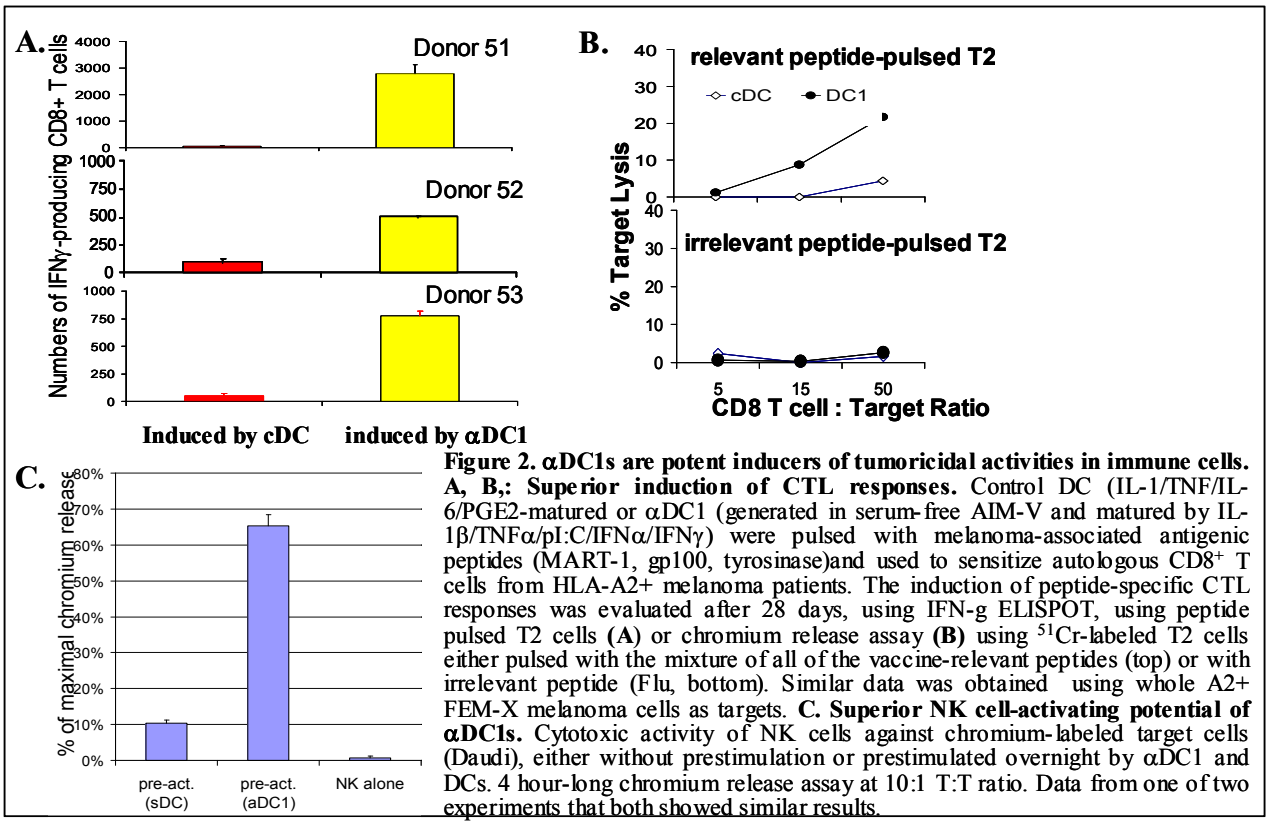


Figure 2. αDC1s are potent inducers of tumoricidal activities in immune cells. A, B,: Superior induction of CTL responses. Control DC (IL-1/TNF/IL-6/PGE2-matured or αDC1 (generated in serum-free AIM-V and matured by IL-1β/TNFα/pI:C/IFNα/IFNγ) were pulsed with melanoma-associated antigenic peptides (MART-1, gp100, tyrosinase)and used to sensitize autologous $CD8^+$ T cells from HLA-A2+ melanoma patients. The induction of peptide-specific CTL responses was evaluated after 28 days, using IFN-g ELISPOT, using peptide pulsed T2 cells **(A)** or chromium release assay **(B)** using ^{51}Cr-labeled T2 cells either pulsed with the mixture of all of the vaccine-relevant peptides (top) or with irrelevant peptide (Flu, bottom). Similar data was obtained using whole A2+ FEM-X melanoma cells as targets. **C. Superior NK cell-activating potential of αDC1s.** Cytotoxic activity of NK cells against chromium-labeled target cells (Daudi), either without prestimulation or prestimulated overnight by αDC1 and DCs. 4 hour-long chromium release assay at 10:1 T:T ratio. Data from one of two experiments that both showed similar results.

Importantly for wider clinical applications of DC-based vaccines, we could also show high ability of αDC1 to **cross-present apoptotic tumor** (*Fig. 3*), an important factor allowing to use as a source of tumor-relevant antigens whole tumor cells, rather than defined peptide epitopes, broadening the spectrum of tumor-relevant targets of immunization and eliminating the need to restrict the study to patients with a defined MHC type (usually HLA-A2). This last property of DC1s also facilitates the use of either autologous tumor or allogeneic prostate cancer cell lines as a source of antigenic material. Allogeneic cell lines offer the advantage of standardized preparation of the vaccine, and a potential possibility to further boost the immunogenic properties of vaccine-carrying DCs by promoting their interaction *in vivo* with high numbers of allo-specific $CD8^+$ T cells [6] and NK cells [7] in tumor-bearing hosts.

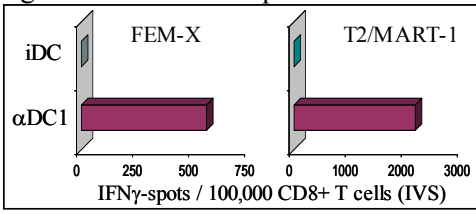


Figure 3. αDC1 effectively cross-present apoptotic tumor cells to $CD8^+$ T cells αDC1 matured in the presence of UV-irradiated apoptotic tumor cells (FEM-X) were used to sensitize $CD8^+$ T cells from HLA-$A2^+$ donor Priming with immature DC was used as a negative control. Frequencies of $CD8^+$ T cells responsive to FEM-X cells, or to MART-1 (27-37 epitope) pulsed on T2 cells were determined by IFNγ ELISPOTs.

Until recently, our effort concentrated on using this strategy to develop more effective vaccines against skin cancers: malignant melanoma and cutaneous T cell lymphoma. These studies, performed in collaboration with the groups of Dr. Walter Storkus, Dr. Michael Lotze, Dr. Luis Falo, and Dr. John Kirkwood, resulted in the design and FDA approval (**BB-IND 11,754)** a clinical protocol in melanoma (**UPCI 03-118**). In the meantime, our collaborators from the Department of Dermatology (Drs. L. Geskin and L. Falo) used our platform to vaccinate patients with advanced cutaneous T cell lymphoma (Sezary Syndrome), observing lack of serious side effects and the ability of tumor-loaded αDC1s to induce clinical responses. While our past attempts concentrated on the development of the clinical trials with the immunologic primary efficacy endpoint, the recently established collaboration between our two groups (of Dr. Pawel Kalinski and Dr. Gurkamal Chatta), allow us to extend our unique vaccination platform (developed and currently used exclusively in the University of Pittsburgh Cancer Institute) in prostate cancer: the leading cause of cancer-related deaths in men in the US.

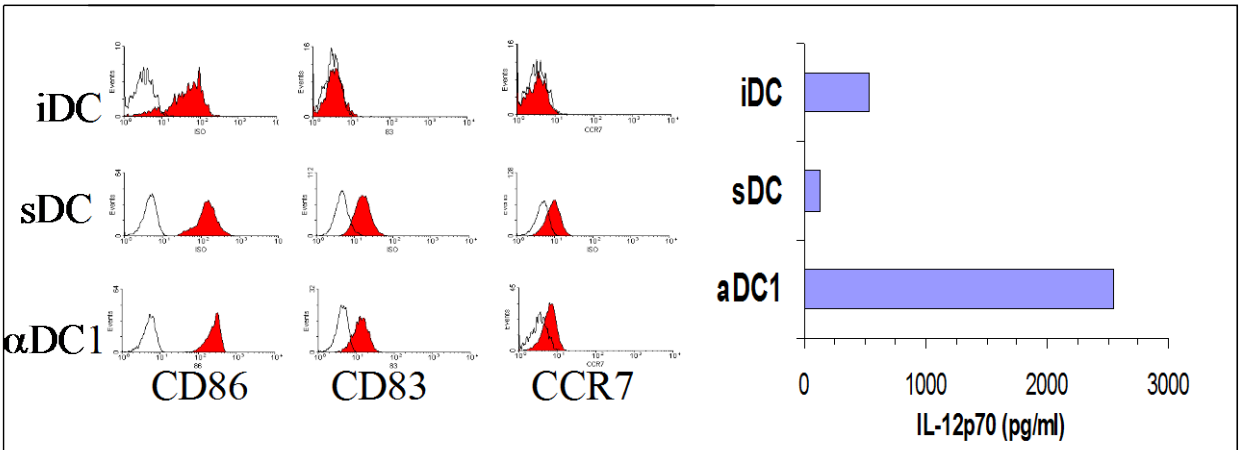


Figure 4. Successful generation of αDC1s from prostate cancer patients: a fully-mature status an elevated ability to produce IL-12 of αDC1s. Left. Phenotype of DC1. **Right.** αDC1 show high IL-12p70 producing capacity. αDC1s, sDCs (IL-1β/TNFα/IL-6/PGE_2-matured DC), or immature DCs were stimulated with CD40L-transfected J558 cells.

Our data demonstrate the **feasibility of inducing αDC1s from the monocytes of prostate cancer patients**. As shown in ***Fig. 4***, αDC1s from such patients share fully mature status ($CD86^{high}$, $CD83^{+}$, $CCR7^{+}$ phenotype) with the elevated ability to produce high levels of IL-12p70 (bioactive form of IL-12). These features positively distinguish αDC1s from both immature DCs ($CD86^{low}$, $CD83^{-}$, $CCR7^{-}$ and IL-12p70 ^{intermediate}) and from ($CD86^{high}$, $CD83^{+}$, $CCR7^{+}$ but IL-12p70^{low}) sDCs (***see Fig 4***).

Numerous tumor-produced factors, including prostate-produced prostaglandin E2 (PGE_2), an immunosuppressive factor produced by prostate cancer [8-18], are known to suppress the IL-12-producing capacity of maturing DCs[2,19-21]. On the other hand, our data with the non-clinical grade DC1s obtained in the presence of serum demonstrated their resistance to PGE_2 [2], suggesting that such cells may be able to resist prostate cancer-associated immune suppression. In order to verify that the loading of αDC1s with apoptotic LNCap will not prevent the development of high IL-12 producing function of αDC1s (generated under the clinically-desirable serum-free condition), we compared the expression of DC maturation markers and IL-12p70 producing capacity in αDC1s generated in the absence and in the presence of UV-irradiated apoptotic LNCap cells. As shown in **Fig 5**, in accordance with our expectations, the presence of apoptotic LNCap cells did not prevent the development of αDC1s characterized by mature phenotype and sgtrongly elevated ability to produce bioactive IL-12p70. xpression to induce autoimmune prostatitis as a novel immunotherapy for prostate cancer, through dendritic cell-induced stimulation of $CD8^{+}$ cytotoxic T cell (CTL) responses against prostate autoantigens. This is based on the premise that a vigorous autoimmune response against prostate-specific proteins, capable of destroying normal prostate tissue, will also destroy malignant prostate tissue, provided the malignant tissue expresses these proteins. Since the prostate gland is routinely removed or ablated as a part of the treatment for early prostate cancer, any remaining/recurring prostatic tissue could be destroyed immunologically. Three prostate tissue specific antigens are currently being evaluated in clinical trials: prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and prostate specific membrane antigen (PSMA). However the immunogenicity of these proteins is not well understood, and a priori, there is no perfect prostate-specific immunologic target. Hence our focus on either autologous tumor (where available) or allogenic tumor cell lines. Additionally, since advanced prostate cancer in humans, is initially treated with androgen ablation, we will also incorporate limited (3 to 6 months) androgen deprivation (with LHRH analogues) in the treatment protocol. It has recently been reported that androgen ablation[22] in patients with prostate cancer, induces a T cell infiltrate in to the prostate, and could potentially augment the immune response, following immunization.

Our target population for treatment will be men with prostate cancer, who have relapsed following surgery and/or radiation, and now have a rising PSA with a PSA doubling time (PSA-DT) of less than 12

months. This patient population is **ideal for an immunologic intervention** given that: i) this is the largest sub-population of men with prostate cancer in the U.S.; ii) there is no standard treatment for this sub-group; and iii) this sub-group has minimal systemic disease burden. We will carefully monitor immunological responses to vaccination at different time points, but the primary endpoint of vaccine efficacy will be stabilization of PSA levels or failure of the PSA to progress. Several recent studies have validated failure of PSA progression as a good surrogate endpoint for lack of progression in clinical trials in prostate cancer.

Within the 12 months funding period of the currently-proposed CTDA, we will **1) develop the clinical protocol** to test the safety and efficacy of αDC1-based vaccines for patients against advanced prostate cancer, will **2) discuss the trial design** with our PRC and with FDA (in form of a pre-IND meeting), and will subsequently **3) obtain the FDA and IRB approval**s for the developed protocol. As the part of the proposed trial development process, we will **4) develop and submit a Clinical Trial Award DOD application or an R21-type NIH application** (PAR 03-005: Quick Funding of Clinical Trials) to request the appropriate funding for the clinical implementation of the developed protocol.

B. Relevance

DC-based immunotherapy of cancer has demonstrated immunologic efficacy, ability to induce clinical responses in individual patients [23-44] and can result in an improved survival benefit in a subset of patients with hormone refractory prostate cancer [37].

Despite these encouraging observations, the overall clinical efficacy of this type of immunotherapy is still below expectations. Based on the high ability of DC-based vaccines to produce IL-12, the factor critically important for the induction and persistence of the tumoricidal activities of CTLs, TH1 and NK cells [45] should boost the effectiveness of DC-based vaccination, and our preliminary data showing superior activation by DC1s of CTLs,Th1 cells, and NK cells, in melanoma models and in healthy volunteers ([2,3,6,7] and Figs 1-3), and preliminary clinical results observed in CTCL patients, we expect that the introduction of the **FDA-approved αDC1s (BB-IND 11,754)** in the immunotherapy of prostate cancer will strongly increase its clinical efficacy.

We expect that the established resistance of polarized DC1 against the immunosuppressive activity of PGE_2 [2] makes these cells **particularly suitable as a therapeutic tool against prostate cancer** (PCa), associated with overproduction of PGE_2 [8-18], and the pronounced immunosuppressive impact on endogenous DC [46]. In addition, the availability of PSA as a marker of prostate cancer progression, makes prostate cancer a good system to **monitor the biologic, rather than immunological responses** to vaccination as a primary endpoint of the vaccine efficacy, facilitating the overall progress in the field of cancer immunotherapy.

C. INTERVENTIONS

The core of the proposed immunointervention (αDC1-based vaccine)[3] will be similar to the **current FDA (BB-IND 11,754)- and IRB-approved protocol UPCI 03-118** ***(see Part 12 of this application)*** recently developed for melanoma patients. The study will evaluate 2 patient treatment groups in a phase I/II evaluation of DC-based intralymphatic (a single priming cycle) and intradermal (four booster cycles) immunizations against metastatic prostate cancer. The patients will be vaccinated with tumor-loaded αDC1[3], used as a single treatment, or followed up (at 6 weeks) by **subsequent limited androgen ablation (for 3 months).** The rationale for the latter intervention lies in the ability of androgen ablation[22] to induce both apoptosis as well as a T cell infiltrate in the prostate of patients with prostate cancer. Thus we predict that the proposed sequence of therapeutic interventions will result in their **synergistic activity by concentrating the αDC1-induced CTLs and Th1 cells into the prostate cancer tissue.**

Based on our preliminary discussions and the previously-established collaboration with Cell Genesys Inc (San Francisco, CA), **w**e will prioritize the use of allogeneic prostate cancer cell lines PC-3 cells (CG1940) and LNCaP cells (CG8711), as a source of apoptotic bodies loaded on αDC1. These cellular vaccines are generated in a FDA-approved GMP facility by Cell Genesys Inc., and to date approximately

182 patients with prostate cancer have been treated under BB-IND 7285 and BB-IND 8041. This feasible source of prostate-cancer antigen showed promise both in preclinical murine studies [47,48], or in previously-performed clinical trials, either used as a self-standing vaccine, or pulsed on or fused with conventional dendritic cells [33,36,49]. Dr. Chatta is the PI on the **currently ongoing UPCI 04-149 trial, involving both cell lines** as a stand-alone vaccine (rather than as a component of the DC-based vaccine). The use of such standardized antigenic material has emerged during our discussions as not only being convenient and standardized, but also being superior to the patients autologous tumor material, HLA-A2-restricted peptide antigens, or recombinant PSA and/or PMSA proteins, that would respectively, restrict our study to the patients with available tumor tissue, patients with a defined HLA-$A2^+$ allotype, or to targeting of mostly $CD4^+$ T cell responses against PCa.

In order to maximize the efficacy of vaccine delivery, to assure the optimal timing of their interaction with lymph node-based T cells, and to allow them to act as a medium of prolonged feedback interactions between the expanding populations of $CD4^+$ and $CD8^+$ T cells, the first priming dose of the αDC1-based vaccine will be administered via the intralymphatic route, over the period of four days, similar to our melanoma protocol UPCI 03-118 (***see section E below and Part 12).*** This priming cycle of immunization will be administered in our GCRC on an inpatient basis, and will be followed by four doses of intradermal administration of the vaccine, alone, or in combination with androgen depletion (performed on outpatient basis; ***see section E below)***.

D. PATIENT POPULATION

220,000 men are newly diagnosed with prostate cancer (PCa) each year, and 30,000 men die of the disease annually[1]. The risk for PCa increases with age. A number of plausible scenarios exist that could explain this: i) hormones, growth factors, and cytokines within the prostate tissue itself may become deregulated with age; and ii) aging maybe associated with changes in immune surveillance. Standard treatment of organ-confined PCa includes surgery or radiation therapy, and is effective in the short term, but up to one third of patients relapse. Current systemic therapy (androgen ablation and chemotherapy) for PCa is limited, primarily palliative [502], and is associated with significant morbidity. Prostate-specific vaccine therapy, if effective and if initiated early, i.e., at the time of initial relapse, offers the prospect of prolonging survival with minimal morbidity. Thus, we have chosen to focus on men with PCa, with a PSA relapse after either surgery and/or radiation therapy. Men with PSA-only disease, represent the largest sub-poulation of patients with PCa, for whom there is no standard treatment. Furthermore, they also represent a heterogenous group, with widely varying PSA-doubling times. Once again the focus in this trial will be on patients with a PSA-doubling time of less than 12 months.

E. CLINICAL PROTOCOL:

The core of the proposed trial will be similar to our **currently PRC- FDA- and IRB-approved protocol UPCI 03-118 (BB-IND 11,754:** ***see part 12),*** recently developed for melanoma patients.

We propose to evaluate two patient treatment groups in a phase I/II evaluation of DC-based intralymphatic (a single priming cycle) and intradermal (four booster cycles) immunizations against metastatic prostate cancer. The patients will be vaccinated with tumor-loaded alpha-type-1-polarized DC (αDC1), used as a single treatment, or followed up (at 6 weeks) by the **subsequent androgen-depleting therapy**. The rationale for limited androgen ablation is described above.[22] We expect that the proposed sequence of therapeutic interventions will result in their **synergistic activity by concentrating the αDC1-induced CTLs and Th1 cells into the prostate cancer tissue.**

Study schema *(may be modified in the course of the protocol development):*

Group 1 (up to 14 patients): αDC1 vaccine alone:	4 day long intralymphatic priming course (week 1), and four booster intradermal single doses of αDC1: weeks 6, 10, 14, 18)

Group 2 (up to 14 patients): αDC1 vaccine (see group 1) plus limited (for 3 months) androgen ablation (initiated at week 6 of treatment)

Any patient with a demonstrable clinical response (first assessed at 8 weeks) will have an option of undergoing re-treatment with αDC1 only for up to 10 rounds of treatment. The priming cycle of immunization (four day long intralymphatic administration of αDC1-based vaccines) will be administered on an inpatient basis in our GCRC. The rationale for the prolonged administration of the first cycle of vaccine is to allow for the prolonged DC-mediated interaction between the expanding populations of the vaccine induced tumor-specific $CD4^+$ and $CD8^+$ T cells, as well as to assure the efficient and rapid delivery of αDC1s to the T cell areas of the lymph nodes, in order to assure their optimal IL-12-producing activity during the interaction with T cells (see the comprehensive justification of this approach in our approved melanoma protocol UPCI 03-118; ***see part 12***). The above vaccination strategy has been extensively discussed and eventually approved in case of our melanoma protocol UPCI 03-118. We expect that it will also be accepted by our PRC and IRB, as well as by the FDA, in which case we plan to request a pre-IND meeting, to discuss our proposal and to receive the FDA feedback, prior to the formal IND filing.

Overall Design and Endpoints: We propose to conduct a 2-arm clinical trial to evaluate the administration of tumor-loaded alpha-type-1-polarized DC (αDC1) with or without androgen ablation in men with PSA-only disease. Patients will be sequentially accrued to receive either DC alone or DC followed by aandrogen ablation, 6 weeks later. The primary endpoint will be the stablization of PSA levels, and lack of PSA progression, as defined by the PSA working group.[51] The secondary (immunologic) endpoint of efficacy will be the induction of Ag-specific IFNγ-producing $CD8^+$ T cells against the tumor cell lines used in the vaccine. IFNγ ELISPOT assays will be employed as a principal readout system (performed as immunomonitoring by the Lab of Dr T. Whiteside: IMCPL. The selection of this endpoint is based on our (and the others) observations, that they (in contrast to e.g. cytotoxic tests) can be reliably performed with frozen aliquots of the cells, allowing for the simultaneous analysis of the samples from multiple donors/time points, and eliminating the problem of inter-assay variability. As tertiary endpoints of efficacy, we will analyze (using tumor-pulsed autologous DCs) the changes in the frequencies of the IFNγ□ versus IL-5-producing (Th1/Th2 profile) tumor-specific $CD4^+$ T cells, the cytotoxicity of circulating tumor-specific CTLs, and NK cells, against the cell lines used in vaccines, and in any patients with available tumor tissue, also against autologous tumor cells.

Sample Size: To test the hypothesis of sufficient PSA response, we will apply a Simon two stage design to each cohort. A response probability of .05 was selected as too low to be of further interest (and comparable to the lack of activity). In order to declare that this vaccine strategy warrants sufficient additional study we would like to detect a PSA response rate of 40%. In the first stage we will accrue 5 patients. If none of the patients demonstrate an immunologic response accrual will cease at that dose tier. If we observe a single response among the first 5 patients we will continue to the second stage accruing 9 more patients or a total of 14. The same two stage design will be applied to both the arms. Therefore a minimum of 10 and a maximum of 28 patients are required to successfully screen this vaccination strategy.

Characteristics of the Design: Each of the sequential two stage designs have been selected to reject a low immunologic response rate of 5% at □ = .05. The d
if the underlying response rate is 40%. We note 1) that the probability of rejecting the strategy at the first stage is 77% if the response rate is no better than 5%, and 2) the probability of falsely declaring either strategy to be effective is at most 10%.

Data Analysis: We anticipate completing the clinical trial within 12 months of starting. The proportion of patients with a PSA response will be calculated with a 95% confidence interval. With regard to the secondary endpoints of the efficacy CTL responses, we will compute a two-sample Wilcoxon test using the variable net increase in IFN-γ spots to compare the 14 patients who received αDC1 administration alone and the 14 patients who also received androgen-depleting therapy. We will perform a one-sided test

at significance level 0.05 of a hypothesis of no difference vs. an alternative of an improvement for the combination therapy. The test will have power 0.80 to detect a difference in means of 1 standard deviation (if the data are normally distributed). The analysis of the tertiary endpoints will be performed in an exploratory manner. Most of these indicators are quantitative or ordered categorical data that will be analyzed for change from pre to post vaccine by either the signed rank test or McNemars' test. The step-down Bonferroni method will be used to control for overall error rate in reporting the results of several tests.

F. MANAGEMENT PLAN:

The proposed trial will be performed under the direct supervision of Dr. Chatta (the clinical PI on the developed protocol), the PI on several ongoing clinical trials in prostate cancer and other genito-urinary tumors, in a close collaboration with Dr. Kalinski (laboratory/scientific co-PI, playing the same role on the melanoma protocol UPCI 03-118), who, jointly with Dr. Storkus (co-I), will be also responsible for the evaluation of the secondary (immunologic) endpoints of efficacy. The priming cycle of immunization (four day long intralymphatic administration of αDC1-based vaccines) will be administered on an inpatient basis in our GCRC by Dr. Howard Edingon (co-I), who plays a similar role on our melanoma protocol (UPCI 03-118), while the follow-up booster vaccinations and anti-androgen therapy will be administered on an outpatient basis at our GCRC at the Hillman Cancer Center. The vaccine will be generated in the cGMP facility of the UPCI (IMCPL), directed by Dr. Whiteside, the co-investigator on the current application with extensive expertise in cell therapy, who is also a co-investigator on several or our currently-ongoing clinical trials.

The investigators and clinical research coordinator meet weekly to review and discuss study data from this site, to review subject safety issues and subject recruitment, accrual and retention. Decisions to continue or close the trial to accrual are also discussed during these meetings. Any modifications necessary to ensure patient safety are discussed and modifications will be submitted to the IRB. The IRB will be notified of any change in the risk/benefit ratio which would affect whether the study should continue. If any literature becomes available which suggests that conducting this trial is no longer ethical, the study will be terminated and the IRB will be notified of the new findings. 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. All study data reviewed and discussed during these meetings will be kept confidential. In addition, the University of Pittsburgh Cancer Institute's Data and Safety Monitoring Committee (DSMC) will provide oversight for this study. The DSMC will ensure that the investigator is assessing and reporting all serious adverse events to the FDA and IRB in accordance with their respective guidelines. The DSMC meets monthly to review all active protocols. Findings of these meetings are reported to the IRB at the time of renewal.

F. INSTITUTIONAL SUPPORT: The University of Pittsburgh Cancer Institute (UPCI) is one of the first centers in the world attempting immunotherapy of cancer with dendritic cells. For this reason, our center is both particularly committed to and is in a special position to test and implement novel strategies of DC-based vaccination against cancer. This environment is supportive both for the logistics of the proposed protocol (αDC1-based vaccines are being currently prepared within the Hillman Cancer Center in the cGMP facility (IMCPL) directed by Dr. Whiteside, the co-investigator on current application), as well as for obtaining the funding for the clinical implementation of the developed protocol (multiple PO1-type and SPORE-type programs, existing and under development, or the institutional bridge funds which were used in the past by the PI of this application to obtain the clinical grade peptides for our melanoma protocol, prior to the subsequent award of the NIH funding (1R21CA114131). Any developments in this field can be rapidly utilized in our center to also treat patients with other types of malignancies, solidifying the position of Pittsburgh and the University of Pittsburgh Cancer Institute as the leading center in cancer treatment, and can be widely disseminated by the network of our collaborations.

8. REFERENCES:

1. Kalinski, P., Hilkens, C.M., Wierenga, E.A. & Kapsenberg, M.L. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* **20**, 561-7 (1999).
2. Vieira, P.L., de Jong, E.C., Wierenga, E.A., Kapsenberg, M.L. & Kalinski, P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J Immunol* **164**, 4507-12 (2000).
3. Mailliard, R.B. et al. alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* **64**, 5934-7 (2004).
4. Kalinski, P., Schuitemaker, J.H., Hilkens, C.M., Wierenga, E.A. & Kapsenberg, M.L. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J Immunol* **162**, 3231-6 (1999).
5. Langenkamp, A., Messi, M., Lanzavecchia, A. & Sallusto, F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat Immunol* **1**, 311-6 (2000).
6. Mailliard, R.B. et al. Complementary dendritic cell-activating function of CD8+ and CD4+ T cells: helper role of CD8+ T cells in the development of T helper type 1 responses. *J Exp Med* **195**, 473-83 (2002).
7. Mailliard, R.B. et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J Immunol* **171**, 2366-73 (2003).
8. Liu, X.H., Kirschenbaum, A., Yu, K., Yao, S. & Levine, A.C. Cyclooxygenase-2 suppresses hypoxia-induced apoptosis via a combination of direct and indirect inhibition of p53 activity in a human prostate cancer cell line. *J Biol Chem* (2004).
9. Gupta, S. et al. Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. *Cancer Res* **64**, 3334-43 (2004).
10. Badawi, A.F. et al. Age-associated changes in the expression pattern of cyclooxygenase-2 and related apoptotic markers in the cancer susceptible region of rat prostate. *Carcinogenesis* **25**, 1681-8 (2004).
11. Lin, D.W. & Nelson, P.S. The role of cyclooxygenase-2 inhibition for the prevention and treatment of prostate carcinoma. *Clin Prostate Cancer* **2**, 119-26 (2003).
12. Nithipatikom, K. et al. Requirement of cyclooxygenase-2 expression and prostaglandins for human prostate cancer cell invasion. *Clin Exp Metastasis* **19**, 593-601 (2002).
13. Boutemmine, D. et al. Molecular characterization of canine prostaglandin G/H synthase-2 and regulation in prostatic adenocarcinoma cells in vitro. *Endocrinology* **143**, 1134-43 (2002).
14. Liu, X.H. et al. Prostaglandin E(2) stimulates prostatic intraepithelial neoplasia cell growth through activation of the interleukin-6/GP130/STAT-3 signaling pathway. *Biochem Biophys Res Commun* **290**, 249-55 (2002).
15. Attiga, F.A., Fernandez, P.M., Weeraratna, A.T., Manyak, M.J. & Patierno, S.R. Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. *Cancer Res* **60**, 4629-37 (2000).
16. Chen, Y. & Hughes-Fulford, M. Prostaglandin E2 and the protein kinase A pathway mediate arachidonic acid induction of c-fos in human prostate cancer cells. *Br J Cancer* **82**, 2000-6 (2000).
17. Tjandrawinata, R.R., Dahiya, R. & Hughes-Fulford, M. Induction of cyclo-oxygenase-2 mRNA by prostaglandin E2 in human prostatic carcinoma cells. *Br J Cancer* **75**, 1111-8 (1997).
18. Shaw, M.W. et al. Immunobiology of the Dunning R-3327 rat prostate adenocarcinoma sublines: plasma and tumor effusion prostaglandins. *Am J Reprod Immunol Microbiol* **8**, 77-9 (1985).
19. Kalinski, P., Vieira, P.L., Schuitemaker, J.H., de Jong, E.C. & Kapsenberg, M.L. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* **97**, 3466-9 (2001).
20. Kalinski, P., Schuitemaker, J.H., Hilkens, C.M. & Kapsenberg, M.L. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are

determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol* **161**, 2804-9 (1998).
21. Kalinski, P., Hilkens, C.M., Snijders, A., Snijdewint, F.G. & Kapsenberg, M.L. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* **159**, 28-35 (1997).
22. Mercader, M. et al. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. *Proc Natl Acad Sci U S A* **98**, 14565-70 (2001).
23. Prostate cancer vaccine--Northwest Biotherapeutics: CaPVax, DC1/HRPC, DCVax--Prostate. *BioDrugs* **16**, 226-7 (2002).
24. Barrou, B. et al. Vaccination of prostatectomized prostate cancer patients in biochemical relapse, with autologous dendritic cells pulsed with recombinant human PSA. *Cancer Immunol Immunother* **53**, 453-60 (2004).
25. Engleman, E.G. Dendritic cell-based cancer immunotherapy. *Semin Oncol* **30**, 23-9 (2003).
26. Fong, L., Brockstedt, D., Benike, C., Wu, L. & Engleman, E.G. Dendritic cells injected via different routes induce immunity in cancer patients. *J Immunol* **166**, 4254-9 (2001).
27. Fukao, T. Dendritic-cell-based anticancer vaccination: has it matured? *Trends Immunol* **23**, 231-2 (2002).
28. Gilboa, E. & Vieweg, J. Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev* **199**, 251-63 (2004).
29. Havranek, E.G., Whelan, M.A., Greenhalgh, R., Dalgleish, A.G. & Pandha, H. Advances in prostate cancer immunotherapy. *Surg Oncol* **11**, 35-45 (2002).
30. Hernando, J.J., Park, T.W. & Kuhn, W.C. Dendritic cell-based vaccines in breast and gynaecologic cancer. *Anticancer Res* **23**, 4293-303 (2003).
31. Horig, H., Lee, C.S. & Kaufman, H.L. Prostate-specific antigen vaccines for prostate cancer. *Expert Opin Biol Ther* **2**, 395-408 (2002).
32. Kuratsukuri, K., Nishisaka, N., Jones, R.F., Wang, C.Y. & Haas, G.P. Clinical trials of immunotherapy for advanced prostate cancer. *Urol Oncol* **5**, 265-273 (2000).
33. Lundqvist, A. et al. Allogeneic tumor-dendritic cell fusion vaccines for generation of broad prostate cancer T-cell responses. *Med Oncol* **21**, 155-65 (2004).
34. Murphy, G.P. et al. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. *Prostate* **38**, 73-8 (1999).
35. Murphy, G.P. et al. Phase II prostate cancer vaccine trial: report of a study involving 37 patients with disease recurrence following primary treatment. *Prostate* **39**, 54-9 (1999).
36. Pandha, H.S. et al. Dendritic cell immunotherapy for urological cancers using cryopreserved allogeneic tumour lysate-pulsed cells: a phase I/II study. *BJU Int* **94**, 412-8 (2004).
37. Price, N. Improved survival benefit of autologous dendritic cell-based vaccine for hormone refractory prostate cancer: a retrospective subset analysis of the d9901 trial. *Clin Prostate Cancer* **2**, 212 (2004).
38. Ragde, H., Cavanagh, W.A. & Tjoa, B.A. Dendritic cell based vaccines: progress in immunotherapy studies for prostate cancer. *J Urol* **172**, 2532-8 (2004).
39. Simons, J.W. et al. Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. *Cancer Res* **59**, 5160-8 (1999).
40. Small, E.J. et al. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. *J Clin Oncol* **18**, 3894-903 (2000).
41. Tjoa, B.A., Elgamal, A.A. & Murphy, G.P. Vaccine therapy for prostate cancer. *Urol Clin North Am* **26**, 365-74, ix (1999).
42. Tjoa, B.A. et al. Follow-up evaluation of a phase II prostate cancer vaccine trial. *Prostate* **40**, 125-9 (1999).
43. Valone, F.H. et al. Dendritic cell-based treatment of cancer: closing in on a cellular therapy. *Cancer J* **7 Suppl 2**, S53-61 (2001).

44. Vonderheide, R.H. et al. Vaccination of cancer patients against telomerase induces functional antitumor CD8+ T lymphocytes. *Clin Cancer Res* **10**, 828-39 (2004).
45. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46 (2003).
46. Tourkova, I.L. et al. Murine prostate cancer inhibits both in vivo and in vitro generation of dendritic cells from bone marrow precursors. *Prostate* **59**, 203-13 (2004).
47. Todryk, S.M. et al. Heated tumour cells of autologous and allogeneic origin elicit anti-tumour immunity. *Cancer Immunol Immunother* **53**, 323-30 (2004).
48. Hrouda, D. et al. Allogeneic whole-tumour cell vaccination in the rat model of prostate cancer. *BJU Int* **86**, 742-8 (2000).
49. Eaton, J.D. et al. Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. *BJU Int* **89**, 19-26 (2002).
50. Small, E.J. & Vogelzang, N.J. Second-line hormonal therapy for advanced prostate cancer: a shifting paradigm. *J Clin Oncol* **15**, 382-8 (1997).
51. Scher, H.I. et al. Eligibility and outcomes reporting guidelines for clinical trials for patients in the state of a rising prostate-specific antigen: recommendations from the Prostate-Specific Antigen Working Group. *J Clin Oncol* **22**, 537-56 (2004).

APPENDIX II: MAILLIARD ET AL, 2004

α-Type-1 Polarized Dendritic Cells: A Novel Immunization Tool with Optimized CTL-inducing Activity

[CANCER RESEARCH 64, 5934–5937, September 1, 2004]

Advances in Brief

α-Type-1 Polarized Dendritic Cells: A Novel Immunization Tool with Optimized CTL-inducing Activity

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Abstract

Using the principle of functional polarization of dendritic cells (DCs), we have developed a novel protocol to generate human DCs combining the three features critical for the induction of type-1 immunity: (*a*) fully mature status; (*b*) responsiveness to secondary lymphoid organ chemokines; and (*c*) high interleukin-12p70 (IL-12p70)-producing ability. We show that IFN-α and polyinosinic:polycytidylic acid (p-I:C) synergize with the "classical" type-1-polarizing cytokine cocktail [tumor necrosis factor α (TNFα)/IL-1β/IFNγ], allowing for serum-free generation of fully mature type-1-polarized DCs (DC1). Such "α-type-1-polarized DC(s)" (αDC1) show high migratory responses to the CCR7 ligand, 6C-kine but produce much higher levels of IL-12p70 as compared to TNFα/IL-1β/IL-6/prostaglandin E_2 (PGE_2)-matured DCs (sDC), the current "gold standard" in DC-based cancer vaccination. A single round of *in vitro* sensitization with αDC1 (*versus* sDCs) induces up to 40-fold higher numbers of long-lived CTLs against melanoma-associated antigens: MART-1, gp100, and tyrosinase. Serum-free generation of αDC1 allows, for the first time, the clinical application of DCs that combine the key three features important for their efficacy as anticancer vaccines.

Introduction

Dendritic cells (DCs) are increasingly applied as vaccines for cancer patients (1, 2). Several features of DCs, including their maturation status, migratory potential, and cytokine production, were shown important for the ability of DC-based cancer vaccines to induce high numbers of Th1-type $CD4^+$ T cells and $CD8^+$ CTLs. Effective induction of antitumor CTL responses requires fully mature DCs that express high levels of costimulatory molecules (3, 4) and that can migrate in response to lymph-node-produced CCR7 ligands (5). In addition, high interleukin-12p70 (IL-12p70) secretion dramatically enhances the ability of DCs to induce tumor-specific Th1 cells and CTLs, and promotes tumor rejection in therapeutic mouse models (6–8). Unfortunately, because the maturation stage of DCs obtained in the current protocols inversely correlates with their ability to produce IL-12p70 (9, 10), the desirable combination of all of the above three features: high immunostimulatory function, high migratory activity, and high capacity to produce IL-12p70, could not be attained by any previous DC-based vaccines. We have reported that the presence of IFN-γ during the IL-1β/tumor necrosis factor α(TNFα)–induced DC maturation overcomes such maturation-associated "exhaustion," yielding stable type-1 polarized DC(s) (DC1) that produce up to 100-fold higher levels of IL-12p70 (*versus* control DCs) on interaction with CD40L-expressing $CD4^+$ Th cells, and that exhibit a dramatically improved capacity to induce Th1-type responses (11). Here, we report that the inclusion of IFN-α and polyinosinic:polycytidylic acid (p-I:C) to our original DC1-inducing cytokine cocktail, composed of IL-1β, TNFα, and IFN-γ (11), allows for the generation of DC1 in clinically relevant serum-free AIM-V medium. For the first time, this allows for the clinical use of DCs combining a fully mature status and high migratory functions with a strongly elevated, instead of exhausted, ability to produce IL-12p70. When directly compared with the current "gold standard" DCs (sDCs matured by IL-1β/TNFα/IL-6/prostaglandin E_2 (PGE_2); ref. 12), such "α-type-1-polarized DC" (αDC1) induce up to 40-fold higher numbers of melanoma-specific CTLs in a single round of *in vitro* sensitization.

Materials and Methods

Media and Reagents. Iscove's modified Dulbecco's medium with 10% fetal calf serum (both from Life Technologies, Inc., Grand Island, NY) or 2% human serum (Atlanta Biologicals, Norcross, GA) or serum-free AIM-V medium (Life Technologies, Inc., Grand Island, NY) were used to generate DCs. In preliminary experiments (over 30 experiments with blood of different donors), we have also tested (with similar results) 1, 2, 5, and 10% concentrations of fetal calf serum and human serum from different suppliers. The following factors were used to generate mature DCs: *rhu* granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (gifts from Schering-Plough, Kenilworth, NJ); IFN-α (Intron A- IFN-α-2b, Schering-Plough); rhuTNFα, rhuIL-1β, and rhuIFN-γ (all Strathmann Biotech, Hannover, Germany); rhuIL-6 (Genzyme Cambridge, MA); lipopolysaccharide (*Escherichia coli* 011:B4, Sigma, St. Louis, MO); PGE_2 (Sigma); and p-I:C (Sigma). To assure that endotoxin contamination did not contribute to the observed activity of p-I:C, we performed control experiments with END-X B15 endotoxin removal columns (Seikagaku America, Falmouth, MA), with no differences observed. IL-2 (gift from Chiron Corp. Emeryville, CA) and rhuIL-7 (R&D Systems, Minneapolis, MN) were used to support T-cell expansion in *in vitro* sensitization cultures. Flow cytometry analyses were performed with Beckman Coulter Epics XL, after labeling with CD86, CD3, CD8, CD4, CD14, CD1a, and isotype control monoclonal antibodies (all BD-PharMingen, San Jose, CA), CD83 (Coulter, Miami, FL), and CCR7 (R&D).

Generation of Dendritic Cells. Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors or melanoma patients (all stage IV, apart from a single stage-II donor) were isolated with lymphocyte separation medium (Cellgro Mediatech, Herndon, VA). Monocytes were isolated on density gradients, with Percoll (Sigma; refs. 9, 11) or Isolate (Irving Scientific, Santa Ana, CA), followed by plastic adherence, or with $CD14^+$ magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany), in all cases with similar results. Monocytes were cultured for 6 days in 24-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at 5×10^5 cells per well in *rhu* GM-CSF and IL-4 (both 1,000 IU/mL). On the basis of preliminary experiments (data not shown), we have used the following optimal concentrations of the maturation factors: IL-1β (25 ng/mL); TNFα (50 ng/mL), IFNγ

Received 4/8/04; revised 5/25/04; accepted 6/29/04.

Grant support: Grants from the National Cancer Institute, NIH, 1RO1CA82016 and 1RO1CA 095128 (to P. Kalinski), and 1RO1CA57840 (to W. Storkus); The Pittsburgh Foundation (to P. Kalinski); and The Melanoma Foundation (to P. Kalinski).

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(1,000 units/mL); IL-6 (1,000 units/mL); PGE_2 (10^{-6} mol/L); p-I:C (20 μg/mL); IFNα (3,000 units/mL); lipopolysaccharide (250 ng/mL), all added at day 6 until day 8.

Interleukin-12p70 Production. Dendritic cells were harvested, washed, and plated in 96-well plates at 2×10^4 cells/well. To mimic the interaction with CD40L-expressing Th cells, CD40L-transfected J558 cells [a gift from Dr. P. Lane, University of Birmingham, United Kingdom, that in previous studies proved equivalent to activated $CD4^+$ T cells and soluble CD40L (9–11, 13)] were added at 5×10^4 cells/well. Twenty-four-hour supernatants were analyzed by IL-12p70 ELISA (Endogen, Woburn, MA).

Chemotaxis. Dendritic cell migration, induced by 6C-kine (Biosource, Camarillo, CA), was measured in 96-well 5 μm pore ChemoTx system (Neuro Probe, Gaithersburg, MD). DCs (25×10^3 in 25 μL of AIM-V medium) were placed on the membrane surface and incubated for 90 min at 37°C, before the enumeration of migrated DCs in bottom chambers (in four random areas). Results were expressed as mean DC numbers ± SEM in the four areas in duplicate wells. To determine the IL-12p70-producing ability of migrated DCs, CD40L-J558 cells were added directly to the bottom chambers, containing the migrated DCs, for 24 hours (see above).

CTL Induction. $CD8^+$ T cells (96–98% pure) from $HLA\text{-}A2^+$ donors were negatively isolated with the StemSep system (StemCell Technologies Inc., Vancouver, British Columbia, Canada). $CD8^+$ T cells (5×10^5 cells) were sensitized by autologous αDC1 or sDC (5×10^4 cells) pulsed with the HLA-A2-restricted peptides MART-1 (27–35), gp100 (209–217 and 154–162), and tyrosinase (368–376D). 3,000 Rad γ-irradiated CD40L-J558 cells (5×10^4) were added as surrogate of CD40L-expressing $CD4^+$ Th cells (9, 11, 13). In preliminary experiments, we have also used (with similar results; data not shown) the Staphylococcus Enterotoxin B (SEB)-driven model (9, 11) of CTL induction, with SEB-pulsed DC1 (or sDCs), $CD8^+$ T cells, and irradiated $CD4^+$ T cells as an alternative source of CD40L-mediated "helper signals." In all cases, rhuIL-2 (50 units/mL) and IL-7 (10 ng/mL) were added at day 4. $CD8^+$ T-cell cultures were expanded by an additional stimulation (day 14) with irradiated peptide-pulsed autologous PBMCs. At day 28, the differentially induced $CD8^+$ T-cell lines were stimulated with peptide-pulsed $HLA\text{-}A2^+$ T2 cells to monitor the frequency of melanoma-specific $CD8^+$ T cells with IFN-γ enzyme-linked immunospot (ELISPOT). The numbers of nonspecific spots, obtained with unpulsed T2 cells (nonspecific controls) were subtracted. CTL activity was determined by ^{51}Cr-release assays by using T2 cells, pulsed with individual peptides, with unpulsed T2 cells as nonspecific controls.

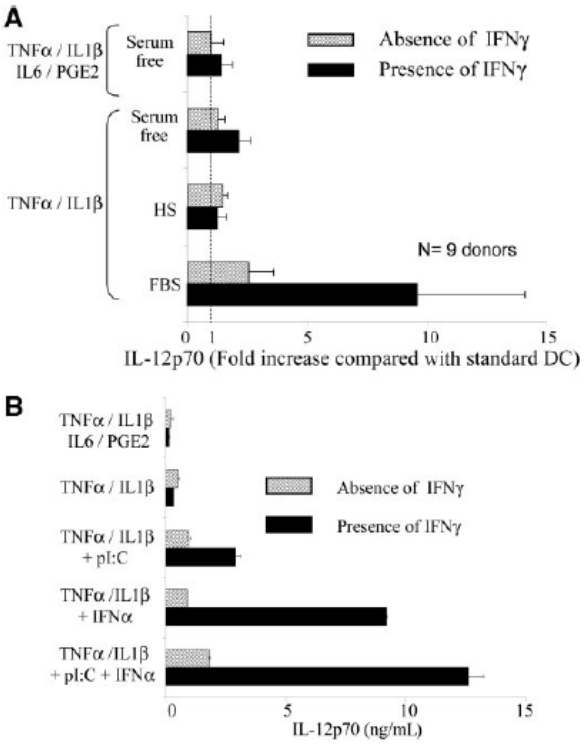

Fig. 1. IFNα and pI:C support DC1 induction in serum-free medium. In *A*, DCs generated from healthy donors in serum-free or serum-supplemented medium and matured by the combination of IL-1β and TNFα (11) or the combination of IL-1β/TNFα/IL-6/PGE_2 (sDCs; ref. 12), in the absence or presence of IFNγ, were harvested and stimulated with CD40L. The data from nine donors are expressed as a fold-increase in IL-12-producing capacity, compared with sDCs generated in parallel cultures from each individual donor (mean ± SEM). In *B*, DC1 from a healthy donor, generated in serum-free medium with the type-1 polarizing cocktail (IL-1β/TNFα/IFNγ) supplemented with IFNα, pI:C, or their combination, are superior producers of IL-12p70. Similar data were obtained when we used the blood of seven additional donors.

Results

IFNα and p-I:C Support the Generation of Fully Mature DC1 in Serum-free Conditions. As we reported previously (11), the inclusion of IFNγ in the maturation cocktail containing IL-1β and TNFα resulted in the development of stable type-1-polarized DC (DC1) in fetal calf serum-supplemented medium, characterized by high ability to produce IL-12p70 upon subsequent stimulation (Fig. 1). However, this "traditional" type-1-polarizing cocktail (IL-1β, TNFα, and IFNγ) was ineffective in serum-free AIM-V medium, or in human serum-supplemented medium (Fig. 1*A*). Similarly, the addition of IFNγ to a widely used "complete cytokine cocktail" (IL-1β/TNFα/IL-6/PGE_2; ref. 12) also proved ineffective in inducing DC1 in serum-free conditions.

Whereas neither IFNα nor P-I:C alone (nor in combination with IL-1β and TNFα) were priming DCs for subsequent production of high levels of IL-12p70 (Fig. 1*B*; refs. 11, 13), the addition of IFNα to the cocktail of IL-1β, TNFα, and IFNγ allowed for the generation of DC1 that produced very high levels of IL-12p70 from normal donors (Fig. 1*B*) and from patients with advanced melanoma (Fig. 2). Additional exposure to p-I:C further improved the IL-12p70-producing ability.

α-Type-1-polarized DCs (αDC1), generated in serum-free conditions in the presence of all five factors (IL-1β, TNFα, IFNγ, IFNα, and p-I:C), demonstrated a fully mature surface phenotype, similar to IL-1β/TNFα/IL-6/PGE_2-matured standard DC (sDCs), expressing similar levels of the maturation-associated CD83 and CD86, and of the predictive marker of lymph-node-migratory ability of DCs, (5, 14, 15), CCR7 (Fig. 2; please note that sDC show higher autofluorescence). The induction of a fully mature DC phenotype (αDC1) was consistently observed only when the complete α-type-1-polarizing cocktail, containing both IFNα and p-I:C, was used (Fig. 2*A*). Interestingly, although p-I:C was inducing only incremental enhancement of the IL-12-producing function and of the expression of CD83, and CD86, it was critical for the expression of high levels of CCR7 on maturing DCs.

α Type-1 DC (αDC1) Combine Fully Mature Status with the Ability to Produce Elevated Levels of IL-12p70 after Migrating in Response to CCR7 Ligand. Because PGE_2, the critical component of the complete cytokine cocktail (12), has been shown important not only for the expression of CCR7 but also for the responsiveness of DCs to CCR7 ligands (14, 15), we tested whether αDC1 can migrate in response to the CCR7 ligand, 6C-kine. As shown in Fig. 2*B*, αDC1 efficiently (although slightly less efficient than sDCs) migrated in response to 6C-kine, indicating that IFNα and p-I:C can provide an alternative (to PGE_2) signal instructing DC to migrate to the lymph nodes.

Importantly, subsequent CD40L stimulation of αDC1 that had migrated in response to the 6C-kine gradient, revealed the persistence of their high ability to produce IL-12p70 (Fig. 2*C*). These data demonstrate that the migratory and IL-12-producing DC functions are expressed by the same individual cells and predict high ability of αDC1 to produce IL-12p70 *in vivo*, on migration to vaccine site-draining lymph nodes.

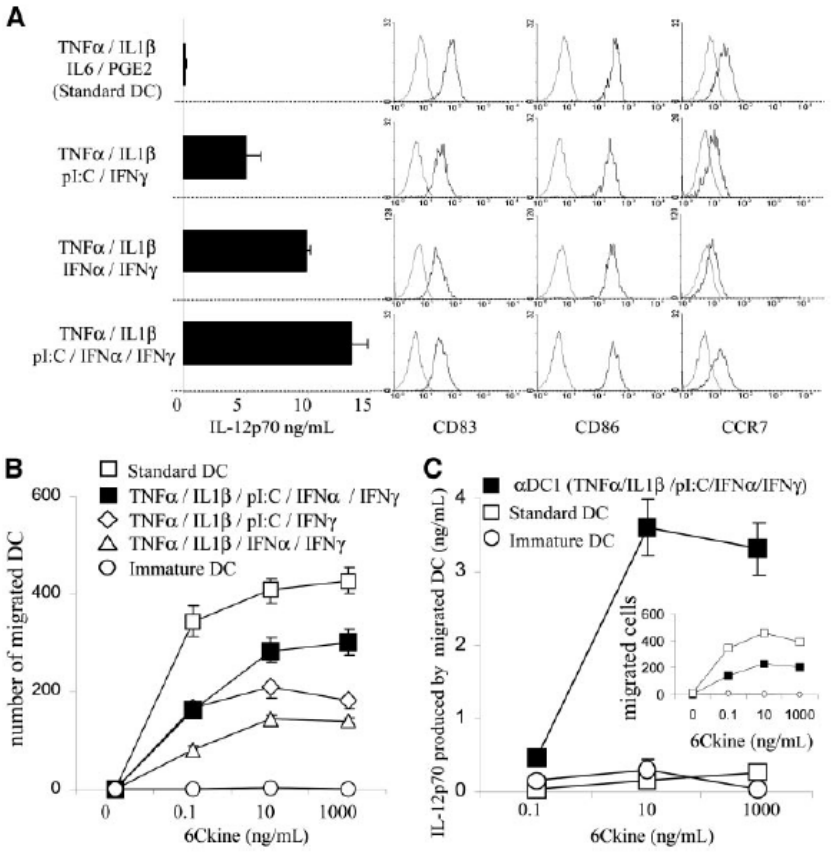

Fig. 2. αDC1 from melanoma patients are fully mature and retain high IL-12-producing capacity after the 6C-kine-induced migration. *A*, production of IL-12p70 and expression of the maturation-associated markers on sDC and DC1 induced in serum-free cultures. The data from a representative of 12 experiments, that all gave comparable results. The optimal induction of CCR7 on αDC1 required the inclusion of all five factors. *B*, 6C-kine-induced migration of differentially matured DCs. *C*, αDC1 (but not sDCs nor immature DC) retain their ability to produce high levels of IL-12p70 in response to CD40L, after migration in 6C-kine gradients. *Inset*, the corresponding relative numbers of migrated DCs.

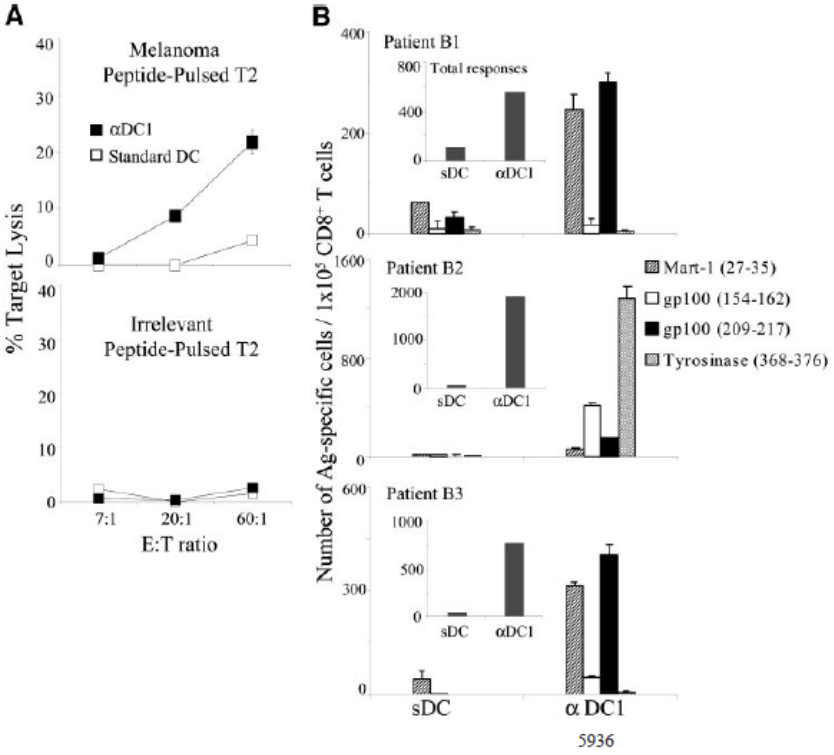

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Fig. 3. αDC1 exhibit superior capacity to induce $CD8^+$ T-cell responses against melanoma. αDC1 or sDC from $HLA\text{-}A2^+$ melanoma patients were pulsed with HLA-A2-presented melanoma-associated CTL epitopes and were used to sensitize autologous $CD8^+$ T cells. *A*, cytotoxic activity of the CTLs induced by standard DC or by αDC1 against control or peptide-pulsed T2 cells. Data from one of three experiments with the blood from melanoma donors that each gave similar results. *B*, frequencies of $CD8^+$ T cells responsive to individual peptides in three $HLA\text{-}A2^+$ melanoma patients (Patient B1, stage II; 14 months after resection. Patients B2 and B3, stage IV). The level of non-specific background (obtained with nonpulsed T2 cells) was subtracted in all cases. *Insets*, total numbers of melanoma peptide-specific $CD8^+$ T cells induced by *in vitro* vaccination with sDC or αDC1 in each of the patients; respectively, 101 *versus* 564 (Patient B1), 51 *versus* 1902 (Patient B2), and 66 *versus* 774 (Patient B3). Similar differences between αDC1- and sDC-sensitized cultures have also been observed in eight experiments with the blood of $HLA\text{-}A2^+$ healthy donors, which demonstrated an advantage of αDC1 in inducing MART-1-specific responses.

αDC1 Show Strongly Elevated Ability to Induce Melanoma-specific CTL Responses. To analyze the comparative ability of αDC1 to promote the development of tumor antigen-specific CTLs, αDC1 or sDCs were pulsed with melanoma-associated antigenic peptides and were used as an *in vitro* vaccine to stimulate autologous peripheral blood $CD8^+$ T cells from HLA-$A2^+$ melanoma patients. Long-term $CD8^+$ T cell lines obtained by further expansion with autologous PBMCs were harvested at day 24 and were used as responder cells against HLA-$A2^+$ T2 cells pulsed with individual peptides. As shown in Fig. 3*A*, when compared with sDCs, αDC1 proved superior in the induction of the melanoma-specific responses of cytotoxic T cells (CTLs). The superior activity of αDC1, was evident in case of each of the HLA-A2-restricted melanoma-associated CTL epitopes (MART-1-, gp100-, and tyrosinase-specific), and was observed in each of the three melanoma patients evaluated (Fig. 3*B*), as well as in eight healthy donors (in whom MART-1-specific responses were analyzed; data not shown). Whereas sDCs were also effective in sensitizing the melanoma-specific responses, αDC1 induced an average of 20-fold higher CTL levels (see the legend to Fig. 3 for the overall numbers of sDC-induced and αDC1-induced CTLs).

Discussion

In attempt to boost the ability of DCs to induce anticancer responses, we have developed a novel serum-free culture procedure yielding DCs that combine, within a single cell, three features important for their efficacy as carriers of anticancer vaccines: (*a*) fully mature status; (*b*) high migratory responsiveness to lymph-node-associated chemokines (CCR7 ligands); and (*c*) ability to produce high levels of IL-12p70, after the migration in response to 6C-kine. αDC1 efficiently migrate in response to 6C-kine, produce high levels of IL-12p70 after migration and subsequent CD40 ligation, and promote superior CTL induction *in vitro*. Although the sensitization with standard mature DCs (12), commonly used in current clinical trials, was also clearly effective, the data obtained with the cells from three melanoma patients (stage II-IV), indicate that one round of *in vitro* sensitization with αDC1 yields, as an average, 20-fold higher numbers of long-lived melanoma-specific CTLs.

The present data demonstrate that DC maturation does not necessarily need to be associated with the exhaustion of their ability to produce IL-12 (9, 10), and opens the possibility of clinical application of fully mature DCs with elevated IL-12-producing capacity, obviating the inclusion of either fetal calf serum (16) or PGE_2 (12), used in the currently available protocols. In contrast to previously applied DC-based vaccines, which relied on either immature DCs (with high ability to produce IL-12 but low stimulatory and migratory capacities) or on mature DCs (with high stimulatory and migratory functions, but reduced IL-12 production), the currently described αDC1 protocol allows us for the first time to combine all of these desirable features within a single DC.

The present data show that IFNα and p-I:C (an IFNα-inducing factor) synergize with the IFN-γ-based type-1-polarizing cocktails in promoting the development of fully mature DC1 under serum-free conditions. The ability of p-I:C to supplement the action of recombinant IFNα suggests that its activity is partially independent from the induction of IFNα/β, in accord with observations that, in IFNα/β receptor-deficient mice, p-I:C fails to induce several maturation-associated DC markers, but does induce CCR7 (17). The molecular mechanism(s) of the synergism of IFNα, p-I:C, and IFNγ in the induction of DC1, is a subject of our ongoing analysis. By analogy with other cell systems, it is likely to involve mitogen-activating protein kinase (MAPK)-, extracellular signal-regulated protein kinase (ERK)-, signal transducers and activators of transcription (STAT)-, and interferon regulatory factor (IRF)-signaling pathways (18–19).

Although high IL-12p70 production plays a key role in the ability of DC1 to induce Th1 responses (8, 11, 13), other factors may be also involved. DCs matured with p-I:C alone (without IFNγ) show increased ability to induce polyclonal Th1 responses, in the absence of an elevated IL-12p70 production (11, 13), suggesting that additional, as-yet-unidentified factors, may also contribute to high CTL-inducing ability of αDC1.

The *in vivo* effectiveness of αDC1, as prospective cancer vaccines, will need to be evaluated in comparative clinical trials. Superior CTL-inducing activity of αDC1 *in vitro*, combined with their high *in vitro* migratory function (60–95% of the migratory potential of sDCs in different donors) suggests their high *in vivo* efficacy. However, we need to test which $CD4^+$ T-cell antigens, including "heterologous helper antigens" such as KLH (2, 16), are best suited to provide the optimal level of $CD4^+$ T-cell help to assure the optimal performance of αDC1.

In addition to their prospective use as vaccine carriers, αDC1 may also be used to develop additional therapies for cancer and chronic infections with pathogens resistant to standard treatments, such as HIV. Their superior ability to activate Ag-specific T cells *in vitro* may also allow the use of αDC1 as *ex vivo* inducers of tumor-specific T cells for adoptive immunotherapy, or as a tool to identify new factors and mechanisms involved in the development of type-1 immunity.

References

1. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature (Lond) 1998;392(6673):245–52.
2. Engleman EG. Dendritic cell-based cancer immunotherapy. Semin Oncol 2003; 30(3Suppl 8):23–9.
3. Albert ML, Jegathesan M, Darnell RB. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. Nat Immunol 2001;2:1010–7.
4. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 2001;193:233–8.
5. Dieu MC, Vanbervliet B, Vicari A, et al. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med 1998;188:373–86.
6. Zitvogel L, Mayordomo JI, Tjandrawan T, et al. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. J Exp Med 1996;183:87–97.
7. Nishioka Y, Hirao M, Robbins PD, Lotze MT, Tahara H. Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. Cancer Res 1999;59:4035–41.
8. Xu S, Koski GK, Faries M, et al. Rapid high efficiency sensitization of $CD8^+$ t cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12-dependent mechanism. J Immunol 2003;171:2251–61.
9. Kalinski P, Schuitemaker JH, Hilkens CM, Wierenga EA, Kapsenberg ML. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-γ and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. J Immunol 1999;162:3231–6.
10. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of Th1, Th2 and nonpolarized T cells. Nat Immunol 2001;1:311–6.
11. Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kalinski P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 2000;164:4507–12.
12. Jonuleit H, Kuhn U, Muller G, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 1997;27:3135–42.
13. de Jong EC, Vieira PL, Kalinski P, et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell- promoting dendritic cells in vitro with diverse Th cell-polarizing signals. J Immunol 2002;168:1704–9.
14. Scandella E, Men Y, Gillessen S, Forster R, Groettrup M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. Blood 2002;100:1354–61.
15. Luft T, Jefford M, Luetjens P, et al. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E2 regulates the migratory capacity of specific DC subsets. Blood 2002;100:1362–72.
16. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–32.
17. Honda K, Sakaguchi S, Nakajima C, et al. Selective contribution of IFN-α/β signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. Proc Natl Acad Sci USA 2003;100:10872–7.
18. Takaoka A, Mitani Y, Suemori H. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. Science (Wash DC) 2000;288:2357–60.
19. Harcourt JL, Offermann MK. Multiple signaling cascades are differentially involved in gene induction by double stranded RNA in interferon-alpha-primed cells. Eur J Biochem 2001;268:1373–81.

APPENDIX III: CGMP-GRADE ALLOGENEIC CELL LINE LNCAP

BioReliance Test Results

SUMMARY

Cell expansion was initiated on November 12, 2001 from five (5) vials of ATCC CRL-1740, P/N: 1.40233, L/N: 1735226. The cell line was passaged five (5) additional times. On December 18, 2001, the ATCC CRL-1740 LNCaP.FGC (MCB) was cryopreserved at passage six (6) in 90% FBS + 10% DMSO. A total of 172 vials were produced, containing an average of 1.13×10^7 cell(s)/vial at a viability of 85%. Following one post-bank passage, the cell viability was 89%. Mycoplasma and Sterility samples for this bank were tested under AA53TN.102003.BSV and AA53TN.510036.BSV respectively. Included in this report are tables listing the raw materials used, the post-bank cell yields and viabilities, as well as a flow chart describing how the cells were handled during the preparation of the bank.

Materials Used for Production of ATCC CRL-1740 LNCap.FGC (MCB)

P/N: 5.40233 L/N: 3006-102836

MATERIAL	SOURCE	LOT NUMBER
RPMI-1640	Life Technologies	1104226
Glucose	Life Technologies	1114523
L-Glutamine	BioWhittaker	0M1684
Sodium Pyruvate	BioWhittaker	1M0503
Hepes Buffer	BioWhittaker	1M0856
Sodium Bicarbonate	Sigma	090K0818
PBS	BioWhittaker	0M1542
Trypsin Versene	BioWhittaker	0M1041/1M0254
WFI	Baxter	C505230
Cryovials	Nalge Nunc	053342
DMSO	Sigma	090K2340

NOTE: See Appendix A for Vendor Certificate of Analysis.

BIORELIANCE

CELL BANK CERTIFICATION

PRODUCT: ATCC CRL-1740 LNCap.FGC (MCB)

PART NUMBER: 5.40233

LOT NUMBER: 3006-102836

Test	Specification	Actual
Viability (Post Bank, Thaw) (Trypan blue dye exclusion)	≥30%	85%
Viability (Post Bank, passaged) Trypan blue dye exclusive	≥ 80%	89%
Mycoplasma Testing (P102003.BSV)	Negative	Negative
Sterility Testing (P510036.BSV)	Pass	Pass
Number of Vials Produced	150	172
Cell Suspension Volume/Vial (at fill)	1.0 mL	1.1 mL
Cell Concentration (post bank thaw)	Report Results	1.13×10^7 cells/vial
Cryopreservative	90% FBS + 10% DMSO	90% FBS + 10% DMSO

John A. Gilly, Ph.D.
Vice President, Manufacturing

February 25, 2002
Date

Dana Evans
Quality Assurance

2/25/02
Date

BIORELIANCE

Final Report

TEST FOR THE PRESENCE OF AGAR-CULTIVABLE AND NON AGAR-CULTIVABLE MYCOPLASMA

Study Number:	AA49NG.102003.BSV
Test Article ID:	LNCaP P/N: 1.40233 ATCC Lot No. 1735226
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

The test article, LNCaP P/N: 1.40233 ATCC Lot No. 1735226, was found to be negative for the presence of agar-cultivable mycoplasma when cultured on agar and broth and for non agar-cultivable mycoplasma when cultured in Vero cells and treated with the Hoechst stain.

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CELL CULTURE IDENTIFICATION AND CHARACTERIZATION

Study Number:	AA53TN.380001.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street, Suite 205 Dallas, TX 75204
Authorized Representative:	Kathy Brooks

CONCLUSION

The test article, ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836, was characterized by isoenzyme analysis. The isoenzyme migration distances observed in the case of ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 were most consistent with those obtained for cells of human origin.

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IN VITRO ASSAY FOR THE PRESENCE OF VIRAL CONTAMINANTS

Study Number:	AA53TN.003000.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204
Authorized Representative:	Kathy Brooks

CONCLUSION

Adventitious viral contaminants were not detected when the test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was inoculated onto indicator cells, and the cells were tested for hemadsorption, hemagglutination, and observed for 28 days for cytopathic effect.

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TRANSMISSION ELECTRON MICROSCOPIC EVALUATION OF CULTURED CELLS

Study Number:	AA53TN.013000.PAI
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204
Authorized Representative:	Kathy Brooks

CONCLUSION

Transmission electron microscopic examination of the test article cells, ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836, revealed no identifiable virus-like particles nor any other microbial agents in the study reported herein.

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RT-PCR ASSAY FOR THE DETECTION OF HEPATITIS C VIRUS (HCV)

Study Number:	AA53TN.105025.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5 µg) of RNA isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was analyzed for the presence of Hepatitis C virus (HCV) RNA by the reverse-transcriptase polymerase chain reaction (RT-PCR)[1] technique. The assay can detect 100 copies of HCV in the presence of 0.5 µg of genomic RNA.

The results presented herein indicate that the test article tested negative for the presence of HCV RNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF HUMAN T-CELL LYMPHOTROPIC VIRUS TYPES I AND II (HTLV-I/II) IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105013.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) µg of DNA (representing approximately 7.5 x 10^4 cells) isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was analyzed for the presence of human T-cell lymphotropic virus types I and II (HTLV-I/II) proviral DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of HTLV-I/II proviral DNA in the presence of 0.5 µg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of HTLV-I/II DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF EPSTEIN BARR VIRUS (EBV) IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105011.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) μg of DNA isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 (representing approximately 7.5 x 10^4 cells) was analyzed for the presence of Epstein Barr virus (EBV) DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 10 copies of EBV in the presence of 0.5 μg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of EBV DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF HUMAN HERPESVIRUS 7 (HHV-7) IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105029.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) µg of DNA isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 (representing approximately 7.5 x 10^4 cells) was analyzed for the presence of Human Herpesvirus 7 (HHV-7) DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of HHV-7 in the presence of 0.5 µg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of HHV-7 DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF HEPATITIS B VIRUS IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105042.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) μg of DNA isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 (representing approximately 7.5×10^4 cells) was analyzed for the presence of Hepatitis B virus (HBV) DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of HBV in the presence of 0.5 μg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of HBV DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF CYTOMEGALOVIRUS (CMV) IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105012.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) µg of DNA isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 (representing approximately 7.5 x 10^4 cells) was analyzed for the presence of human cytomegalovirus (CMV) DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of CMV in the presence of 0.5 µg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of CMV DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF HUMAN HERPESVIRUS 6 (HHV-6) IN BIOLOGICAL SAMPLES

Study Number:	AA53TN.105020.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

One-half (0.5) µg of DNA (representing approximately 7.5 x 10^4 cells) isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was analyzed for the presence of human herpesvirus 6 (HHV-6) viral DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of HHV-6 (variants A and B) viral DNA in the presence of 0.5 µg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of HHV-6 DNA.

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1 The Polymerase Chain Reaction (PCR) is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Hoffmann-LaRoche, Inc., licensed by BioReliance from Perkin-Elmer Cetus Instruments.

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POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF HUMAN IMMUNODEFICIENCY VIRUS TYPES 1 AND 2 (HIV-1/2) IN BIOLOGICAL SAMPLES

Study Number: AA53TN.105010.BSV

Test Article ID: ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836

Sponsor: Baylor Institute for Immunology Research
3434 Live Oak Street Suite 205
Dallas, TX 75204 United States

Authorized Representative: Kathy Brooks

CONCLUSION

One-half (0.5) μg of DNA (representing approximately 7.5 x 10^4 cells) isolated from test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was analyzed for the presence of human immunodeficiency virus types 1 and 2 (HIV-1/2) proviral DNA by the polymerase chain reaction (PCR)[1] technique. The assay can detect 100 copies of HIV-1/2 proviral DNA in the presence of 0.5 μg of genomic DNA.

The results presented herein indicate that the test article tested negative for the presence of HIV-1/2 DNA.

[1] The Polymerase Chain Reaction (PCR) process is covered by U.S. patents Nos. 4,683,195 and 4,683,202 owned by Roche Molecular Systems, Inc. and F. Hoffman-LaRoche Ltd.

Final Report

TEST FOR THE PRESENCE OF AGAR-CULTIVABLE AND NON AGAR-CULTIVABLE MYCOPLASMA

Study Number:	AA53TN.102003.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

The test article, ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836, was found to be negative for the presence of agar-cultivable mycoplasma when cultured on agar and broth and for non agar-cultivable mycoplasma when cultured in Vero cells and treated with the Hoechst stain.

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IN VITRO ASSAY FOR THE PRESENCE OF FIVE BOVINE VIRUSES

Study Number: AA53TN.032004.BSV

Test Article ID: ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836

Sponsor: Baylor Institute for Immunology Research
3434 Live Oak Street Suite 205
Dallas, TX 75204 United States

Authorized Representative: Kathy Brooks

CONCLUSION

Bovine viruses were not detected when the test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was examined for the presence of five specific bovine viruses in the study reported herein.

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IN VITRO ASSAY FOR THE PRESENCE OF PORCINE PARVOVIRUS IN CELL LYSATES

Study Number:	AA53TN.033004.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Street Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

Porcine parvovirus was not detected when the test article ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836 was examined in the study reported herein.

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TEST FOR THE PRESENCE OF INAPPARENT VIRUSES

Study No.: AA53TN.005002.BSV

Test Article: ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836

Final Report
For

Baylor Institute for Immunology Research
3434 Live Oak Street Suite 205
Dallas, TX 75204 United States

By
BioReliance
9900 Blackwell Road
Rockville, Maryland 20850

MAILED
4/30/02

BIORELIANCE

SUMMARY

The purpose of this assay is to detect viruses which do not cause a discernable effect in cell culture systems. The test article or the negative control article was injected into adult mice, guinea pigs and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue after 14 days into a new group of suckling mice followed by an additional 14 day observation period. All animals were observed for signs of illness and any that became sick or showed any abnormalities were examined in an attempt to establish the cause of illness or death. Embryonated hens' eggs were injected with the test or the negative control article by the allantoic route followed by a subpassage of allantoic fluid via the same route. Allantoic fluid from the original and subpassage eggs was tested for hemagglutination at 4°C and room temperature using guinea pig, human O, and chick erythrocytes. A second group of embryonated hens' eggs was injected with the test article or the negative control article into the yolk sac, followed by a subpassage of the yolk sac material into a new set of eggs, via the yolk sac route. All embryos were examined for viability.

No evidence of contamination with adventitious viral agents was observed due to the test article, ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836.

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STERILITY TEST IN ACCORDANCE WITH 21 CFR, USP 24 AND/OR EUROPEAN PHARMACOPOEIA [EP] FOR FINAL PRODUCT

Study Number:	A.A53TN.510036.BSV
Test Article ID:	ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836
Sponsor:	Baylor Institute for Immunology Research 3434 Live Oak Suite 205 Dallas, TX 75204 United States
Authorized Representative:	Kathy Brooks

CONCLUSION

The test article, ATCC CRL-1740 LNCaP.FGC (MCB) P/N: 5.40233, L/N: 3006-102836, was found to be satisfactory when tested for bacterial and fungal contamination in the study reported herein.

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