UNIVERSITY OF VIRGINIA
HUMAN IMMUNE THERAPY CENTER

UVA-MEL-63
A TRIAL TO EVALUATE THE IMMUNOGENICITY AND SAFETY OF A MELANOMA HELPER PEPTIDE VACCINE PLUS NOVEL ADJUVANT COMBINATIONS

Cyclophosphamide, Hiltonol, And Melanoma Peptides (CHAMP)

ATTACH TO THE FRONT OF EVERY COPY OF THIS PROTOCOL

Signature Page

Sponsor-Investigator

_____	_____	_____
Name	**Signature**	**Date**

SPONSOR-INVESTIGATOR'S AGREEMENT
I confirm that I have read this protocol and I agree to conduct the study as outlined herein. I agree to conduct the study in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with Good Clinical Practices, as outlined in ICH E6, and the applicable laws and regulations.

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Mel-63
A TRIAL TO EVALUATE THE IMMUNOGENICITY AND SAFETY OF A MELANOMA HELPER PEPTIDE VACCINE PLUS NOVEL ADJUVANT COMBINATIONS

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Protocol Synopsis

Title

A trial to evaluate the immunogenicity and safety of a melanoma helper peptide vaccine plus novel adjuvant combinations (MEL63)

Investigational Drugs

- 6 melanoma helper peptides (6MHP)(Table 1)
- Montanide ISA-51 (incomplete Freund's adjuvant)
- polyICLC
- cyclophosphamide

Table 1. Peptides used in the 6 Melanoma Helper Peptide (6MHP) vaccine

Amino Acid Sequence	Epitope (source protein, residues)	ref
AQNILLSNAPLGPQFP	Tyrosinase $_{56\text{-}70}$ (alanine added#)	(1)
FLLHHAFVDSIFEQWLQRHRP	Tyrosinase $_{386\text{-}406}$	(2)
RNGYRALMDKSLHVGTQCALTRR	Melan-A/MART-1$_{51\text{-}73}$	(3)
TSYVKVLHHMVKISG	MAGE-3 $_{281\text{-}295}$	(4)
LLKYRAREPVTKAE	MAGE-1,2,3,6 $_{121\text{-}134}$	(5)
WNRQLYPEWTEAQRLD	gp100 $_{44\text{-}59}$	(6,7)

FDA approved drugs for the intended indication

N/A

Indication

Part 1: High-risk resected melanoma: Stage IIB-IV, plus stage IIA with high risk features on Melanoma DecisionDx assay.

Part 2: Stage IIIB-IV melanoma with one or more tumor deposits accessible for biopsy and/or excision.

Objectives and Endpoints

Primary

Part 1:

1. Safety: to determine whether it is safe to administer a multipeptide vaccine comprised of 6 melanoma-associated class II MHC-restricted helper peptides (6MHP) plus Montanide ISA-51 (Incomplete Freund's adjuvant, IFA), or IFA + polyICLC (Hiltonol), with or without oral metronomic cyclophosphamide (mCy). This will be evaluated by adverse event assessments, including CTCAE 4.03 and subclassified by irAE categories.

2. Immunogenicity: To determine which of the following treatments is most effective for inducing $CD4^+$ T cell responses to 6MHP after 6MHP vaccination (assessed in PBMC and vaccine-draining nodes) as measured initially by ELIspot assay:
   a. Montanide ISA-51 (IFA)
   b. IFA + systemic metronomic cyclophosphamide (mCy)
   c. IFA + polyICLC
   d. IFA + polyICLC + mCy

Part 2:

3. To obtain preliminary data on whether treatment with the recommended optimal combination (determined in Part 1) in patients with one or more tumor deposits accessible for biopsy or excision modifies the tumor microenvironment (increases infiltration of $CD4^+$ and $CD8^+$ T cells into tumor metastases).

Secondary

4. To determine which of the following treatments is most effective for inducing $CD8^+$ T cell responses to melanoma antigens (assessed in PBMC and vaccine-draining nodes) to 6MHP vaccination as measured initially by ELIspot assay:
    a. Montanide ISA-51 (IFA)
    b. IFA + systemic metronomic cyclophosphamide (mCy)
    c. IFA + polyICLC
    d. IFA + polyICLC + mCy

Exploratory

5. To assess whether the vaccine adjuvant most effective for inducing CD4+ T cell responses:
    a. induces Th1-dominant cytokine responses in the VSME and SIN.
    b. induces IgG antibodies to 6MHP.

6. To obtain preliminary data about the vaccine-site microenvironment (VSME) induced by the vaccines administered in different adjuvants. This will include assessment of Th1/Th2/Th17 bias of cells in the VSME, T cell retention in the VSME, and induction of retention integrins.

7. To assess whether immune responses to melanoma antigens will be associated with survival in each study cohort and overall. These include measures of:
    a. $CD4^+$ T cell response to 6MHP.
    b. IgG antibody to 6MHP.
    c. $CD8^+$ T cell response to a panel of melanoma proteins.

8. To estimate clinical outcomes for participants in each study arm, and overall, including disease-free survival (DFS) or progression-free survival (PFS), as appropriate, and overall survival.

Design

This is an open-label, single center phase I/II study

Regimen

Part 1: All participants will receive the 6MHP vaccine. Participants will be randomized to receive the vaccine with the following:

a. Montanide ISA-51 (IFA)
b. IFA + systemic metronomic cyclophosphamide (mCy)
c. IFA + polyICLC
d. IFA + polyICLC + mCy

Part 2: Participants will receive the 6MHP vaccine with the optimal adjuvant combination identified from Part 1 of the study.

Figure 1A: Protocol Schema: Part 1

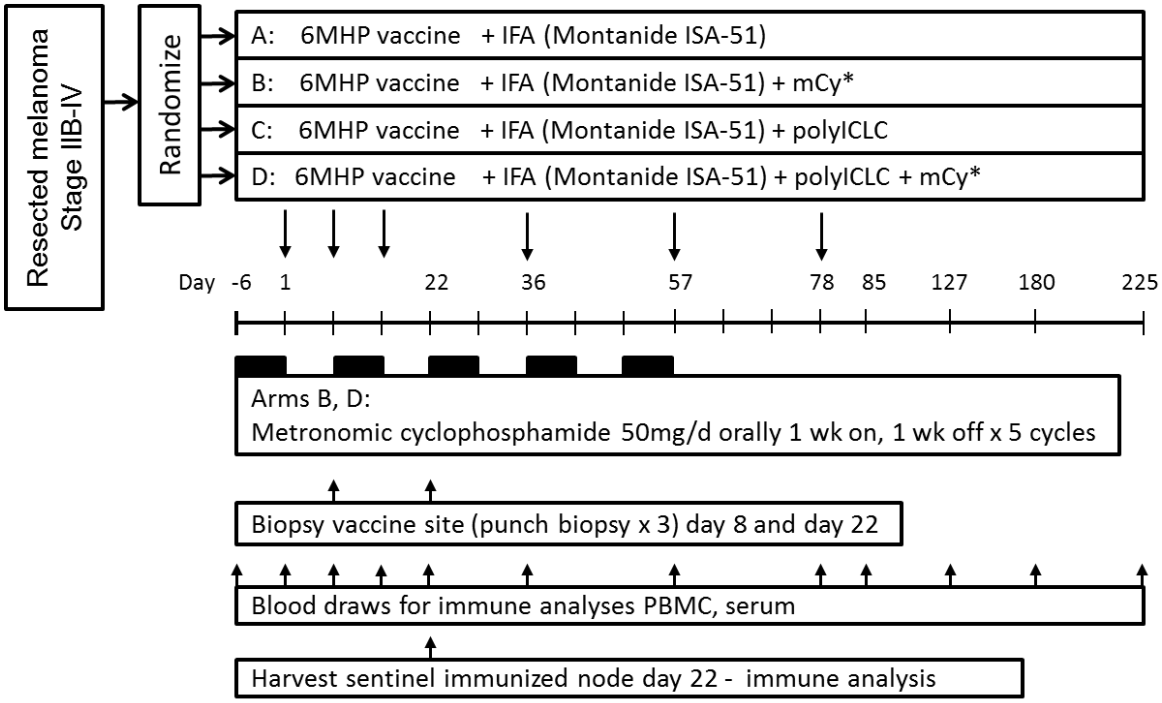
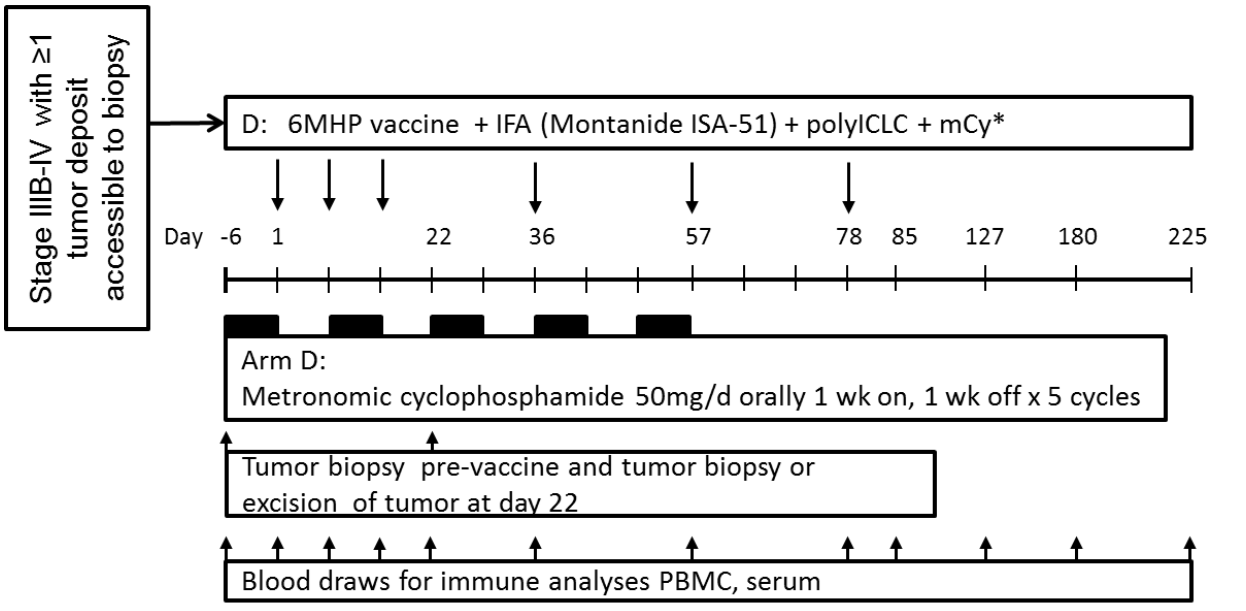


Figure 1B: Protocol Schema: Part 2
Optimal adjuvant combination identified from Part 1 of the study.



Biopsies

Part 1:

Vaccine Site Biopsies

Each participant enrolled in Part 1 will undergo a biopsy of a vaccine site at two time points (days 8 and 22; one week after vaccines 1 and 3). The biopsies will consist of three 4-mm punch biopsies of skin.

Sentinel Immunized Node

A lymph node draining the 3rd vaccine site (sentinel immunized node (SIN) will be biopsied on day 22.

Tumor Biopsy

Optional at the time of recurrence or later as clinically indicated.

Part 2:

Tumor Biopsies

Each participant enrolled in Part 2 will undergo a tumor biopsy and/or tumor excision at two time points:

- Pre-vaccine: Optimally, patients will have a tumor biopsy within 3 weeks of starting treatment. However, tumor tissue from a prior biopsy can serve as the pre-treatment sample provided: 1) there is no intervening treatment in between the pre-study biopsy and study treatment, and 2) formalin-fixed tumor tissue is available and adequate to provide at least 20 unstained slides with sufficient tumor for analysis.
- Day 22. The day 22 biopsy will typically include complete resection in accord with clinical indications for disease control.

Optional biopsies may be completed at the time of progression or later as clinically indicated.

Population

The main criteria for inclusion include:

Part 1:

- Age 18 years or older.
- A diagnosis of melanoma at high risk of recurrence/metastasis after surgical or ablative therapy (stage IIB-IV). Patients with stage IIA melanoma who are found to be high-risk based on the DecisionDx Melanoma test may also be eligible.

Part 2:

- Age 18 years or older.
- A diagnosis of stage IIIB-IV melanoma with one or more tumor deposits accessible for biopsy and/or excision.

Accrual Goal

Maximum total target accrual should not exceed 74 patients for Parts 1 and 2 but is estimated to be approximately 65 patients.

List of Abbreviations

Abbreviation	Full text
β–HCG	Beta Human chorionic gonadotropin (pregnancy test)
6MHP	6 melanoma-derived class II MHC-restricted helper peptides
12-MP	12 melanoma-derived class I MHC-restricted peptides
AE	adverse event
AGC	absolute granulocyte count
AJCC	American Joint Committee on Cancer
ALT	alanine aminotransferase
ANC	absolute neutrophil count
APC	antigen presenting cell
AST	aspartate aminotransferase
BRAFi	BRAF inhibitor
BSA	body surface area
BUN	urea nitrogen
CC	Cancer Center
cc	cubic centimeter
CFR	Code of Federal Regulations
cm	centimeter
CO_2	carbon dioxide
CRF	case report form
CT	computed tomography
CTA	cancer-testis antigens
CTCAE	Common Terminology Criteria for Adverse Events
CTL	cytotoxic T lymphocyte
CTO	Clinical Trials Office
Cy	cyclophosphamide
DC	dendritic cells
dL	deciliter
DLT	dose limiting toxicity
DSMC	Data and Safety Monitoring Committee
DTH	delayed type hypersensitivity
ECOG	Eastern Cooperative Oncology Group
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
g	gram
GCRC	General Clinical Research Center
GM-CSF	granulocyte-macrophage stimulating colony
GMP	good manufacturing practice
Hgb	hemoglobin
HGBA1C	hemoglobin a1c
HITC	Human Immune Therapy Center
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
IBW	ideal body weight
id	intradermal
IFN	interferon
IL-2	interleukin-2
IL-7	interleukin-7
IL-15	interleukin-15

IML	Immune Monitoring Laboratory
In.	inch
IND	investigational new drug
ip	intraperitoneal
IRB	Institutional Review Board
IU	international unit
IV	intravenous
kg	kilogram
KLH	keyhole limpet hemocyanin
LC	Langerhans cells
LDH	lactate dehydrogenase
Lf	flocculation value
m	meter
mcCi	microcurie
mcg	microgram
mcl	microliter
mCy	metronomic cyclophosphamide
MDP	melanocyte differentiation proteins
mg	milligram
MHC	major histocompatability complex
mIU	million international units
ml	milliliter
mm	millimeter
MRI	magnetic resonance imaging
NBT/BCIP	Nitro blue tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate
NCI	National Cancer Institute
NOS	not otherwise specified
NSAID	non-steroidal anti-inflammatory drug
OCS	Office of Collaborative Studies
PAP	pulmonary alveolar proteinosis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PD-1	Programmed-death 1
PET	positron emission tomography
PHA	phytohemagglutinin
PI	principal investigator
PMA	phorbol myristate acetate
ppm	parts per million
PRC	protocol review committee
RPMI	Roswell Park Memorial Institute
sc	subcutaneous
SD	standard deviation
SIN	sentinel immunized node
TAA	tumor associated antigens
tet	tetanus helper peptide
TFA	trifluoroacetic acid
T_h	$CD4^+$ helper T cells
TIL	tumor infiltrating lymphocytes
TNF	tumor necrosis factor
TPF	Tissue Procurement Facility
ULN	upper limits of normal
USP	United States Pharmacopeia
UVA	University of Virginia
WBC	white blood cell
w/v	weight to volume
v/v	Volume to volume

1.0 BACKGROUND AND SCIENTIFIC RATIONALE

1.1 Introduction

Cancer immunotherapy for solid tumors is coming of age, with FDA-approved immuno-therapeutics in prostate cancer, melanoma, and renal cell cancer. Interleukin-2 (IL-2) and the CTLA4 antibody ipilimumab are approved for melanoma; both induce durable clinical regressions. Recent data also show that blockade of PD-1/PD-L1 induces durable clinical regressions of melanoma, renal cell cancer and non-small cell lung cancer (NSCLC) (8,9). Furthermore, antigen-specific adoptive T cell therapy induces clinical regressions that are durable in about 20% of treated patients(10). There is excitement about this growing armamentarium of systemic immunotherapeutics, whose effects are mediated predominantly by T lymphocytes. Despite the effectiveness of those therapies, still about 70-80% of patients fail them, and go on to develop progressive disease. There is a need for new combination approaches that build on the demonstrated clinical value of immune therapy.

Cancer vaccines inducing antigen-specific T cell responses are emerging as a component of combination immunotherapy. In the past few years, a cancer vaccine has been approved for prostate cancer, based on two randomized trials showing improved survival (11), and a randomized prospective trial showed that adding a peptide vaccine to high-dose IL-2 significantly prolonged progression-free survival (PFS) when compared to IL-2 alone(12). Thus, after several decades of development and optimization, there is now evidence that cancer vaccines may improve clinical outcomes, in particular in combination with other active therapy.

We have developed a vaccine incorporating 6 intermediate-length peptides that induce $CD4^+$ helper T cell (T_H) responses (6 helper peptides, 6MHP), which has clinical activity in patients with advanced melanoma. The current proposal is to optimize the 6MHP vaccine (Aim 1), by testing two local adjuvants and one systemic adjuvant. This clinical trial also will incorporate correlative studies of immune responses in blood, skin, and lymph nodes, to obtain a more complete understanding of the host: tumor relationship in the context of these vaccination approaches. This study holds promise to optimize the immunogenicity of vaccines comprising class II MHC peptides with improved adjuvants, which may have relevance across a spectrum of cancers,

1.2 Study Rationale

1.2.1 Evidence for the Role of the Immune System in Protecting Against the Development of Solid Tumors

There has long been evidence of immune responses to cancer, but evidence of impact on tumor progression has not been well demonstrated until recently. The most convincing evidence of the importance of immune surveillance in preventing the development of solid tumors is provided by recent work in murine models, in which 50-100% of knockout mice lacking STAT1 and/or IFNγ receptor function developed spontaneous solid tumors of various histologies within 12-15 months, whereas normal mice never developed malignancies during the same time period (13). These studies strongly support the role of cellular immune function in the control of cancer progression.

1.2.2 The Role of $CD4^+$ Helper T Lymphocytes in Anti-tumor Immune Responses

Initially, the majority of cancer vaccines were designed to activate the $CD8^+$ cytotoxic T cell arm of the host immune system. However, more recent approaches target the activation of $CD4^+$ T_h cells. This is based in part on results from earlier studies which demonstrated depletion of $CD4^+$ T-cells abrogates all or part of protective immune response to vaccines (14). Furthermore, adoptive therapy with $CD4^+$ T-cells has been shown to induce tumor protection in some model systems (15) (16). Thus, the protective immunity induced by tumor cell vaccines appears to be mediated both by $CD8^+$ T-cells and by $CD4^+$ T-cells.

T_h cells can activate dendritic cells (DC) for heightened antigen presentation, causing the DC to secrete IL-2 and other cytokines that may help to direct the immune response. Furthermore, strong T_h1 help produces the proper cytokine milieu which is critical to the induction of immune-mediated tumor destruction (17,18). In addition, T_h responses are believed to be involved in the establishment of memory responses.

1.2.3 Safety of Peptide-based Vaccines

Peptide-based vaccines have been administered safely to humans in a number of clinical trials, with toxicity limited usually to local injection site reactions and transient grade I-II systemic reactions. In our experience, other systemic toxicities are attributable to cytokines added as adjuvants, but the peptides themselves appear to be very well tolerated (19). In previous studies conducted by the UVA-HITC and by the Eastern Cooperative Oncology Group (ECOG), vaccines containing 6MHP have been administered to over 200 patients and have been well-tolerated(20-22). In fact, injection site reactions have been lower with 6MHP than with Class I MHC associated peptides (23).

1.3 6MHP

1.3.1 Selection of Class II MHC-restricted Epitopes for Melanoma-reactive T_h Cells

In the current protocol we are including class II MHC-restricted peptides derived from melanoma proteins in an effort to generate melanoma-specific T_h responses. The melanoma specific class II MHC-restricted peptides (Table 1) are derived from melanocytic differentiation proteins (MDP) and cancer-testis antigens (CTA). The peptides were originally reported to bind to HLA-DR1, -DR4, -DR11, -DR13, and/or -DR15, and approximately 90% of the melanoma patient population will express at least one of those class II alleles. Our prior work has demonstrated that these peptides, like other HLA-DR restricted peptides are also presented promiscuously on many other HLA-DR molecules (24); thus, we do not restrict enrollment based on HLA expression.

The melanoma-associated class II MHC-restricted peptides in the 6MHP vaccine include 4 from MDPs (tyrosinase (2), gp100 (1), MelanA/MART-1 (1)), and 2 from CTAs (MAGE proteins). The first report of HLA-DR-restricted peptides recognized by T-cells on melanoma identified $tyrosinase_{56\text{-}70}$ and $tyrosinase_{448\text{-}462}$ (1). Both peptides require high concentrations to induce T cell responses, but the former peptide has a higher binding affinity for HLA-DR4 than the latter; therefore, $tyrosinase_{56\text{-}70}$ (QNILLSNAPLGPQFP) was chosen for use in this study. A DR15-restricted peptide, $tyrosinase_{386\text{-}406,}$ was reported also to be an antigen for T_h cells and was selected for use in this study (2). Peptides

presented by HLA-DR4 from MART-1/Melan-A have been identified and we have chosen to include Melan-A/MART-1_{51-73} in this trial. The last MDP represented in the vaccine is gp100, from which a peptide at residues 44-59 has been identified. T-cells sensitized against this peptide can recognize melanoma cells, and this epitope has been demonstrated to be naturally processed and presented in the context of HLA-DR4 (6,7,25).

The peptides from CTA to be included are from MAGE proteins. The peptide MAGE-A3 $_{281-295}$ can stimulate peptide reactive CTL in vitro and is strongly recognized by DR11-restricted MAGE-3 reactive CTL (4). Another MAGE peptide is homologous with MAGE-1, 2, 3, and 6, and is restricted by DR13. It represents MAGE $_{121-134}$ (5).

1.3.2 Immune Monitoring Assays for $CD4^+$ T-cells

An essential step in the development of effective cancer vaccines is identification of the immune response parameters that effectively measure relevant immunologic endpoints, such as immunogenicity. Ideally, these endpoints will be associated with clinical response. A number of immunologic assays have been evaluated over the years for their ability to serve as sensitive and reliable tools for immune monitoring purposes.

One method for measuring epitope-specific helper T cell responses is the ELIspot assay, which was derived to evaluate functional antigen-specific responses by permitting direct counting of T-cells reacting to antigen by production of IFNγ or other cytokines (26) (27,28). T-cells that are not anergized should secrete IFNγ after exposure to their cognate antigen, especially if they have a memory phenotype (29). ELIspot assays can reproducibly detect functional T cell responses to defined antigens at levels below 0.01% (26,30), and they do not require prolonged *in vitro* culture prior to evaluation.

We have also found measures of proliferation, by incorporation of tritiated thymidine, are effective for detection of helper responses, and that multiparameter flow cytometry enables detection of multifunctional T cells and differentiating CD4+ and CD8+ T cell responses. For the present study, we propose to use flow cytometry as the primary assay, and ELIspot assays as a secondary assay. Characterization of the helper T cell response may aid in detection of differences in the immunologic milieu when the vaccines are administered. This can be achieved by measuring cytokines secreted into the media by $CD4^+$ T cells proliferating in response to antigen, using an ELISA assay (31) or flow cytometry, with calculation of the $T_h1/T_h2/T_h17$ balance.

1.4 Montanide ISA-51

1.4.1 Montanide ISA-51 as a Vaccine Adjuvant

Montanide ISA-51 adjuvant has been effective at inducing immune responses against murine viral antigens when administered with a synthetic peptide epitope (32,33) and is widely used as a vaccine adjuvant in veterinary practices. The product consists of a mineral oil base similar to incomplete Freund's adjuvant (IFA). However, the Arlacel A emulsifying agent of incomplete Freund's, which has caused reactions in the past, has been replaced with a purified manoside monooleate called "montanide", which appears to be safer. The UVA HITC has sponsored studies where peptide-based vaccines in Montanide ISA-51 have

been safely administered to more than 600 participants. Immunological responses against the immunizing peptides have been detected in most participants (20-22,34-36).

Montanide ISA-51 may or may not be an optimal adjuvant; concerns have been raised about its usefulness as an adjuvant with a short peptide vaccine, attributed to changes from a prior formulation to the current one (34), but in our hands, we found that both formulations were very similar in their effectiveness as vaccine adjuvants with peptide vaccines (34). Another concern is that vaccination with short (9-mer) peptides in IFA induces chronic inflammation at the site of vaccination, with retention of antigen-reactive T cells at the vaccine site, both in mice and humans (37,38). However, this does not appear to be the case with longer peptides (37). We believe, thus, that these 14-23 mer peptides in 6MHP are not likely susceptible to retention at the vaccine site; so that IFA is a good adjuvant to use.

1.5 6MHP Administered in Montanide ISA-51

1.5.1 Immunogenicity and Clinical Activity

This protocol builds on 3 prior NIH-funded clinical trials performed at the University of Virginia and collaborating centers: 1) Mel41 (NCT00089219): Phase I/II first-in-humans trial of 6MHP vaccine in stage III/IV melanoma (supported by R21 CA105777)(22); 2) Mel44 (NCT00118274): Multi-peptide Vaccine Administered with Cyclophosphamide for High-Risk Melanoma (supported by R01 CA118386)(21), and 3) ECOG trial E1602 (NCT00071981) testing multi-peptide vaccines to stimulate $CD8^+$ and $CD4^+$ T cells in advanced melanoma (supported by R01 CA104362)(39). Together these trials demonstrate the safety, immunogenicity, and clinical activity of the 6MHP vaccine.

Table 2. Clinical response rates to 6MHP vaccines in patients with advanced melanoma in Mel41 and E1602 trials (RECIST)

Study	N	CR+ PR	CR+PR +SD	RR	DCR
Mel41	17	2	4	12%	24%
E1602 Arm D	42	3	15	7.1%	36%
E1602 Arm C	32	2	8	6.3%	25%
All studies	**91**	**7**	**27**	**7.7%**	**30%**
Mel41+Arm D	59	5	19	8.5%	32%

The 6MHP vaccine incorporates a set of 6 intermediate-length peptides that are promiscuous in binding to Class II MHC molecules (Table 1); thus, they are presented by a wide range of MHC molecules, and can be applied to patients without pre-testing MHC expression(22). Vaccination with 6MHP has been immunogenic in 81% of patients with stage III-IV melanoma, when administered with incomplete Freund's adjuvant (IFA) + GM-CSF as the vaccine adjuvant, and when measuring immune responses in vaccine-draining nodes (sentinel immunized nodes)(22). When immune responses are measured in peripheral blood, they are detected in 40-60% of patients (21,22,39).In patients with advanced melanoma, this vaccination strategy has been associated also with clinical regressions in 7-12% of patients and with stable disease for an additional 12-29% (mean disease control rate, DCR, 30%, Table 2. In the Mel41 trial, durations of SD and clinical responses have ranged from 1 to 7 years (22).

We have found in a separate study that GM-CSF did not increase immunogenicity compared to IFA alone. Instead, we found that IFA alone is a better adjuvant than IFA+GMCSF for induction of $CD4^+$ T cell responses to an intermediate length helper peptide (35). Though GM-CSF continues to be used as a vaccine adjuvant in other settings, studies by us and others (40) provide randomized prospective data on its use in humans in combination with other adjuvants - in both cases, the addition of GM-CSF was associated with lower immunogenicity, worse clinical outcome, or both (35,40). Thus, in another trial, we tested patients with no clinical evidence of disease (resected stage IIB-IV), with the 6MHP vaccine in Montanide ISA-51, without GM-CSF (21): immune responses to 6MHP in the blood were detected in 48% of patients.

We tested, in two trials (Mel44 and E1602), whether co-administration of the 6MHP vaccine with a peptide vaccine that stimulates $CD8^+$ T cells (12 MHC Class I restricted peptides, 12MP (36)) would induce greater $CD8^+$ T cell reactivity and increased clinical responses than vaccination with 12MP alone. In the Mel44 trial, patients were vaccinated with 12MP+6MHP (MELITAC 12.6) or 12MP+tetanus helper peptide (MELITAC 12.1), with IFA alone as the adjuvant, in patients with resected stage IIB-IV melanoma and no measurable disease. That trial was designed to enroll 160 eligible patients, plus allowances for ineligibility and over-enrollment. 170 patients were enrolled at 3 centers (3/05–1/08). Three (1.8%) were ineligible on post review. Immune responses were evaluable for 161 (96%) of 167 eligible patients. T cell responses were measured by IFNγ ELIspot assay directly ex vivo, among $CD8^+$ T cells for 12MP, and among $CD4^+$ T cells for tetanus helper peptide (arms A and B), and 6MHP (arms C and D).

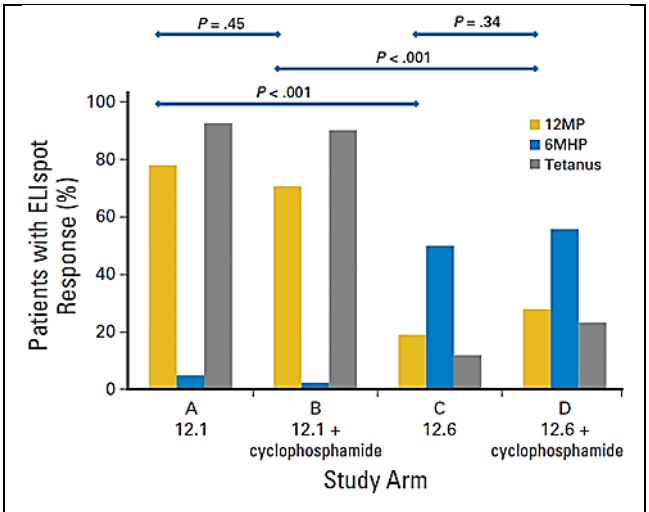


Figure 2. Mel44 trial. Immune response rates for 12MP, 6MHP, and tetanus peptide by ELISpot assay for each study arm, by evaluation of PBMC through day 50. *P* values for the response to 12MP are noted for four pairs of study arms. 12.1 [MELITAC 12.1], vaccines with 12MP plus tetanus peptide AQYIKANSKFIGITEL; 12.6 [MELITAC 12.6], vaccines with 12MP plus 6MHP.

The combination of 6MHP with 12MP paradoxically reduced the circulating $CD8^+$ T-cell response rate (Figure 2) (21). Responses to the tetanus helper peptide were detected in 91% of patients vaccinated with it, and the mixture of 6 melanoma helper peptides (6MHP) induced T_H cell responses in 52% of those vaccinated with it (21). Patients on the Mel44 trial also were randomized to pre-treatment with one dose of cyclophosphamide (CY, 300 mg/m2), which had no effect on $CD8^+$ or $CD4^+$ T cell responses (Figure 2). Clinical outcome was not altered by adding 6MHP or CY to 12MP.

In the E1602 trial, patients with advanced measurable melanoma were vaccinated in a 4-arm randomized phase II design: Patients on arms A, B, C, and D were vaccinated with 12MP, 12MP+tetanus peptide, 12MP+6MHP, or 6MHP alone, respectively, and with IFA+GMCSF as vaccine adjuvant(39). Similar to the finding in the Mel44 trial, the combination of 12MP+6MHP did not induce greater $CD8^+$ T cell responses or better clinical outcome. However, T cell responses were observed in the peripheral blood in about 40% of patients vaccinated with 6MHP (Figure 3), and objective clinical responses were observed in arms C and D: 2 partial responses (PR) in Arm C (6.3%) and 3 in Arm D (7.1%) (Table 2). There is additional evidence of clinical benefit, as the 1 year survival for Arms C and D exceed the 95% confidence interval from a meta-analysis of outcomes from cooperative group trials (Figure 4A). Importantly, there also is a very significant prolongation of survival for patients who had a helper T cell response to the 6MHP vaccines (Arms C+D, p = 0.005, Figure 4B). That difference persisted in a multivariate analysis (p = 0.038, HR 0.5; report in preparation). The association between immune response to helper peptides was specific for the 6MHP, as no difference in survival was observed with immune response to the tetanus peptide for Arm B (not shown).

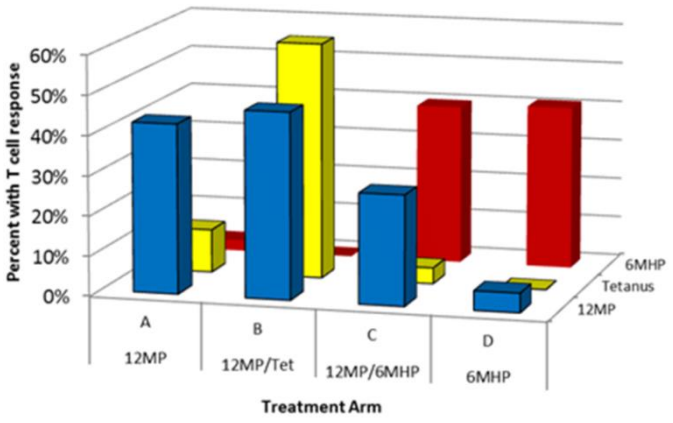


Figure 3. E1602 trial. Immune responses to 12MP, 6MHP, and tetanus peptide. **A**) The proportions of analyzable patients with CTL response to 12MP (blue bars), helper T cell response to tetanus peptide (yellow bars), and helper T cell response to 6MHP (maroon bars) are shown for each study arm.

1.5.2 Summary of Preliminary Immunogenicity and Clinical Response Data with 6MHP Vaccines

A consistent finding from these 3 trials is that 6MHP vaccines are immunogenic, with 40-50% immune response rate, when measured in blood, and 81% when measured also in vaccine-draining nodes. Also, in the 2 trials that enrolled patients with measureable disease, there were durable clinical responses(22), and there was clinical activity with an 8% RECIST-defined response rate, and 30% disease control rate (Table 2), similar to those of ipilimumab (RR 11%, DCR 29%)(41) and durable clinical responses. Importantly, in E1602, there was a strong and specific association between immune response to the 6MHP and survival, support the clinical relevance of immune responses induced to the 6MHP vaccine.

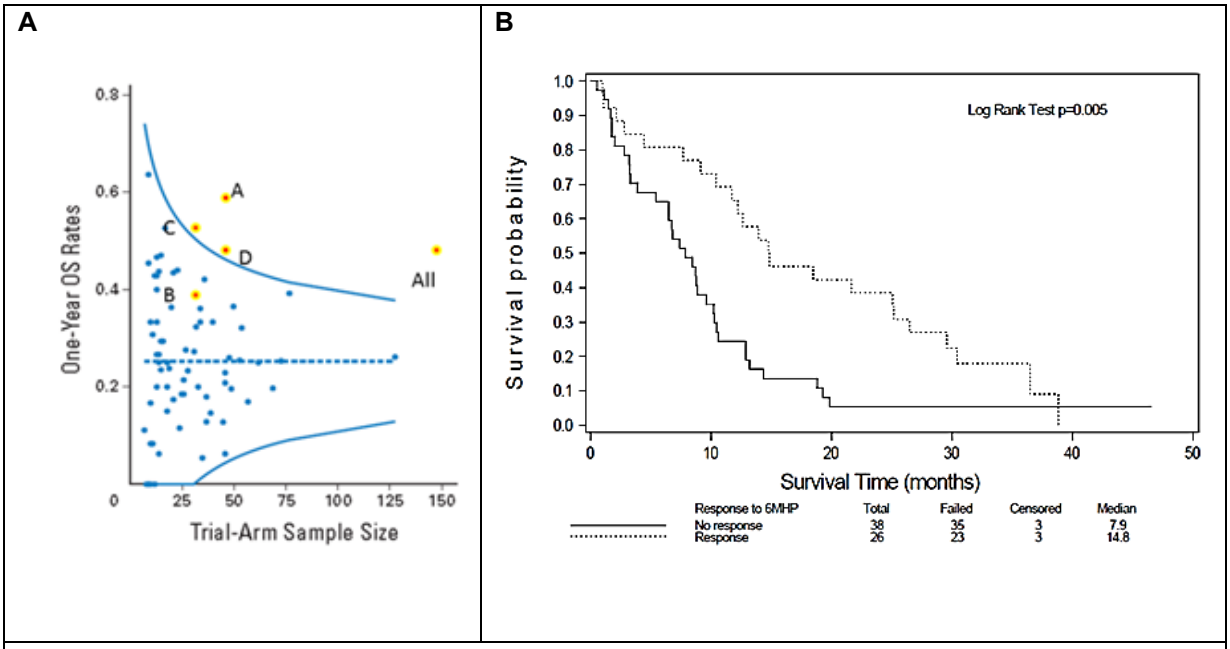


Figure 4. Overall survival on E1602. **(A)** 1 year survival for each treatment arm and for the full study population is shown on the plot of 1 year OS for all melanoma trials as previously published by Korn et al(42), and **(B)** Overall survival by helper T cell response to 6MHP in analyzable patients on arms C and D of E1602 trial (n = 64).

1.5.3 Toxicities Previously Reported for Participants Receiving the 6MHP Vaccine with in Montanide ISA-51 adjuvant, with or without GM-CSF

There is no reason to expect direct toxicity of the melanoma peptides; they are not directly cytotoxic in vitro and have been used safely in prior studies. On the other hand, because some of these peptides are identical or similar to a portion of a normal protein, risks of autoimmunity in humans are important to evaluate. There is no murine system adequately modeling the human immune response to these peptides. The most meaningful evaluation of this peptide vaccine mixture is in patients with melanoma. This trial will focus on participants with high risk resected melanoma (stage IIB/C, III, or IV melanoma without measurable disease; and stage IIA melanoma found to be high risk based on gene expression profiling). These individuals face a high risk (> 35%) of premature death, and the anticipated risk of short-term or long-term toxicity of this vaccine preparation is minimal, while the vaccine may delay or decrease the risk of morbidity and mortality due to melanoma in these patients(20,22).

Potential implications of autoimmunity against cells of melanocytic lineage are illustrated by reported cases of vitiligo occurring coincident with regressions of melanoma (43). Most of these are limited, often occurring in skin surrounding the regressing melanoma, but occasionally occurring systemically. While pathogenesis of this phenomenon can only be hypothesized, it is reasonable to consider this a worst-case scenario. The loss of skin pigment and hair pigment can be striking in these cases, but is not a cause of morbidity or mortality. Of greater potential concern is the theoretical risk of damage to the retinal pigment epithelium; however, visual loss has not been observed with these peptide vaccines, in over 200 patients treated, despite observing vitiligo in up to 10% of

patients (20-22). Depigmentation of the retinal pigment epithelium has been observed in a small number of patients vaccinated with dendritic cells pulsed with MDP-derived peptides; however, this change was asymptomatic and was not associated with loss of visual acuity (personal communication – Frank Haluska). A careful study of the retinal pigment epithelium using monobenzyl ether of hydroquinone to induce pigment cell destruction on a biochemical basis suggests the safety of pigment cell destruction and supports immunotherapy directed against MDP as a strategy for melanoma therapy (personal communication, JM Kirkwood).

Table 3. Reported toxicities for 6MHP vaccines in humans (n=207)

Toxicity (based on max grade)	Gr 1	Gr2	Gr3	Gr4
LOCAL, INJECTION SITE				
Injection site reaction	18%	56%	2.4%	
Ulceration		5%	1.4%	
CONSTITUTIONAL				
Fatigue	43%	8%	3.4%	
Headache	27%	1.4%	0.5%	
Rigors, Chills	24%	1.4%	--	
Nausea	23%	2%	0.5%	
Sweating	19%	1%		
Myalgias	18%	0.5%		
Arthralgias	17%	0.5%		
Fever	16%	2%		
Dizziness	13%			
Anorexia	13%	3%		
Diarrhea	12%	2%		
Cough	13%			
Allergic rhinitis	11%			
Nasal/paranasal reactions	11%			
Pain larynx/throat	10%			
Flushing	10%			
Pruritis	9%	0.5%		
Rash	6%	3.4%		
Dyspnea	5%	1%	0.5%	
Vomiting	5%	1%	0.5%	
Flu-like syndrome	6%			
Mucositis	6%			
Constipation	5%			
Autoimmune reaction	4%	0.5%		
Wound, non-infectious	4%			
Pain, other	2%	1%	0.5%	
Abdominal pain	1.4%		0.5%	
Tinnitus		0.5%	0.5%	
Tumor pain	0.5%		0.5%	
Hearing (without monitoring program)			0.5%	
CLINICAL LABORATORY				
Hyperglycemia (not fasting)	22%	1%		
Hemoglobin, low	17%	1%	0.5%	
Hyperkalemia	13%			
Lymphopenia	9%	2.9%	1%	
Leukocytes	8%	1%		
Hyponatremia	7%			
Increased creatinine	6%	0.5%		
Hypoglycemia	6%			
AST, SGOT	5%			0.5%

ALT, SGPT	4%		1%	
Neutrophils	3%	2%		
Metabolic, Other	3%	0.5%	0.5%	
Alk phos	4%	0.5%		
Bilirubin	3%		0.5%	

Included are events reported in 4% or more of participants or events with one or more grade 3-4 treatment-related AEs. *One patient had a grade 4 ALT elevation (<0.5%)

The 6MHP vaccines have been administered to 207 humans in 3 clinical trials (UVA trials Mel41 and Mel44, and ECOG trial E1602; NCT00089219, NCT00118274, NCT00071981). Treatment-related adverse events were graded using the NCI Common Terminology Criteria for Adverse Events v3.0. Toxicities experienced by at least 4% of participants are included in Table 3. The vast majority of these toxicities are grade 1; a subset list of expected grade 2 or 3 toxicities is in Table 4.

Table 4. Expected toxicities for 6MHP vaccines

Toxicity	Grade 2
Injection site reaction	+
Ulceration	+
Fatigue	+

1.6 polyICLC

1.6.1 Rationale for use of polyICLC as a vaccine adjuvant and with Montanide ISA-51

Polyinosinic-Polycytidylic acid (PolyIC) is a double-stranded RNA (dsRNA) that acts as a TLR3 agonist. However, its short half-life limits its usefulness. To increase half-life and its practical use in clinical settings, it has been stabilized with polylysine and carboxymethylcellulose as polyICLC. Like polyIC, polyICLC is a TLR3 agonist. TLR3 is expressed in the early endosome of myeloid DC; thus polyICLC preferentially activates myeloid dendritic cells, favoring a Th1 cytotoxic T-cell response (44) (45). PolyICLC also activates NK cells and induces cytolytic potential.(45) It has been administered in the emulsion with Montanide ISA-51 plus long peptides, with increased immunogenicity over Montanide ISA-51 plus peptides alone; the combination was safe, with some significant local reactions but no DLTs (46).

1.6.2 Toxicities of vaccination with peptides in IFA + polyICLC

We have performed a clinical trial of a peptide vaccine using 12 short melanoma peptides (12MP) with IFA plus polyICLC (Mel58, NCT01585350), and others have vaccinated with long peptides with IFA plus polyICLC (47). Data from the Mel58 trial are not yet final, but vaccination with the combination of polyICLC and IFA has been well-tolerated, though inflammation at the vaccine sites has been prominent, and - as with IFA and short peptides alone – has occasionally been associated with skin ulceration at the vaccine site. The published experience with long peptides in IFA + polyICLC is that there were injection site reactions and fatigue, and that of 11 patients, 1 developed grade 2 panniculitis at the injection site, and 3 patients developed grade 2 injection site reactions. The injection site reactions were not considered DLTs, but further vaccinations in some patients were held to prevent more severe toxicity. Our own experience with 6 vaccines using polyICLC + IFA is that it has been tolerated well in most patients, though we had one patient who came off early due to grade 3 toxicity (unpublished preliminary data, Mel58 trial). We have used

a lower dose of polyICLC (0.5 ml, 1 mg) than the initial experience with NY-ESO1 peptides (0.7ml).

1.7 Metronomic Cyclophosphamide

1.7.1 Rationale for use of metronomic cyclophosphamide

Cyclophosphamide (CY) has been studied as a systemic adjuvant for cancer vaccines. CY doses lower than those used for tumor lysis have been reported to augment immune responses in mice and humans (48-52) through several potential mechanisms (50,53-59), including decreasing regulatory T cells (60,61), and supporting dendritic cell maturation (62). In preclinical studies, immunopotentiation has been reported with CY administered 1 to 7 days prior to vaccination (63-66). Prior human trials of immunomodulatory properties of CY have tested dose from 75 to 1000 mg/m^2, with variable results (57,58,67-70). For melanoma patients, pre-treatment with 300 mg/m^2 of CY was associated with augmented delayed type hypersensitivity (DTH) responses to an autologous melanoma cell vaccine in sequential non-randomized studies (68,71). Prior human experience suggested that CY increased immunogenicity when administered 3 days prior to a cell-based vaccine, but those studies were non-randomized and were limited by semi-quantitative immunologic endpoints (68,69,71). Other human experience failed to identify changes in regulatory T cells with CY treatment, (72) and one study identified negative effects of CY (200 mg/m^2 or greater) pretreatment on cellular immune responses to a breast cancer cell vaccine, proposing that even lower doses may support immunogenicity (70). The largest experience has been with a dose of 300 mg/m^2 prior to vaccination. Thus, in a prior randomized prospective trial, we evaluated that dose, administered once, 5 days prior to the first vaccine, but found that it had no significant effect on circulating CD4$^+$ or CD8$^+$ T cell responses (21).

Other doses or timing of CY pretreatment may have different effects than those observed in that study. In fact, a very different dosing scheme for use of cyclophosphamide has shown promise, where T cell responses to peptide vaccines in patients with ovarian cancer were about 10-fold higher in patients receiving a metronomic dosing of very low dose cyclophosphamide over a 10-week period in addition to vaccine, compared to patients receiving vaccine alone (73). That trial used 50 mg per day oral dosing, for one week, followed by 1 week with no CY, and repeating that 2 week cycle for a total of 5 cycles (10 weeks) coincident with the vaccines. Metronomic scheduling of various drugs has had differential and beneficial effects in multiple settings, and has been justified in particular for cyclophosphamide (74). A goal of the present study is to evaluate whether T cell responses to the helper peptide vaccines will be increased by combination with this regimen of very low dose CY (50 mg per day) in a metronomic schedule (daily for seven days, followed by no treatment for 7 days, repeated x5, over 10 weeks).

1.7.2 Toxicities of metronomic cyclophosphamide

Cyclophosphamide administered at 52 mg orally per day induced grade 3 nausea in 2/24 patients (8%), with no grade 4 treatment related toxicity. When given at 50-100 mg 3 weeks out of 4, there was neutropenia in 2/13 patients and lymphopenia in 5/13 patients (grade 3 – 4, combined). In another study of 80 patients treated with cyclophosphamide 50 mg/m2/day, grade 3 toxicities

included lymphopenia in 19 (<25%), anemia in 1, leukopenia in 1, and no grade 4 toxicities. Thus, this low dose appears to be safe (74).

1.8 Dosing

1.8.1 6 MHP

The peptide vaccine is sequestered locally, and the immune response occurs primarily locally and in the draining lymph nodes. Thus, the dose of the vaccine does not need to be scaled up proportionately to the size (by weight or body surface area) of the recipient, as might be done for a drug whose effect is related to its distribution in body fluid. Because direct toxicity of the peptide is not expected, dose escalation is not as meaningful as it would be with a drug with a narrow therapeutic index.

1.8.2 polyICLC

PolyICLC also is administered locally with the vaccine with the intention of supporting activation of the immune response at the site of vaccine and in the draining lymph nodes. It has been used safely in cancer patients, with intravenous doses up to 300 mcg/kg.(75) We will administer 1 mg (0.5 mL) per vaccine, as used in other trials (e.g.: NCT01008527). It will be incorporated in the emulsion with peptides and Montanide ISA-51.

1.8.3 Metronomic Cyclophosphamide

Dosing will be 50 mg orally once per day of dosing. Patients will begin taking cyclophosphamide once a day (any time of day) 1 week prior to the first vaccine (day -6), then will not take it for seven days. This 14-day cycle will be repeated 5 times for a total of 35 doses of 50 mg over 10 weeks.

1.9 Summary

We have developed a vaccine incorporating 6 intermediate-length peptides that induce $CD4^+$ helper T cell (T_H) responses (6 helper peptides, 6MHP), which has clinical activity in patients with advanced melanoma. The current proposal is to optimize the 6MHP vaccine, by testing 2 local adjuvants and one systemic adjuvant. Part 1 of this clinical trial is to test whether the immune response to 6MHP can be improved by combining polyICLC with IFA, and/or by co-administration of low-dose metronomic cyclophosphamide. In Part 2 of this study, we will obtain preliminary data on whether vaccination with the optimal combination of 6MHP and adjuvants increases infiltration of $CD4^+$ and $CD8^+$ T cells into tumor.

This clinical trial also will incorporate correlative studies of immune responses in blood, skin, lymph nodes, and tumor to obtain a more complete understanding of the host: tumor relationship in the context of these vaccination approaches. This study holds promise to optimize the immunogenicity of vaccines comprising class II MHC peptides with improved adjuvants, which may have relevance across a spectrum of cancers.

2.0 OBJECTIVES

2.1 Primary Objectives and Endpoints

Part 1:
Safety: to determine whether it is safe to administer a multipeptide vaccine comprised of 6 melanoma-associated class II MHC-restricted helper peptides (6MHP) plus Montanide ISA-51 (Incomplete Freund's adjuvant, IFA), or IFA + polyICLC (Hiltonol), with or without oral metronomic cyclophosphamide (mCy). This will be evaluated by adverse event assessments, including CTCAE 4.03 and subclassified by irAE categories.

Immunogenicity: To determine which of the following treatments is most effective for inducing $CD4^+$ T cell responses to 6MHP after 6MHP vaccination (assessed in PBMC and vaccine-draining nodes) as measured initially by ELIspot assay:

a. Montanide ISA-51 (IFA)
b. IFA + systemic metronomic cyclophosphamide (mCy)
c. IFA + polyICLC
d. IFA + polyICLC + mCy

Part 2:
To obtain preliminary data on whether treatment with the recommended optimal combination (determined in Part 1) in patients with one or more tumor deposits accessible for biopsy or excision modifies the tumor microenvironment (increases infiltration of $CD4^+$ and $CD8^+$ T cells into tumor metastases).

2.2 Secondary Objectives and Endpoints

To determine which of the following treatments is most effective for inducing $CD8^+$ T cell responses to melanoma antigens (assessed in PBMC and vaccine-draining nodes) to 6MHP vaccination as measured initially by ELIspot assay:

a. Montanide ISA-51 (IFA)
b. IFA + systemic metronomic cyclophosphamide (mCy)
c. IFA + polyICLC
d. IFA + polyICLC + mCy

2.3 Exploratory Objectives and Endpoints

To assess whether the vaccine adjuvant most effective for inducing CD4+ T cell responses:

a. induces Th1-dominant cytokine responses in the VSME and SIN.
b. induces IgG antibodies to 6MHP.

To obtain preliminary data about the vaccine-site microenvironment (VSME) induced by the vaccines administered in different adjuvants. This will include assessment of Th1/Th2/Th17 bias of cells in the VSME, T cell retention in the VSME, and induction of retention integrins.

To assess whether immune responses to melanoma antigens will be associated with survival in each study cohort and overall. These include measures of:

a. $CD4^+$ T cell response to 6MHP.
b. IgG antibody to 6MHP.
c. $CD8^+$ T cell response to a panel of melanoma proteins.

To estimate clinical outcomes for participants in each study arm, and overall, including disease-free survival (DFS) or progression-free survival (PFS), as appropriate, and overall survival.

3.0 PARTICIPANT SELECTION CRITERIA

3.1 Inclusion Criteria

3.1.1 **Part 1**

Patients with stage IIB, IIC, III, or IV melanoma at original diagnosis or at restaging after recurrence, rendered clinically free of disease by surgery, other therapy, or spontaneous remission within 6 months prior to registration. Patients with small radiologic or clinical findings of an indeterminate nature may still be eligible.

Patients with high-risk stage IIA melanoma (by DecisionDx Melanoma test, Castle Biosciences, Inc,;Friendswood, TX) also may be eligible. These participants may have had cutaneous, uveal, mucosal primary melanoma, or an unknown primary melanoma. Diagnosis of melanoma must be confirmed by cytological or histological examination. Staging of cutaneous melanoma will be based on version 7 AJCC staging system (Appendix 2).

Part 2

Patients with a diagnosis of stage IIIB-IV melanoma with one or more tumor deposits accessible for biopsy and/or excision. These participants may have had cutaneous, uveal, mucosal primary melanoma, or an unknown primary melanoma. Diagnosis of melanoma must be confirmed by cytological or histological examination. Staging of cutaneous melanoma will be based on version 7 AJCC staging system (Appendix 2).

Patients must have adequate cutaneous,subcutaneous, soft tissue, or nodal metastases of melanoma readily accessible for biopsy to provide a minimum of 0.1 cm^3 of tissue per biopsy (approximately 0.58 cm by 0.58 cm x 0.58 cm or two 2mm core biopsies) and up to about 0.3 cm^3 of tissue per biopsy at each time point, depending on the individual's tumor availability and specifics. We will try to maximize the amount of tissue collected at each time-point, trying to collect up to about 0.3 cm^3 of tissue per biopsy when it is available.

Several scenarios may fulfill the tumor burden requirement. For example, a patient may have one large lesion from which core biopsies can be taken for the first biopsy time point and then the entire lesion excised for the final tissue sample. Alternatively, a patient may have two lesions, each ≥0.1 cm^3, and these lesions would be excised sequentially as biopsies 1 and 2. Other combinations are acceptable.

Optimally, patients will have a tumor biopsy within 3 weeks of starting treatment. However, tumor tissue from a prior biopsy can serve as the pre-treatment sample provided: 1) there is no intervening treatment in between the pre-study biopsy and study treatment and 2) formalin-fixed tumor tissue is available and adequate to provide at least 20 unstained slides with sufficient tumor for analysis.

3.1.2 Participants will be required to have radiological studies to rule out radiologically evident disease. Required studies include:

- Chest CT scan,
- Abdominal and pelvic CT scan, and
- Head CT scan or MRI
- PET/CT fusion scan may replace scans of the chest, abdomen, and pelvis.

3.1.3 Participants who have had brain metastases will be eligible if all of the following are true:

- Each brain metastasis must have been completely removed by surgery or each unresected brain metastasis must have been treated with stereotactic radiosurgery.
- There has been no evident growth of any brain metastasis since the most recent treatment.
- No brain metastasis is > 2 cm in diameter at the time of registration.

3.1.4 The most recent surgical resections or gamma-knife therapy for malignant melanoma must have been completed ≥ 1 week and for Part 1 ≤ 6 months prior to registration.

3.1.5 ECOG performance status of 0 or 1 (Appendix 3)

3.1.6 Ability and willingness to give informed consent

3.1.7 Laboratory parameters as follows:

- ANC > 1000/mm3
- Platelets > 100,000/mm3
- Hgb > 9 g/dL
- HgbA1c ≤ 7.5%
- Hepatic:
- AST and ALT ≤ 2.5 x upper limits of normal (ULN)
- Bilirubin ≤ 2.5 x ULN (except in patients with Gilbert's disease, where bilirubin to 4x ULN is allowed)
- Alkaline phosphatase ≤ 2.5 x ULN
- Renal
- Creatinine ≤ 1.5 x ULN
- Serology (within 6 months of study entry)
- HIV negative
- Hepatitis C negative (no evidence of active virus)

3.1.8 Blood is to be collected for HLA typing (Class I and Class II), which will be analyzed as part of the immunologic endpoints, but HLA type will not be an inclusion/exclusion criterion.

3.1.9 Age 18 years or older at registration.

3.1.10 **Part 1:** Participants must have at least two intact (undissected) axillary and/or inguinal lymph node basins.

Part 2: Participants must have at least one intact (undissected) axillary and/or inguinal lymph node basin.

3.2 Exclusion Criteria

3.2.1 Participants who have received the following medications or treatments at any time within 4 weeks of registration:

- Chemotherapy
- Interferon (e.g. Intron-A®)
- Radiation therapy (Stereotactic radiotherapy, such as gamma knife, can be used ≥ 1 week and ≤ 6 months prior to registration)
- Allergy desensitization injections
- High doses of systemic corticosteroids, with the following qualifications and exceptions:
    - In patients with adrenal or pituitary insufficiency replacement steroid doses are allowed; however, daily doses of 10 mg or more of prednisone (or equivalent) per day administered parenterally or orally are not allowed in patients with normal adrenal and pituitary function.
    - Inhaled steroids (e.g.: Advair®, Flovent®, Azmacort®) are permitted at low doses (less than 500 mcg fluticasone per day, or equivalent) (76,77).
    - Topical, nasal, and intra-articular corticosteroids are acceptable.
- Growth factors (e.g. Procrit®, Aranesp®, Neulasta®)
- Interleukins (e.g. Proleukin®)
- Any investigational medication
- Targeted therapies specific for mutated BRAF or for MEK

3.2.2 Participants who are currently receiving nitrosoureas or who have received this therapy within the preceding 6 weeks

3.2.3 Participants who are currently receiving a checkpoint molecule blockade therapy, or who have received this therapy within the preceding 12 weeks.

3.2.4 Participants with known or suspected allergies to any component of the vaccine.

3.2.5 Participants may not have been vaccinated previously with any of the synthetic peptides included in this protocol. Participants who have received vaccinations containing agents other than the synthetic peptides included in this protocol and have recurred during or after administration of the vaccine will be eligible to enroll 12 weeks following their last vaccination.

3.2.6 Pregnancy. Female participants of childbearing potential must have a negative pregnancy test (urinary or serum beta-HCG) obtained within 2 weeks prior to registration. Males and females must agree, in the consent form, to use effective birth control methods during the course of vaccination.

3.2.7 Female participants must not be breastfeeding

3.2.8 Participants in whom there is a medical contraindication or potential problem in complying with the requirements of the protocol in the opinion of the investigator.

3.2.9 Participants classified according to the New York Heart Association classification as having Class III or IV heart disease (Appendix 4).

3.2.10 Participants with uncontrolled diabetes, defined as having a HgbA1c > 7.5%.

3.2.11 Participants must not have had prior autoimmune disorders requiring cytotoxic or immunosuppressive therapy, or autoimmune disorders with visceral involvement. Participants with an active autoimmune disorder requiring these therapies are also excluded. The following will not be exclusionary:

- The presence of laboratory evidence of autoimmune disease (e.g. positive ANA titer) without symptoms
- Clinical evidence of vitiligo
- Other forms of depigmenting illness
- Mild arthritis requiring NSAID medications

3.2.12 Participants with known addiction to alcohol or drugs who are actively taking those agents, or participants with recent (within 1 year) or ongoing illicit IV drug use.

3.2.13 Body weight < 110 pounds (without clothes) at registration, due to the amount and frequency with which blood will be drawn.

3.3 Registration and Randomization

All participants must sign the consent form prior to determination of eligibility for this study. All participants who meet the inclusion/exclusion criteria may be registered. Registration will occur following verification of eligibility by the treating physician. Participants should receive their first study treatment within 3 weeks of registration.

Part 1:
Treatment allocation will be discussed with participants during the process of informed consent and will occur after registration. Treatment allocation will be based upon an adaptive design until a safety bound has been triggered or target accrual has been met. Arm allocation slots are generated by the study statisticians.

Part 2:
All participants will be assigned to the optimal treatment determined in Part 1.

4.0 STUDY DRUG

4.1 Peptide Synthesis

The vaccine drug product 6MHP to be administered consists of 6 peptides. All peptides were synthesized directly from amino acids by Multiple Peptide Systems (now Polypeptide Group, San Diego, CA) under GMP conditions. Recombinant vectors in bacteria or viruses were not used. The synthetic peptides were purified by HPLC. The identity of the synthetic peptides has been confirmed by verifying their mass and amino acid sequences by mass spectrometry. Details of the synthesis, certificates of analysis, and technical summaries are included in the IND application.

4.2 Storage of Individual Peptides

Each bulk peptide was supplied to the HITC as lyophilized powder without excipients and stored at a temperature ≤ -70°C and protected from light.

4.3 Reconstitution and Vialing of the Vaccine

Lyophilized peptides were reconstituted, mixed and vialed under GMP conditions by Clinalfa (Merck Biosciences AG, Laufelfingen, Switzerland). Lyophilized peptides were supplied to the HITC as individual use vials. Lot release testing of the final vialed peptide has also been completed by Clinalfa in accord with FDA guidelines. Details of the vialing are included in the IND application.

4.4 Vaccine Storage

The vials of lyophilized peptide are stored by the HITC at a temperature ≤ -70°C and protected from light. Once thawed, the vial(s) must be used for preparation of the vaccine within 24 hours.

4.5 Lot Testing

Each lot of peptide vaccine is evaluated as required by the FDA for identity, sterility, general safety, purity, and pyrogenicity. The details of these tests are outlined in the IND application.

4.6 Stability testing

The peptide vaccine will undergo stability testing as described in Appendix 6.

4.7 Labeling

Each vial of lyophilized peptide is labeled with the following information:

- Short name of the product
- Product number
- Proper name of the product
- Name and address of the vialing facility
- Lot number
- Date of manufacture (the date of vialing the reconstituted peptides)
- Serial number
- Quantity of each peptide per vial
- Vial contains no preservative, store at ≤ -70°C
- “Caution: New Drug – Limited by US Federal law to investigational use”

4.8 Montanide ISA-51

Montanide ISA-51 is available from Seppic, Inc. (Fairfield, NJ). A drug master file for Montanide ISA-51 is filed with the FDA and is cross-referenced in the IND application.

Class II MHC-restricted melanoma peptides (6-MHP; 200 mcg) in aqueous solution are mixed 1/1 with Montanide ISA-51 to form water-in-oil emulsions (see vaccine mixing sheets for complete mixing instructions).

4.9 Study Drug Accountability

The investigational drug will be stored in accord with directions specified in Section 4 of the protocol in a secure area with the UVA-HITC laboratories. Study drug accountability is maintained using the InvestMed database.

5.0 polyICLC (Hiltonol)

5.1 Packaging and Labeling

This is provided for used as an adjuvant as a clinical grade reagent in single-use vials containing 1 mL of a 2 mg/mL solution. We will administer 1 mg (0.5 mL) per vaccine, as used in other trials (e.g.: NCT01008527).

5.2 Storage

The polyICLC is stored at 2-8^{o}C.

5.3 Supply

Hiltonol is provided by the Ludwig Institute for Cancer Research and its Cancer Vaccine Consortium at no charge; as they have purchased a lot of it from Dr. Andres Salazar from Oncovir, Inc. (Washington, D.C).

5.4 Study Drug Accountability

Study drug will be kept in the University of Virginia Investigational Pharmacy or Human Immune Therapy Center.

Study drug accountability will be maintained by the University of Virginia Investigational Pharmacy or Human Immune Therapy Center.

6.0 CYCLOPHOSPHAMIDE

6.1 Storage

Cyclophosphamide can be stored at room temperature.

6.2 Supply

Cyclophosphamide is available commercially and is an FDA approved chemotherapy agent for other indications. Generic formulations are available and are acceptable. For this study, 35 doses of 50 mg tablets or capsules of cyclophosphamide (or combination of tablets or capsules adding up to 50 mg) will be administered.

7.0 TREATMENT PLAN

7.1 Management of Participants

This study will be conducted on an outpatient basis, with participants scheduled to be evaluated as needed for clinical care, and as specified in the study calendar (Appendix 1) through 8 months (or more often if needed for testing or medical reasons). Participants will be off treatment follow-up at the end of 8 months, or when another therapy is initiated, whichever occurs first. Once off treatment follow-up, participants will be followed yearly for progression-free survival and overall survival.

7.2 Administration of 6MHP, Montanide ISA-51, and polyICLC

7.2.1 Overview

The peptides will be prepared by combining the 6MHP mixture of 6 peptides, which are provided as 300 mcg (0.3mg) of each peptide per vial with Montanide ISA-51 or polyICLC and Montanide ISA-51. More information is provided below, for each study arm. Details of making emulsions with IFA (Montanide ISA-51) are provided in the vaccine mixing sheets.

All subjects will receive vaccines on days 1, 8, 15, 36, 57 and 78. Each vaccine will be administered subcutaneously (50%) and intradermally (50%) at one skin location. The same skin location will be used for vaccines 1-3. If the vaccine site has severe inflammation or ulceration after 1-2 vaccines, the next vaccine may be placed near the original site. For vaccines 4-6 (after excision of the sentinel immunized node), a different site will be used and may be rotated among extremities or administered in adjacent skin areas on the same extremity.

7.2.2 Dose Calculations

Study Arms A, B (IFA): At each designated time-point, 200 mcg each of the 6 peptides (Table 1) will be emulsified in Montanide ISA-51 adjuvant (2 ml total) and administered.

Study Arms C, D (IFA + polyICLC): At each designated time-point, 200 mcg each of the 6 peptides (Table 1) plus 1 mg of polyICLC will be emulsified in Montanide ISA-51 adjuvant (2 ml total) and administered.

7.2.3 Pre-medications

None required.

7.2.4 Preparation of Study Drug

Directions on how to prepare the investigational drug will be provided in vaccine mixing sheets. Prepared vaccines will be stored in a plastic syringe and delivered to the clinicians in a plastic bag. This bag with the syringe will be stored at room temperature until the vaccine is administered. Ideally, the vaccine should be administered 1-2 hours after mixing. If the vaccine is not administered within 4 hours after mixing, it should be discarded.

7.2.5 Post-Vaccine Observation

All participants will be closely observed for adverse events for at least 20 minutes following each vaccination. Any time thereafter, participants should report any adverse events to the research coordinator or research clinician.

7.3 Administration of Cyclophosphamide

7.3.1 Overview

Study Arms B and D: Cyclophosphamide will be provided in 50 mg tablets or capsules (or combination of tablets or capsules adding up to 50 mg), which will be taken orally once a day for 7 days followed by a 7 day rest period. This will be repeated for 5 cycles. Cyclophosphamide may be taken before or after a vaccine, on days when both vaccine and cyclophosphamide are administered.

Patients will be provided with a daily patient diary on which to document the days on which they took a dose of cyclophosphamide. Cycles will begin on the following days:

- Day -6 (Cycle 1)
- Day 8 (Cycle 2)
- Day 22 (Cycle 3)
- Day 36 (Cycle 4)
- Day 50 (Cycle 5)

7.3.2 Dose Calculations

At each designated time-point, 50 mg of cyclophosphamide will be taken orally.

7.3.3 Pre-medications

None required.

7.4 Dose Modifications

7.4.1 6MHP, Montanide ISA-51, and polyICLC

There will be no dose modifications of vaccine components.

7.4.2 Cyclophosphamide

There will be no dose modifications of cyclophosphamide.

7.5 Dose Delays

7.5.1 Dose Delays Due to Toxicity

- In circumstances where assessment of an AE is limited, such as by intercurrent illness, or when laboratory studies are required to assess for other causes of toxicity, the vaccine schedule may be interrupted for up to 21 days. Delay of one vaccine administration by up to 21 days will not be considered a protocol violation if due to an AE, regardless of attribution. If more than one vaccine is delayed by 21 days due to an AE, regardless of attribution, vaccine treatment should be discontinued.

- Dose delays for cyclophosphamide

  Toxicity of cyclophosphamide is expected to be very mild. However, if a patient does not tolerate cyclophosphamide or has an expected grade 2- 3 toxicity attributable to this drug, the mCy may be held until toxicities resolve to grade 1 or lower. If a grade 3 toxicity that is not expected occurs, the mCy should be discontinued. Reasons for discontinuation should be noted. If mCy is resumed, it should resume as close to the original schedule as possible and not be extended beyond week 10.

  If there is a change in the schedule of other protocol interventions for toxicity or reasons other than toxicity (Table 5, section 7.5), the mCy should either:

  - begin at the time of the other interventions of the protocol, and continue for 7 sequential calendar days, or

- may continue as originally scheduled. This latter choice is preferable if the shift in schedule of other interventions is temporary.

For example, a temporary shift may occur when visits through day 15 are on a Wednesday, but the visit scheduled day 22 is moved to a Monday or Tuesday (1-2 days earlier) to perform the SIN biopsy or to avoid a holiday, followed by resuming with subsequent treatment visits on Wednesdays. In that case, starting the mCy on a Wednesday each week is preferred.

Missed doses of cyclophosphamide should not be repeated, but will be recorded.

7.5.2 Delayed Visits for Reasons Other Than Toxicity

A schedule for return visits should be established at the first visit. If a participant misses a treatment, the missed treatment will be administered as soon as possible, so that subsequent treatments will be given in the appropriate intervals. Treatment may be continued for an additional time period, if needed. Participants who are treated outside of the established schedule should return to the original schedule as soon as possible

Table 5 defines what constitutes a delayed visit, whether the participant should continue to be treated, and whether a protocol violation should be reported and recorded. The range of days is counted from the original scheduled date.

Table 5. Delayed Visit for Reasons other than Toxicity

Treatment Period	Range of Days	Participant Treatment	Protocol Deviation
Pre-study Biopsy(Not applicable to those patients who had a prior biopsy and have left-over tissue available			
Day -6	Day -27 through Day -6	Pre-study biopsy	No
	-28 or less	Pre-study biopsy	Yes
	-5 or greater	Pre-study biopsy	Yes
*Initiate mCy**			
Day -6	± 2 days	Treatment/Labs/mCy	No
	± 3 to 7 days	Treatment/Labs/mCy	Yes
	± 8 or more days	Labs	Yes
Vaccine 1			
Day 1	± 2 days	Vaccine/Labs	No
	± 3 to 7 days	Vaccine/Labs	Yes
	± 8 or more days	Labs	Yes
*Vaccine 2/Biopsy**/start 1 week mCy*			
Day 8	± 2 days	Vaccine/Labs/Biopsy/mCy	No
	± 3 to 7 days	Vaccine/Labs/Biopsy/mCy	Yes
	± 8 or more days	Labs	Yes
*Vaccine 3**			
Day 15	± 2 days	Vaccine/Labs	No

Table 5. Delayed Visit for Reasons other than Toxicity

Treatment Period	Range of Days	Participant Treatment	Protocol Deviation
	± 3 to 7 days	Vaccine/Labs	Yes
	± 8 or more days	Labs	Yes
*Assessment/Biopsy/start 1 wk mCy**			
Day 22	± 2 days	Biopsy/Labs/mCy	No
	± 3 to 7 days	Biopsy/Labs/mCy	Yes
	± 8 or more days	Labs	Yes
*Vaccine 4**/start 1 wk mCy*			
Day 36	± 7 days	Vaccine/Labs/mCy	No
	± 8 to 14 days	Vaccine/Labs/mCy	Yes
	± 15 or more days	Labs	Yes
Day 50*/start 1 wk mCy**	± 2 days	Vaccine/Labs/Biopsy/mCy	No
	± 3 to 7 days	Vaccine/Labs/Biopsy/mCy	Yes
	± 8 or more days	Labs	Yes
Vaccines 5-6			
Days 57, 78	± 7 days	Vaccine/Labs	No
	± 8 to 14 days	Vaccine/Labs	Yes
	± 15 or more days	Labs	Yes
Assessment			
Week 12 (day 85)	± 7 days	Labs/Scans	No
	± 8 to 14 days	Labs/Scans	Yes
	± 15 or more days	Labs	Yes
Follow-up			
Week 18 (day 127)	± 7 days	Labs	No
	± 8 to 30 days	Labs	Yes
Week 26 (day 180)	± 7 days	Scans/Labs	No
	± 8 to 30 days	Scans/Labs	Yes
Week 32 (day 225)	± 7 days	Labs	No
	± 8 to 30 days	Labs	Yes

* mCy = metronomic cyclophosphamide. This will be administered orally for seven sequential calendar days (eg days -6 through day 0, then days 8-14, 22-28, 36-44, 50-56.

** A participant will be taken off protocol treatment if more than one vaccination is delayed [± 3 to 7 days] during the treatment period.

7.6 Discontinuation of Therapy

Protocol treatment will be discontinued for any of the following reasons:

- Any dose-limiting toxicity as defined in section 10.9.
- Disease progression requiring other therapy (e.g. surgery under general anesthesia, radiation, chemotherapy, or steroid therapy). The appearance of small metastases or recurrent tumor deposits will not be a basis for discontinuing the vaccinations. Biopsy to determine the nature of new lesions, biopsies completed as part of this study, or minor surgical procedures to excise a new lesion, will not be a basis for discontinuing vaccinations. Even surgery under general anesthesia will be acceptable for biopsies done pre-vaccine or day 22, and will not be a basis for discontinuing protocol treatment.
- Initiation of cytotoxic chemotherapy (other than metronomic cyclophosphamide), radiation therapy, surgery for resection of disease, steroid therapy, or other immunosuppressive therapy.
- Any other potential adverse reaction deemed sufficiently serious to warrant discontinuation of therapy by the Principal Investigator or one of the Associate Investigators.
- Noncompliance with the requirements of the study.
- Therapy may be discontinued at the participant's request.
- Therapy may be discontinued at the discretion of an Investigator.
- Pregnancy. Pregnant participants will continue to be followed for the duration of the pregnancy.

  Participants who discontinue treatment will be followed according to the follow-up schedule, unless a participant has withdrawn consent.

7.7 Replacement of Study Participants

A participant who is enrolled but who does not receive study drug or any of the study related procedures may be replaced. Every attempt will be made to evaluate any data from these participants for endpoint assessment.

7.8 Concomitant Medications

Medications taken in the month prior to registration will be recorded on the baseline case report form. This includes prescription medications, over-the-counter medications, injected medications, biological products, blood products, imported drugs, or street drugs. Participants should be maintained on drugs that they were taking prior to entry unless a change in regimen is medically indicated.

<u>The following are non-permitted medications or treatments</u>

- Cytotoxic chemotherapy
- Interferon therapy (e.g. Intron-A®)
- Radiation therapy
- Nitrosoureas
- Allergy desensitization injections
- Corticosteroids, as detailed in section 3.2.1.
- Growth factors (e.g. Procrit®, Aranesp®, Neulasta®)
- Interleukins (e.g. Proleukin®)
- Antibodies to PD-1 or other immune checkpoint blockade therapies (e.g. Keytruda®)

- Other investigational medications
- Street drugs

7.9 Permitted Medications or Treatments

- Nonsteroidal anti-inflammatory agents
- Anti-histamines (e.g. Claritin®, Allegra®)
- Topical corticosteroids or steroids
- Short-term therapy for acute conditions not specifically related to melanoma
- Chronic medications except those listed in section 7.8
- Influenza vaccines are permitted, but should be administered at least 2 weeks prior to or at least 2 weeks after a study vaccine.

7.10 Treatment Compliance

Treatment compliance may be evaluated through drug accountability assessments and through the evaluation of subject medical records and CRF documents.

7.11 Biopsies

Part 1

Vaccine Site Biopsies
Each participant will undergo a biopsy of a vaccine site at two time points (days 8 and 22; one week after vaccines 1 and 3). The biopsies will consist of three 4-mm punch biopsies of skin.

Sentinel Immunized Node
A lymph node draining the 3rd vaccine site (sentinel immunized node (SIN) will be biopsied on day 22.

Tumor Biopsy
Optional at the time of recurrence or later as clinically indicated.

Part 2

Tumor Site Biopsies
Each participant enrolled in Part 2 will undergo a tumor biopsy and/or tumor excision at two time points:

- Pre-vaccine: Optimally, patients will have a tumor biopsy within 3 weeks of starting treatment. However, tumor tissue from a prior biopsy can serve as the pre-treatment sample provided: 1) there is no intervening treatment in between the pre-study biopsy and study treatment and 2) formalin-fixed tumor tissue is available and adequate to provide at least 20 unstained slides with sufficient tumor for analysis.
- Day 22. The day 22 biopsy will typically include complete resection in accord with clinical indications for disease control.

Optional biopsies may be completed at the time of progression or later as clinically indicated.

8.0 CLINICAL AND LABORATORY EVALUATIONS

The following evaluations will be performed on an outpatient basis. Please refer to the study calendar Appendix 1 for scheduling.

8.1 Physical Exams and Evaluations

- Medical History
- Complete Physical Exam (includes vital signs, weight, performance status, medication review, neurologic function-general)
- Assessment of skin and nodal basins for evidence of disease recurrence or metastasis
- Assessment of skin for vitiligo
- Assessment of hair and eye color
- Visual acuity (Snellen chart) (baseline only)
- Color vision exam (Ishihara eye chart) (baseline only)
- Assessment of baseline symptoms (baseline only)
- Designation of vaccination sites (at screening only)
  Evidence suggests nodes proximal to a tumor site may be relatively immunosuppressed; therefore, the vaccination sites will be selected to be distant from the sites of tumor whenever possible. In general, participants will be vaccinated in upper arm or thigh sites with intact draining nodes. Vaccines will be administered at the designated site(s).

8.2 Pathology Review

- Review of pathology at the University of Virginia

8.3 Performance Status

- ECOG performance status criteria will be used in the evaluations (Appendix 3)

8.4 Clinical Labs

- CBC with differential, including automated lymphocyte count (0.3 ml)
- Comprehensive chemistry panel to include sodium, potassium, creatinine, glucose, calcium total bilirubin, ASL, ALT, and alkaline phosphatase. NOTE: fasting blood sugars, when required, may be evaluated 4 hours or more after last eating. (0.9 ml)
- Urinalysis
- β-HCG for women of childbearing potential (2 ml)
- HgB-A1C (3 ml)
- HIV screening (antibody screen); reflexive testing to determine whether active disease is present. (3 ml)
- Hepatitis C Virus screening (antibody screen); reflexive testing to determine whether active disease is present (combined with HIV)
- ANA and Rf

8.5 Toxicity Assessments

- Assessment of adverse events. The NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 will be used for the characterization and grading of adverse events.
- Toxicity diaries will be distributed to participants and reviewed by study personnel.

8.6 Research Blood Samples

Blood should be obtained prior to the vaccine injection if a vaccine is scheduled to be administered. Results of research blood tests are not required prior to administering the vaccine on that date.

The following blood samples for research will be collected and processed by the UVA Biorepository and Tissue Research Facility (BTRF).

- 80 cc -120 cc blood collected in heparinized green top tubes for lymphocytes.
- 20 cc blood collected in red top tubes for serum

8.7 Vaccine Site Biopsies

8.7.1 Sampling

Each participant will undergo biopsy of a vaccine site at two time points (days 8 and 22; one week after vaccines 1 and 3). The biopsies will consist of three 4-mm punch biopsies of skin. The biopsies on day 8 will be performed on skin at the site of vaccine #1. Vaccines 2 and 3 are to be administered immediately adjacent to the site of vaccine #1, so that they are likely to drain to the same lymph node(s). Vaccine 3 is to be administered to the same site as vaccine #2. The biopsies on day 22 will be of the skin at the sites of vaccines 2 and 3.

8.7.2 Procedure

Three 4-mm punch biopsies will be obtained under local anesthesia. As each is removed, it will be passed off to staff of the BTRF, who will place one specimen in liquid nitrogen, one in formalin, and one in RNA-later.

8.7.3 Evaluations

The skin of the vaccine sites may be evaluated for immune activation and cellular infiltrates using multiple assays, including, but not limited to:

a) Formalin-fixed paraffin-embedded specimen: Immunohistochemistry for infiltration by immune cells (CD8, CD4, dendritic cells), for Th1/Th2/Th17/Treg bias (T-bet, GATA3, RORγt, FoxP3).
b) Quick frozen tissue: Luminex or other protein assays for cytokines and chemokines
c) RNA-later: Gene expression profiling of vaccine sites for immune signatures.

8.8 Sentinel Immunized Node Biopsy

8.8.1 Procedure

The node (sentinel immunized node, SIN) will be identified by radiocolloid (usually technetium 99 sulfur colloid) injection, with or without lymphoscintigraphy imaging, and with use of a handheld gamma probe during the procedure. This will be performed under local anesthesia in the clinic, in conjunction with the vaccine site biopsy, by a qualified surgeon.

Lymphatic mapping will be initiated, usually in the nuclear medicine suite, after intradermal injection with radiocolloid (typically technetium 99-sulfur colloid). The node excision will be performed under local anesthesia (usually lidocaine HCl 1-2%, with or without epinephrine 1:100,000 injection, with or without 8.4% sodium bicarbonate), in the outpatient clinic or comparable procedure room, using sterile technique. A handheld gamma probe will be used.

When possible, the node will be sectioned into 5 sections: a central section (10-20% of the node), leaving two adjacent sections of about 40% each. These latter two sections will be bisected. They will be allocated into various preservation conditions:1 central section will be fixed in formalin, then paraffin-embedded

a. (for histology/immunohistology)

b) 1 section will be placed in RNA-later. (for RNA/RT PCR)

c) 1 section will be quick-frozen (for immunohistology/protein studies)

d) 2 sections (40%) will be processed for single cell suspension by mechanical disaggregation, then enzymatic digestion (collagenase, hyaluronidase, DNAase). The resulting suspensions will be cryopreserved in FBS and DMSO (for cellular immune function and flow cytometry).

e) If there is additional tissue, it may be processed for additional immunologic or angiogenic studies.

The incisions will be sutured closed.

Toxicities related to the biopsies will be recorded.

8.9 Tumor Biopsies

Tumor biopsies will be completed in subjects enrolled in part 2 of the study. Biopsy sites may be in nodes, skin, soft tissue, liver, or other sites that can be accessed by needle biopsy, incisional or excisional biopsy. Biopsies may be completed with or without image guidance.

Size Requirements

A critical component of this protocol is the histologic and cytologic evaluation of changes in immune effectors and the tumor microenvironment after vaccination and systemic therapy. A minimum of 0.16 cm^3 but ideally 0.3 cm^3 or more of tumor tissue will be needed for each biopsy time point as described in the inclusion criteria. Biopsies may be taken from a single lesion or multiple lesions at each of the time points depending on the size of each lesion. If taken from multiple lesions, those lesions should be similar. For example, three non-ulcerated skin metastases would be considered similar; one bleeding small bowel metastasis would not be considered similar to a subcutaneous nodule).

Sampling

The biopsies will vary based on the clinical scenario and may include six core biopsies, an incisional biopsy or an excisional biopsy.

Procedure

When appropriate (and we anticipate the majority of cases) the biopsies will be performed under local anesthesia (typically lidocaine HCl 1% and epinephrine 1:100,000 injection + or - 8.4% sodium bicarbonate), in the outpatient clinic or comparable procedure room, using sterile technique. In cases when clinical standard of care requires a larger procedure the biopsies may be performed in the operating room under standard technique.

To minimize errors in analysis due to sampling error and specimen heterogeneity, each study biopsy specimen will be divided into several components and randomly allocated into various preservation conditions. Ideally, tissue will be divided into the following preservation conditions, using core needle biopsies (19 mm long and 2 mm diameter;

about 80 mm^3), or incisional or excisional biopsies with at least the same minimum tissue volume:

It is most critical to obtain the following:

- Formalin: 1 core biopsy or similar tissue volume (about 80 mm^3 or greater) will be fixed in formalin, then paraffin-embedded (for histology/immunohistology)
- Quick-frozen: 2 core biopsies or similar tissue volume (each about 80 mm^3 or greater) quick-frozen processed for protein studies, histology, or nucleic acid studies. If only one core can be obtained, this portion should be provided as two specimens (eg cut the core biopsy specimen in half).

When sufficient tissue is available, the following should also be obtained:

- RNA-later: 1 core biopsy or similar tissue volume (about 80 mm^3 or greater) will be placed in RNA-later (for RNA/RT PCR)
- Viable cell suspension: 2 core biopsies or similar tissue volume (total about 160 mm^3 or greater) will be processed for single-cell suspension by mechanical disaggregation, then enzymatic digestion (collagenase, hyaluronidase, DNAase). The resulting suspensions will be cryopreserved in FBS serum and DMSO (for cellular immune function and flow cytometry).
- If there is additional tissue, it may be processed for additional immunologic studies.

The incisions will be sutured closed. Toxicities related to the biopsies will be recorded.

Based on our experience in prior clinical trials (Mel48 (NCT00705640), Mel51 (NCT00977145), and Mel53 (NCT01264731)), it is adequate, for each specimen, to have at least the equivalent of a core biopsy specimen that is 19 mm long and 2 mm in diameter (about 80 mm^3), or a cubic specimen 5 mm in width. Thus, for 1 FFPE and 1-2 QF specimens, we need about 160-240 mm^3 (2-3 cores, or 1 lesion about 7-10 mm in diameter). For those with larger specimens (>1 cm diameter), some will also be saved as viably cryopreserved single cell suspensions.

A 5 micron section of each tumor specimen will be stained by H&E and reviewed to assess the extent and quality of viable tumor. For FFPE specimens, only those with at least 4 mm^2 viable tumor (on cross-section) will be considered evaluable for histologic and immunohistologic studies. FFPE tissue will be evaluated for immunotype and for immune cell infiltrates. For QF specimens, those with at least 70% tumor will be considered evaluable.

If during the study, participants develop metastases or recurrences, or progress, these may be removed, and following receipt by pathology, may be evaluated by the study research team.

Tissue samples may be screened for antigen expression or protein profiles using tests such as Western blots, immunohistochemistry, PCR, flow cytometry or gene chip analysis. Tumor escape mechanisms may also be evaluated. Specimens will be used in immunological assays to assess T cell infiltration, T cell function or antibody response. Assays generally used for this type of testing include, but are not limited to, immunohistochemistry, flow cytometric analyses, T cell receptor sequencing, ELIspot assays, ELISAs, chromium-release assays, proliferation assays and intracellular cytokine staining. Specimens may be used to study the immunologic aspects of the tumor microenvironment or as targets or controls in laboratory assays. Specimens may be used to establish cell lines for long-term studies.

This tissue may also be compared to lesions resected prior to enrollment, which will be requested from the pathology department of each institution as paraffin-embedded tissue samples, and these tissues may be banked for use in future studies. If participants are removed from the study or progress during or after follow-up, tissue may be collected for use as part of this study, as described above, or banked for use in future studies.

8.10 Assessments

8.10.1 Anti-tumor Activity

Anti-tumor activity will be assessed by the following:

Tumor Imaging

Tumor imaging may include CT/PET-CT scans and/or MRI. These will complement physical exam and other imaging as required, but the primary measures of clinical response will be based on CT/PET-CT and/or MRI. For each participant, the same method of assessment will be used to evaluate tumor burden at baseline and throughout the course of the study.

Tumor Measurements

RECIST 1.1 Criteria will be used to evaluate tumor burden. These are summarized in Appendix 5.

8.10.2 Immunologic Assessments

Assessments of T cell function may include, but are not limited to the following:

- ELIspot assays: measure of primary response
  Analysis of T cell responses by IFNγ ELIspot assay, comparing pre-vaccine to post-vaccine. The magnitude of T cell response will be defined by the ratio of responding cells at the time tested over background reactivity (and corrected for any pre-existing response. A 5-fold reactivity over background at two or more time points by day 85 will be considered a positive response. Flow cytometry may be performed to assess T cell responses as well, measuring multifunctional T cell responses.

- ELISAs
- Chromium-release assays
- Proliferation assays
- Intracellular cytokine staining
- T cell receptor sequencing.
- Cytokine bead array
- Flow cytometry and/or CY-TOF mass cytometry
- HLA typing

Characterization of cellular populations may include, but are not limited to the following:

- Immunohistochemistry
- Gene expression analysis
- Flow cytometry and/or CY-TOF mass cytometry
- ELISAs

- Western-blot analysis
- Intracellular cytokine staining
- Cytokine bead array

8.11 Study Calendar

See Appendix 1.

9.0 STATISTICAL CONSIDERATIONS

9.1 Overview

This is an early phase study evaluating the safety and immunogenicity of a vaccine comprised of a mixture of 6 synthetic melanoma helper peptides (6MHP) administered with one of 2 local adjuvant combinations (IFA or IFA + polyICLC), alone or with systemic low-dose cyclophosphamide (mCy). The trial is designed to find the range of optimal treatment combinations, defined by a combination with early and durable immunologic response and an acceptable level of toxicity. An adaptive design will be used to guide accrual decisions with toxicity assessments and the potential for a durable immune response characterizing the primary decision measures.

An additional study objective is to obtain preliminary data on whether treatment with the recommended optimal combination in patients with one or more tumor deposits accessible for biopsy or excision modifies the tumor microenvironment as quantified as a significant increase in infiltration of $CD4^+$ or $CD8^+$ T cells into tumor metastases.

9.2 Study Design

Part 1:

The primary objective is to determine the range of optimal combinations (one or more), among the set defined in Table 6, and then to expand accrual within the acceptable range to determine which overall treatment strategy is best. An optimal combination is a combination that is estimated to have an acceptable toxicity profile as measured by dose limiting toxicities (DLT) and a high rate of early and durable immune response (dRsp) as measured by CD4+ T cell response to 6MHP during the time period of vaccine administration.

Table 6: **Design Definitions**

Zone	Arm/Combination	6MHP+
1	A	IFA
2	B	IFA + mCy
2	C	IFA + PolyICLC
3	D	IFA + PolyICLC + mCy

A DLT is defined in section 10.9. An early dRsp is defined as at least a 5-fold increase in immune response to the 6MHP peptide as measured by $CD4^+$ T cells (see section 8.10.2) over two consecutive time periods during vaccination (days 0 to 85). As data accumulates each patient will be classified as experiencing a DLT (yes/no) and experiencing a dRsp (yes/no). Although DLTs are not anticipated they will be used to guide accrual decisions and protect against the unanticipated. Treatment-related grade 3 or higher adverse event (AE) data from our prior studies will be used to gauge DLT rates. Patients on Mel 44 that received the 6MHP + IFA vaccine (arms C & D), patients

on Mel 55 that received AS-15 and patients on Mel 58 that received IFA + PolyICLC experienced grade 3 or higher treatment-related AEs in 3.5%, 0% and 16% of patients, respectively. Using these results the DLT tolerance level was chosen to be 25% (i.e., any optimal combination that we are satisfied has an estimated DLT probability ≤25% to be considered "acceptable" in terms of safety). Data from the 6MHP arms of Mel 44 resulted in potential durable immune responses in 18% (90% CI(11, 26%)) of patients and provides the baseline to evaluate durable immune response.

Part 2:

To obtain preliminary data on the effects of the optimal treatment to modify the tumor microenvironment as quantified as a significant increase in infiltration of $CD4^+$ or $CD8^+$ T cells into tumor metastases.

9.3 Accrual Allocation for Determination of the Recommended Optimal Combination (Part 1 only)

Accrual to arms will occur in two stages. The initial stage will accrue eligible patients in cohorts of two on each arm, until a patient experiences a DLT. The second stage will allocate eligible patients based upon a continual reassessment method (CRM) for combinations of agents (78,79). The minimum follow-up period for escalation between Zones is 3 weeks after the initial vaccine.

9.3.1 First Stage Patient Allocation

The escalation plan for the 1^{st}-stage is based on grouping treatment combinations into "zones." With this design patients can be accrued and assigned to other open arms within a zone but escalation will not occur outside the zone until the minimum follow-up period is observed for the first patient accrued to an arm. Initial allocation within a zone will be based upon random allocation (1:1) between the possible arms. Escalation to a higher zone occurs only when all arms in the lower zone have been tried, and no DLT has been observed. Patient allocation to subsequent arms within the new Zone will follow the same accrual strategy. This allocation strategy is followed for accrual to increasing zones until a patient experiences a DLT or a stopping rule is triggered (Section 9.6). Once a DLT has been observed, the 2^{nd}-Stage using CRM modeling begins.

9.3.2 Second Stage Patient Allocation

The 2^{nd}-stage will allocate eligible patients based upon a CRM that accounts for both toxicity and immune response in combinations of agents. Toxicity assessment is based upon the occurrence of DLT's and immune response assessment is based on achievement of dRsp. The modeling stage uses (a) a selected set of possible orderings for both the DLT and dRsp probabilities and (b) a working model for both the DLT and dRsp probabilities under each ordering.

Table 7: Possible orderings of DLT probabilities

Order	Orderings	Working models**

		A	B	C	D
1	A-B-C-D	0.01	0.05	0.11	0.17
2	A-C-B-D	0.01	0.11	0.05	0.17
**Working models were chosen according to the algorithm of Lee and Cheung, 2009 (80)					

The orderings of immune response are formulated under two different assumptions (1) the probabilities are increasing with increasing zone, or (2) the probabilities increase initially and then plateau after a certain zone.

Table 8: Possible orderings of dRsp probabilities

Under the assumption of increasing dRsp probabilities					
Order	Orderings	Working models**			
		A	B	C	D
1	A-B-C-D	0.25	0.40	0.55	0.70
2	A-C-B-D	0.25	0.55	0.40	0.70
3	A-B-D-C	0.25	0.40	0.70	0.55
4	A-C-D-B	0.25	0.70	0.40	0.55
5	A-D-B-C	0.25	0.55	0.70	0.40
6	A-D-C-B	0.25	0.70	0.55	0.40
Under the assumption of plateau dRsp probabilities					
Order	Orderings	Working models**			
		A	B	C	D
7	A-B-C-D	0.45	0.55	0.70	0.70
8	A-B-C-D	0.55	0.70	0.70	0.70
9	A-B-C-D	0.70	0.70	0.70	0.70
10	A-C-B-D	0.45	0.70	0.55	0.70
11	A-D-B-C	0.45	0.70	0.70	0.55
**Working models were chosen according to the algorithm of Lee and Cheung, 2009(80).					

The 2nd-stage allocation scheme begins once a DLT is observed regardless of which Zone it occurs in. Within each ordering, the CRM is fit for both toxicity and immune response, using the working model and the accumulated data. The 2nd-stage will accrue eligible patients in cohorts of one and use a CRM model fit to estimate DLT probabilities and immune response probabilities at each arm combination. For each order, $m = 1, 2$, in Table 7, the DLT probabilities for each arm combination $i = 1, \cdots, 4$, are modeled via a one-parameter power model, $Pr(\text{DLT at combination } i) \approx p_{mi}^{\theta_m}$, where the p_{mi} are the working model values for order m given in Table 8. After accrual of each patient into the trial, the parameter θ_m is estimated for each ordering by maximum likelihood estimation where the likelihood is given by $\prod_{i=1}^{4} \left(p_{mi}^{\theta_m}\right)^{y_i} \left(1 - p_{mi}^{\theta_m}\right)^{n_i - y_i}$ where y_i= the number of DLTs and n_i=the number of treated patients in arm combination i. The order with the largest likelihood is chosen and, within this ordering, DLT probability estimates are updated for each combination. If there is a tie between the likelihood values of two or more orderings, then the selected order is randomly chosen from among the tied orderings.

These DLT probabilities will be used to define a set of "acceptable" combinations. For arm combinations B-D, a one-sided 80% confidence interval is calculated using the estimated DLT probability for that arm, based on confidence interval estimation for CRM models (81). If the lower bound of this confidence interval exceeds the maximum toxicity tolerance of 25%, then this arm is deemed

too toxic and excluded from the acceptable set of combinations. If arm A is excluded from the acceptable set then no arm is considered acceptable and the trial is stopped for safety, therefore, for arm A the level of confidence is set at 95% instead of 80%. The toxicity tolerance of 25% was chosen based on the expectedness of adverse events. Note this process is performed for each new accrual. For each dRsp working model, $h = 1, \cdots, 11$, in Table 8, the dRsp probabilities are modeled via a one-parameter power model $\Pr(\text{dRsp at combination i}) \approx q_{hi}^{\exp(\beta_h)}$, where the q_{hi} are the working model values for order h given in Table 8. After accrual of each patient into the trial, the parameter β_h is estimated for each working model by maximum likelihood estimation where the likelihood is given by $\prod_{i=1}^{4} \left(q_{hi}^{\exp(\beta_h)}\right)^{z_i} \left(1 - q_{hi}^{\exp(\beta_h)}\right)^{n_i - z_i}$ where z_i= the number of immune responses and n_i=the number of treated patients in combination i. Again, the model with the largest likelihood is chosen and, within this working model, dRsp probability estimates are updated for each combination. If there is a tie between the likelihood values of two or more models, then the selected model is randomly chosen from among the tied models.

9.3.3 Accrual Deviations

If the minimum follow-up period is not satisfied at the time a new patient is ready to be put on-study, then the patient may be accrued to any arm, by random allocation, which has accrued at least one patient and is in the set of possible optimal combinations. Data from these patients will be used in the modeling stage as their data becomes available for DLT and dRsp determination.

9.3.4 Arm Recommendation

Once the set of "acceptable" combinations is determined, the recommended combination will be based upon how many patients have been entered into the study to that point. For the first third of the trial (i.e. 1/3 the maximum sample size), the combination recommendation is based on randomization using a weighted allocation scheme. The recommended combination for the next entered patient is chosen at random from the "acceptable" combinations with each acceptable combination weighted by its estimated immune response probability. That is, acceptable combinations with higher estimated dRsp probabilities have a higher chance of being randomly chosen as the next recommended combination. For the latter third of the trial (i.e. final 2/3 of maximum sample size), the recommended combination for the next entered patient is defined as the "acceptable" combination with the highest estimated dRsp probability. After each patient, a new recommended combination is obtained, and the next entered patient is allocated to the recommended combination. The trial will stop once sufficient information about the optimal dose range has been obtained, according to the stopping rules outlined in Section 9.6.

9.4 Statistical Properties

Simulation results were run (R-package, pocrm) to display the performance of the design characteristics, see Table 9. For each scenario, 1000 simulated trials were run. Displayed in the table in each scenario for each arm is the true DLT probability, the true dRsp response rate, the percentage of trials in which the arm was recommended as the optimal combination, and the percentage of patients treated. Displayed in the last four columns is the average and selected percentiles for the trial size at study closure, the

percentage of times in the simulations that the trial closed due to safety concerns, the percentage of simulated patients that had a DLT, and the percentage of simulated patients that had a dRsp. The following scenarios were chosen to display the operating characteristics with the optimal combination(s) indicated in bold type in the table.

Scenario 1: All true DLT probabilities are safe (i.e. less toxic than 25%) and the highest Zone has the combination with the highest dRsp rate.

Scenario 2: All true DLT probabilities are safe (i.e. less toxic than 25%) and the dRsp probabilities begin to plateau at arm C in Zone 2.

Scenario 3: Two combinations (C and D) have true DLT probabilities more toxic than 25% and combination B has the highest dRsp rate among safe combinations.

Scenario 4: All true DLT probabilities are safe (i.e. less toxic than 25%) and equal dRsp for combination B and D.

Scenario 5: Two combinations (B and D) have true DLT probabilities more toxic than 25% and combination C has the highest dRsp rate among safe combinations.

Scenario 6: All combinations are too toxic (i.e. more toxic than 25%).

Table 9: Design performance

Maximum sample size set at 70, stop when the optimal arm has accrued 30 patients

Scenario: / **Zone:** / **Regimen:**	True probabilities (DLT, dRsp) % optimal regimen recommended % patient allocation				Avg size, percentiles	% stop	% DLT	% dRsp
	1	**2**		**3**				
	A	**B**	**C**	**D**				
1:	(0.02, 0.19) 0.05 0.15	(0.07, 0.30) 0.09 0.17	(0.05, 0.40) 0.11 0.18	**(0.17, 0.70)** **0.75** **0.50**	48, 25th = 44 50th = 46 75th = 49 90th = 58 95th = 64	0.000	0.107	0.501
2:	(0.01, 0.35) 0.15 0.20	(0.03, 0.45) 0.24 0.25	**(0.05, 0.60)** **0.44** **0.34**	**(0.10, 0.60)** **0.17** **0.21**	50, 25th = 45 50th = 47 75th = 54 90th = 63 95th = 67	0.002	0.046	0.513
3:	(0.14, 0.19) 0.28 0.31	**(0.20, 0.40)** **0.62** **0.43**	(0.44, 0.50) 0.07 0.20	(0.44, 0.70) 0.02 0.06	49, 25th = 42 50th = 48 75th = 56 90th = 65 95th = 70	0.020	0.242	0.373
4:	(0.05, 0.15) 0.13 0.20	**(0.05, 0.40)** **0.67** **0.46**	(0.17, 0.20) 0.04 0.14	**(0.17, 0.40)** **0.17** **0.20**	48, 25th = 44 50th = 46 75th = 50 90th = 59 95th = 64	0.003	0.090	0.332
5:	(0.05, 0.20) 0.10 0.21	(0.40, 0.40) 0.12 0.22	**(0.20, 0.50)** **0.75** **0.47**	(0.45, 0.70) 0.03 0.10	52, 25th = 45 50th = 50 75th = 60 90th = 69 95th = 70	0.005	0.237	0.438
6:	(0.60, 0.20) 0.00 0.92	(0.70, 0.40) 0.00 0.04	(0.80, 0.50) 0.00 0.04	(0.90, 0.70) 0.00 0.01	8, 25th = 2 50th = 7 75th = 12 90th = 17 95th = 21	0.997	0.620	0.215

9.5 Sample Size and Accrual

9.5.1 Optimal Combination (Part 1):

Target sample size for the optimal combination is based upon acquiring sufficient information to assess the objective of estimating dRsp rates, assuming at least one optimal combination has been found. Based upon results from the Mel44 clinical trial (NCT00118274), 30 eligible patients treated at the optimal combination will provide adequate data to assess dRsp. The target of thirty patients was chosen based on having sufficient information to determine if the optimal arm shows an increase dRsp rate compared to the baseline rate observed in the 6MHP arms of Mel 44 of 18% (90% CI(11, 26%)). If at least 13/30 (43% 90% CI(28, 60%)) patients on the optimal arm experience a dRsp the results will be considered promising since the lower limit of the confidence interval exceeds the upper limit from the Mel 44 estimated rate. Total study sample size is estimated from the simulations, but in reality is an outcome determined by the stopping rules in Section 9.6. We set the maximum total sample size to 70 eligible patients; however, as indicated in the simulation results the maximum average trial size over all scenarios is 52 patients. Adjusting for a 5% drop-out/ineligibility rate, maximum total target accrual to part 1 should not exceed 74 patients but based upon the simulations is estimated to be approximately 50 patients.

9.5.2 Assessment of the tumor microenvironment (Part 2):

Published data from the Mel51 clinical trial provides preliminary estimates of variability and baseline change in infiltration of $CD4^+$ or $CD8^+$ T cells into tumor metastases (82). Shown in the following table is the alternative mean change and effect size that could be detected with accrual of 10 to 16 eligible participants based upon assessment of mean change estimated from Mel51 data in 9 participants. The alternative was estimated assuming a one-side 10% level non-parametric test with 90% power. Based upon these results, a target sample of 14 eligible participants treated at the recommended combination would provide for determination of a moderate to large effect size, ES=0.737, a feasible and meaningful result. Adjusting for a 5% drop-out/ineligibility rate, maximum total target accrual to part 2 should not exceed 15 participants.

Infiltrate measure in tumor	N	Null Mean Change	Alternative Mean Change	StD	Effect Size
CD4	10	208.0	1291.1	1199.0	0.903
	12	208.0	1176.1	1199.0	0.807
	14	208.0	1091.7	1199.0	0.737
	16	208.0	1026.3	1199.0	0.682
CD8	10	993.0	3060.7	2289.0	0.903
	12	993.0	2841.3	2289.0	0.807
	14	993.0	2680.1	2289.0	0.737
	16	993.0	2555.1	2289.0	0.682

9.6 Stopping Rules (Part 1 only)

Accrual to the study will be halted and trigger a safety review by the study investigators and DSMC to determine if the study should be modified, or permanently closed to further accrual according to the following:

- Accrual will be halted for safety if the first four entered patients in Zone 1 experience a DLT on all arms in Zone 1 in the 1st-Stage.
- If at any point in the 2nd-Stage, the set of acceptable combinations is empty, the trial will stop for safety.
- Otherwise, accrual to part 1 of the study will end if the recommendation is to assign the next patient to a combination that already has 30 patients treated at that combination.

9.7 Data Analysis Plans

All patients who are placed on-study will be included in the final report.

9.7.1 Safety:

All patients who receive any protocol treatment will be monitored for adverse events. Adverse events will be described and coded based upon the NCI CTCAE v4.03. A DLT is defined as any unexpected adverse event that is possibly, probably or definitely related to treatment and satisfies the criteria in Section 10.9. Occurrence of DLTs will guide escalation and stopping decisions. At study conclusion frequency, proportion and severity of adverse events, and DLTs by arm will be tabulated.

9.7.2 Efficacy:

All eligible patients who receive any protocol treatment will be evaluated for immunologic and clinical endpoints.

a. Immunologic (For all patients unless otherwise specified)

$CD4^+$ T cell responses to 6MHP after 6MHP vaccination as assessed in the PBMC over the treatment course. Those data will be used to define the optimal treatment combination in Part 1 and to describe response overall for both parts of the study. Prolonged duration of immunologic response as measured over the follow-up period will be used to determine whether response persists beyond the treatment period. Point estimates assessing fold increase at each time point will be described and if applicable, repeated measure models will be used to describe the pattern of change over time. Other study endpoints that will be summarized by arm for Part 1 only include $CD4^+$ T cell responses to 6MHP assessed in the vaccine-draining nodes and $CD8^+$ T cell responses to melanoma antigens assessed in vaccine-draining nodes. $CD8^+$ T cell responses to melanoma antigens will also be assessed in PBMC in Parts 1 and 2.

For Part 1, any arm that was in the range of optimal combinations, we will describe whether the vaccine adjuvant contained in the range of optimal combinations induces Th1-dominant cytokine responses in the VSME and SIN, and induces IgG antibodies to 6MHP. Other preliminary data about the vaccine-site microenvironment (VSME) induced by the vaccines administered in different adjuvants will be summarized and include assessment of

Th1/Th2/Th17 bias of cells in the VSME, T cell retention in the VSME, and induction of retention integrins.

b. Clinical:

Patients will be assessed for disease-free survival (DFS) or progression-free survival (PFS), as appropriate, and overall survival (OS). DFS is defined as the time from the date of start of treatment to the date of melanoma recurrence or metastasis, or date of death, whichever occurs first. Patients who have neither recurred/progressed nor died will be censored on the date of last evaluable tumor assessment. PFS is defined as the time from the date of start of treatment to the date of melanoma progression or death, whichever occurs first. Patients who have neither progressed nor died will be censored on the date of last evaluable tumor assessment. OS is defined as the time from the date of start of treatment to the date of death from any cause. Patients who do not experience an event (death) will be censored at date of last follow-up/contact. DFS, PFS and OS distributions will be estimated by the product limit method of Kaplan and Meier.

c. Tumor microenvironment (Part 2 only):

A one-sample sign rank test will be used to assess change (post-pre) in tumor infiltrate measures. Specifically, the tumor biopsy pre vaccine (timing as detailed in section 3.1.1) and, tumor biopsy or excision at week 3 (1 week after 3rd vaccine) will be used to determine whether T cells induced by vaccination infiltrate tumor deposits and whether CD8+ T cell infiltration increases with treatment.

9.7.3 Study Conclusion (Part 1 only)

For Part 1, if more than one combination is contained within the range of optimal combinations, then immune response during the follow-up period, days 85 through 180, may be used to define which patients have prolonged durable responses with the arm with the highest rate defining the better combination. Other secondary endpoints may be used to differentiate which combination is the most worthy of further study. For Part 2, similar assessments will be performed as for Part 1 except that sentinel immunized node and vaccine site biopsies will not be available for Part 2, but assessments of the tumor microenvironment will be performed for Part 2, as detailed above.

10.0 ADVERSE EVENT DATA COLLECTION AND MONITORING

10.1 Definitions

10.1.1 **Adverse event (AE)** – Any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medical treatment or procedure regardless of whether it is considered related to the medical treatment or procedure (attribution of unrelated, unlikely, possible, probable, or definite). Medical conditions or diseases present before starting the investigational drug will be considered as treatment-related AEs if they worsen after starting study treatment.

10.1.2 **Unexpected AE** – Any adverse event not listed in section 10.4.3.

10.1.3 **Serious AE –** Any adverse drug experience occurring at any dose that results in any of the following outcomes:

- death;
- a life-threatening adverse drug experience;
- inpatient hospitalization, or prolongation of existing hospitalization (as defined below in this section);
- a persistent or significant disability/incapacity; or a congenital anomaly/birth defect.
- Important medical events that may not result in death, be life threatening, or require hospitalization may be considered a serious adverse drug experience when, based upon medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.
- Hospitalization for expedited AE reporting purposes is defined as an inpatient hospital stay equal to or greater than 24 hours. Hospitalization is used as an indicator of the seriousness of the adverse event and should be reserved for situations where the adverse event truly fits this definition and not for hospitalizations associated with less serious events. For example, the following are not considered serious adverse events:
    - a hospital visit where a patient is admitted for observation or minor treatment (e.g. hydration) and released in less than 24 hours
    - hospitalization for pharmacokinetic sampling
    - admission to hospice
    - hospitalizations planned before entry into the clinical study
    - hospitalization for elective treatments
    - hospitalizations to work up Grade 1 adverse events

10.1.4 **Unanticipated problem -** An unanticipated problem is any event/experience that meets ALL 3 criteria below:

- Is unexpected in terms of nature, severity or frequency given the research procedures that are described in the protocol-related documents AND in the characteristics of the participant population being studied.
- Is related or possibly related to participation in research. This means that there is a reasonable possibility that the incident may have been caused by the procedures involved in the research study.
- The incident suggests that the research placed the participant or others at greater risk of harm than was previously known or recognized OR results in actual harm to the participant or others.

10.1.5 **Protocol Violation-** A protocol violation is defined as any change, deviation, or departure from the study design or procedures of a research project that is NOT approved by the institution's IRB prior to its initiation or implementation, OR deviation from standard operating procedures, Good Clinical Practices (GCPs), federal, state or local regulations. Protocol violations may or may not be under the control of the study team or UVa staff. These protocol violations may be major or minor violations.

10.1.6 **Suspected Adverse Reaction (as defined in 21 CFR 312.32 (a))-** Any adverse event for which there is a reasonable possibility that the drug caused the adverse event.

10.2 Attribution Assessment

10.2.1 Attribution – The determination of whether an adverse event is related to a medical treatment or procedure. The attribution groups are:

Definite – Applies to those adverse events which, the investigator feels are incontrovertibly related to study drug. An adverse event may be assigned an attribution of definitely related if or when (must have all of the following):

- It follows a reasonable temporal sequence from administration of the test drug.
- It could not be reasonably explained by the known characteristics of the participant's clinical state, environmental or toxic factors, or other modes of therapy administered to the participant.
- It disappears or decreases on cessation or reduction in dose with re-exposure to drug. (Note: This is not to be constructed as requiring re-exposure of the participant; however, the group of definitely related can only be used when a recurrence is observed.)
- It follows a known pattern of response to the test drug.

Probable – Applies to those adverse events for which, after careful consideration at the time they are evaluated, are felt with a high degree of certainty to be related to the test drug. An adverse event may be considered probably related if or when (must have three of the following):

- It follows a reasonable temporal sequence from administration of the test drug.
- It could not be reasonably explained by the known characteristics of the participant's clinical state, environmental or toxic factors, or other modes of therapy administered to the participant.
- It disappears or decreases on cessation or reduction in dose. There are important exceptions when an adverse event does not disappear upon discontinuation of the drug, yet drug-relatedness clearly exists (e.g. bone marrow depression, fixed drug eruptions, tardive dyskinesia).
- It follows a known pattern of response to the test drug.

Possible – Applies to those adverse events for which, after careful consideration at the time they are evaluated, a connection with the test drug administration appears unlikely but cannot be ruled out with certainty. An adverse event may be considered possibly related if or when (must have two of the following):

- It follows a reasonable temporal sequence from administration of the test drug.
- It could not readily have been produced by the participant's clinical state, environmental or toxic factors, or other modes of therapy administered to the participant.
- It follows a known pattern of response to the test drug.

Unlikely – Applies to those adverse events for which, after careful consideration at the time they are evaluated, are judged to be unrelated to the test drug. An

adverse event may be considered unlikely if or when (must have two of the following):

- It does not follow a reasonable temporal sequence from administration of the test drug.
- It could readily have been produced by the participant's clinical state, environmental or toxic factors, or other modes of therapy administered to the participant.
- It does not follow a known pattern of response to the test drug.
- It does not reappear or worsen when the drug is re-administered.

Unrelated – Applies to those adverse events, which after careful consideration, are clearly and incontrovertibly due to extraneous causes (disease, environment, etc.).

10.3 Data collection

Data will be collected using a centralized electronic case report form called **ON**-line **C**linical **O**ncology **R**esearch **E**nvironment = **Oncore**.

10.4 Risks and Safety

10.4.1 Adverse Event Descriptions and Grading Scales

The NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 will be used for the characterization and grading of adverse events.

10.4.2 Time Span for Reporting Adverse Events

Reporting of AEs will begin when the participant is administered the study drug or has a study related biopsy. Events occurring through 30 days after administration of the last dose of 6MHP or mCy, regardless of attribution, will be reported. AEs should be followed to resolution or stabilization. If an AE worsens and becomes an SAE, it should be reported as serious per the guidelines specified for SAE reporting.

AEs that are possibly, probably, or definitely related to any of the study drugs will be recorded until the participant completes treatment follow-up. If, during treatment follow-up, the participant receives an alternative anti-cancer treatment, participants will be off treatment follow-up and will be followed yearly for disease progression and survival.

10.4.3 Agent-Specific Expected Adverse Events List:

Any AE not in this list will be considered an unexpected AE.

Table 10: Toxicities related to 6MHP in prior trials

Toxicity (based on max grade)	Gr 1	Gr2	Gr3	Gr4
LOCAL, INJECTION SITE				
Injection site reaction	18%	56%	2.4%	
Ulceration		5%	1.4%	
CONSTITUTIONAL				
Fatigue	43%	8%	3.4%	
Headache	27%	1.4%	0.5%	

Toxicity (based on max grade)	Gr 1	Gr2	Gr3	Gr4
Rigors, Chills	24%	1.4%	--	
Nausea	23%	2%	0.5%	
Sweating	19%	1%		
Myalgias	18%	0.5%		
Arthralgias	17%	0.5%		
Fever	16%	2%		
Dizziness	13%			
Anorexia	13%	3%		
Diarrhea	12%	2%		
Cough	13%			
Allergic rhinitis	11%			
Nasal/paranasal reactions	11%			
Pain larynx/throat	10%			
Flushing	10%			
Pruritis	9%	0.5%		
Rash	6%	3.4%		
Dyspnea	5%	1%	0.5%	
Vomiting	5%	1%	0.5%	
Flu-like syndrome	6%			
Mucositis	6%			
Constipation	5%			
Autoimmune reaction	4%	0.5%		
Wound, non-infectious	4%			
Pain, other	2%	1%	0.5%	
Abdominal pain	1.4%		0.5%	
Tinnitus		0.5%	0.5%	
Tumor pain	0.5%		0.5%	
Hearing (without monitoring program)			0.5%	
CLINICAL LABORATORY				
Hyperglycemia (not fasting)	22%	1%		
Hemoglobin, low	17%	1%	0.5%	
Hyperkalemia	13%			
Lymphopenia	9%	2.9%	1%	
Leukocytes	8%	1%		
Hyponatremia	7%			
Increased creatinine	6%	0.5%		
Hypoglycemia	6%			
AST, SGOT	5%			0.5%
ALT, SGPT	4%		1%	
Neutrophils	3%	2%		
Metabolic, Other	3%	0.5%	0.5%	
Alk phos	4%	0.5%		

The following toxicities are those greater than grade 1 that are to be considered expected from the standpoint of defining DLTs. They are selected because they occur in at least 4% of patients and are no greater than grade 2.

Table 11: Expected Toxicities for 6MHP vaccines	**Grade 2**
Injection site reaction	+
Ulceration	+
Fatigue	+

Expected toxicities related to polyICLC

The following expected toxicities for polyICLC are based on the data from 45 patients treated with 20 mcg/kg 3x/week. This would be about 1.4 mg per dose and 4.2 mg per week (much higher than we will use).

Table 12: Toxicity Data for PolyICLC

Category	Toxicity	Grade	Comment
Nervous system disorders	Headache	Grade 2	1 of 22 (<5%)
	Tremors	Grade 3	2 of 45 (4%) poss related
Musculoskeletal and connective tissue disorders	Muscle weakness	Grade 2	1 of 45 (<2%) experienced at Grade 3
Respiratory, thoracic and mediastinal disorders	Dyspnea	Grade 2	1 of 45 (<2%) experienced at Grade 3
	Hypoxia	Grade 2	1 of 45 (<2%) experienced at Grade 3
Metabolism and nutrition disorders	Hypernatremia	Grade 2	1 of 45 (<2%) experienced at Grade 3
Investigations	Elevated transaminases (GPT)	Grade 3	4 of 45 (9%) 3 cases possibly related; one case probably related. Typically transient.
	Elevated Alkaline phosphatase	Grade 3	7% of patients, in IB
	Leukocytopenia	Grade 3	2 of 45 (<4%) in published work; 20% in IB
	Thrombocytopenia	Grade 3	14% of patients in IB
	Neutropenia	Grade 3	10% of patients in IB
Blood/lymph disorders	Anemia	Grade 3	13-31% of patients in IB
General disorders and administration site conditions	Vaccine site reaction	Grade 2	1 of 22 (<5%)
	Fever	Grade 3	14% of patients in RCC trial
	Chills	Grade 3	10% of patients in RCC trial
	Fatigue	Grade 3	10% of patients in RCC trial

Thus, this higher dosage of polyICLC induced grade 3 toxicities in a subset of patients. However, in a trial of a peptide vaccine administered in an emulsion with polyICLC and Montanide ISA-51, there were no grade 3 toxicities. For that cohort of 11 patients, the following grade 2 toxicities were reported: injection site reaction (27%), panniculitis (1/11 = 9%)(46). In that trial they administered 1.4 mg of polyICLC with each dose, every 3 weeks x 5 (total 7 mg over 12 weeks). We will administer 1 mg of polyICLC with each dose x 6 (total 6 mg over 11 weeks). Thus, we expect toxicities from polyICLC in this trial to be similar to those in that peptide vaccine trial. The following toxicities are those greater than grade 1 that we consider expected from the standpoint of defining DLTs. Grade 3 toxicities are not expected. Panniculitis is not listed because we have not observed it in our ongoing experience with polyICLC (data not yet summarized for ongoing trials).

Table 13: Expected Toxicity for polyICLC	Grade 2
Injection site reaction	+

Expected toxicities related to mCy.

We anticipate that a low dose of metronomic cyclophosphamide will not carry the same level of risk as a dose that is intended to be used as a chemotherapy medication. Toxicities that have been associated with low-dose administration of cyclophosphamide are summarized in section 1.7.2. At doses and schedules slightly higher than those planned for the present study, a minority of patients (but >4%) experienced grade 3 lymphopenia, neutropenia, and nausea. Because the current proposal will use somewhat lower doses, grade 3 toxicities may not occur. However, if they do occur, cyclophosphamide will be held until the resolve to grade 1 or lower, and cyclophosphamide may be resumed at that time. Expected Grade 2 and Grade3 toxicities for low-dose metronomic cyclophosphamide are listed in Table 14.

Table 14: Expected toxicities related to mCy

	Grade 2	Grade 3
Fatigue	+	
Nausea	+	
Thrombocytopenia	+	
Anemia	+	
Lymphocyte count decreased		+
White blood cell decreased		+
Neutrophil count decreased		+

Expected toxicities from vaccine site and node biopsies.

Below is a list of expected AEs related to vaccine site and SIN biopsies:

Vaccine Site Biopsies

- Bleeding
- Bruising
- Pain
- Very low risk of infection (less than 2%)
- Delayed wound healing
- Scarring

SIN Biopsies

- Bleeding
- Bruising
- Pain
- Infection
- Delayed wound healing
- Scarring
- Very low risk of lymphedema (less than 2%)
- Numbness

10.5 Adverse Event Classifications

Adverse events (AEs) are classified into sections, specified in the CTCAE v4.03. For specific classifications pertaining to the protocol, we specify the following:

Hematologic/Metabolic- Any AE coded under one of the following CTCAE v4.03 categories should be reported under the Hematologic/Metabolic adverse event classification:

Table 15: Hematologic/Metabolic Classifications

Section	AE
Blood and lymphatic	Anemia Leukocytosis
Investigations	**ALL EXCEPT:** Carbon monoxide diffusing capacity decreased Ejection fraction decreased Forced expiratory volume decreased Vital capacity abnormal Weight gain Weight loss
Metabolism and nutrition disorders	**ALL EXCEPT:** Alcohol intolerance Anorexia Dehydration Glucose intolerance Iron overload Obesity Tumor lysis syndrome

Non-hematologic/Non-Metabolic- Any AE not reported under hematologic/metabolic, ocular, or allergic/autoimmune, should be reported under the non-hematologic/non-metabolic adverse event classification.

Ocular – Any AE coded under one of the following CTCAE v4.03 Adverse Event Terms should be reported under the Ocular adverse event classification:

1) A single treatment-related experience of the following adverse events will be classified as a DLT:
   - Eye Disorders: Night blindness (nyctalopia)
   - Eye Disorders: Papilledema
   - Eye Disordersl: Retinopathy

   Participants will be referred for an ophthalmologic exam if any of these ocular adverse events occur.

2) A prolonged treatment-related experience (e.g., lasting > 5 days) of the following non-severe adverse events will be classified as a DLT:

   - Eye Disorders: Blurred vision
   - Eye Disorders: Flashing lights
   - Eye Disorders: Floaters

   Participants will be referred for an ophthalmologic exam if any of these ocular adverse events occur.

Allergic/Autoimmune – Only AEs coded as Immune System Disorder: Allergic reaction, autoimmune disorder, or anaphylaxis should be reported under the Allergic/Autoimmune

adverse event classification. Other AEs coded under Immune System Disorder should be reported under Non-hematologic/Non-metabolic adverse event classification.

10.6 Reporting Adverse Events

10.6.1 Process for Reporting AEs:

Dose-limiting toxicities

DLTs will be entered into Oncore within 5 calendar days of the study team learning of the event. DLT's that are deemed serious and unexpected will be submitted to the IRB per institutional guidelines (see below).

Other AEs

AEs must be recorded into the University of Virginia Cancer Center OnCore database per the following guidelines Table 16.

Table 16: AE reporting

High Risk Studies Reporting requirements for AEs that occur within 30 days of the last dose of protocol specified treatment								
	Grade 1	Grade 2		Grade 3				Grade 4 & 5
	Expected and unexpected	Expected	Unexpected	Expected		Unexpected		Expected and Unexpected
				Without hospitalization	With hospitalization	Without hospitalization	With hospitalization	
Unrelated Unlikely	OnCore 30 days[a]	OnCore 30 days	OnCore 30 days	OnCore 30 days	OnCore 15 days	OnCore 30 days	OnCore 15 days	OnCore 7 days
Possible Probable Definite	OnCore 30 days[a]	OnCore 30 days	OnCore 15 days	OnCore 30 days	OnCore 15 days	OnCore 7 days	OnCore 7 days	OnCore (24-hrs)* 7 days

*Enter into OnCore database within 24 hours if unexpected and definitely related to protocol specified treatment
Hospitalization defined as an inpatient hospital stay or prolongation of a hospital stay equal to or greater than 24 hours
[a] Grade 1 unexpected or expected hematologic/metabolic events will be recorded in the Cancer Center Database; however, regardless of attribution, these events do not have to be reported.

10.6.2 Pregnant-Partner Outcomes

If a male has been exposed to the investigational agent prior to or around the time of conception, this will not be considered an SAE. The HITC will ask permission of the pregnant partner to be followed until term.

Pregnancy

If a female has been exposed to the investigational agent prior to or around the time of conception, this will not be considered an SAE. The HITC will follow the pregnancy until term.

10.6.3 IRB Reporting Requirements

The University of Virginia is responsible for reporting to the UVA IRB-HSR per the following guidelines:

Table 17: UVA IRB-HSR reporting

Type of Event	To whom will it be reported:	Time Frame for Reporting	How reported?
Any internal event resulting in death that is deemed DEFINITELY related to (caused by) study participation (Note: An internal event is one that occurs in a subject enrolled in a UVa protocol.)	IRB-HSR	Within 24 hours	IRB Online and phone call www.irb.virginia.edu/
Internal, Serious, Unexpected adverse event.	IRB-HSR	Within 7 calendar days from the time the study team received knowledge of the event. *Timeline includes submission of signed hardcopy of AE form.*	IRB Online www.irb.virginia.edu/
Unanticipated Problems that are not adverse events or protocol violations This would include a Data Breach.	IRB-HSR	Within 7 calendar days from the time the study team received knowledge of the event.	Unanticipated Problem report form. *http://www.virginia.edu/vprgs/irb/HSR_docs/Forms/Reporting_Requirements-Unanticipated_Problems.doc)*
Protocol Violations (*The IRB-HSR only requires that MAJOR violation be reported, unless otherwise required by your sponsor, if applicable.)* Or Enrollment Exceptions	IRB-HSR	Within 7 calendar days from the time the study team received knowledge of the event.	Protocol Violation and Enrollment Exception Reporting Form *http://www.virginia.edu/vprgs/irb/hsr_forms.html*
Data Breach	The UVa Corporate Compliance and Privacy Office and ITC: if breach involves electronic data- UVa Police if breach includes such things as stolen computers.	As soon as possible and no later than 24 hours from the time the incident is identified. As soon as possible and no later than 24 hours from the time the incident is identified. IMMEDIATELY.	UVa Corporate Compliance and Privacy Office- Phone 924-9741 ITC: Information Security Incident Reporting procedure, http://www.itc.virginia.edu/security/reporting.html Phone- (434) 924-7166

10.6.4 Additional Reporting Requirements for the Sponsor (UVA)

Reporting to the FDA

- Serious and unexpected suspected adverse reactions will be reported to the FDA no later than 15 calendar days after the sponsor determines that the requirements for an IND safety report have been met. The FDA will be notified using an FDA Form 3500a.
- Unexpected fatal or life-threatening suspected adverse reactions will be reported to the FDA no later than 7 calendar days after the Sponsor receives the initial information of the event. The FDA will be notified using an FDA Form 3500a.
- Other adverse event information will be sent to the FDA in the IND annual report.

Reporting to the Ludwig Institute for Cancer Research (LICR)-pertains to arms receiving polyICLC

- All Serious Adverse Events (SAEs), for which a relationship with polyICLC cannot be ruled out. These SAEs should be reported within 15 calendar days of their onset (7 calendar days for life-threatening or fatal SAEs).

10.6.5 Reporting of Participant Withdrawals/Dropouts Prior to Study Completion

Participants who withdraw consent and those dropping out of the study secondary to an AE will be reported to the UVA IRB yearly on the IRB continuation form.

10.7 Adverse Event Review and Monitoring

10.7.1 Capturing Adverse Events

In addition to clinic notes, adverse events will be initially captured using study-specific tools and participant toxicity diaries.

Each participant will be evaluated by a licensed clinician. The following will be performed as designated in the protocol: routine disease-directed physical exam including performance status and blood collection for clinical labs.

Participants should keep a daily diary of toxicities until the next protocol clinic visit. The diaries will be reviewed by a research clinician prior to the next scheduled infusion or vaccine, if one is scheduled. During clinic visits, participants will also be asked about subjective symptoms including headache, malaise, fatigue, dyspnea, nausea, rash, diarrhea, abdominal discomfort, peripheral nerve pain, visual changes, appetite, tremors, night sweats, and ability to concentrate. Additional toxicities will be captured from laboratory tests. For each AE (with the exception of Grade 1 hematologic/metabolic events), date of onset, duration, grade, and attribution will be noted in the participant's study chart, on study documents, or in the clinic note, and will be entered into the UVA Cancer Center database.

After administration of each 6MHP vaccine, participants will be observed for AEs for at least 20 minutes. Vital signs will be collected at the end of the observation period. Follow-up phone calls will be made per the judgment of the research clinicians with regard to individual participant need. Participants will be instructed on how to reach their provider should they have any questions and/or problems during the study.

In the event of an AE, appropriate action will be taken to ensure adequate care for the participant. If the participant is still on protocol, treatment delay or withdrawal from the protocol will be considered according to the protocol guidelines.

10.7.2 Review of Adverse Events by the Study Team

Individual AEs will be reviewed by the treating physician, principal investigator, and the clinical research coordinator(s) (CRC). Other staff on the research team may also review AEs.

SAEs will be reviewed about once per month by the PI and Sponsor during the UVA Melanoma Team Meeting. This meeting will occur at least 20 times in a calendar year. Those present at the meeting may include the sponsor/overall study PI, sub-investigators, protocol development staff, biostatisticians, research nurses, research coordinators, laboratory specialists, and laboratory research managers. These meetings also include the review of individual participants to assess whether they are protocol candidates, whether AEs warrant discontinuation, and whether existing protocols should be continued or closed.

10.8 Recording Laboratory Values

The following laboratory values will be recorded in the UVA Cancer Center database, graded using the CTCAE v4.03 (if a grading category exists), and reported as described in section 10.6:

1. Alk Phosphatase
2. ALT (SGPT)
3. ANA
4. AST (SGOT)
5. Bilirubin, total
6. Creatinine
7. Eosinophil #
8. Hepatitis C serology or virus measures
9. beta-HCG
10. Hgb
11. HIV
12. HLA type
13. Potassium
14. RF
15. Urinalysis
16. WBC

Any abnormal laboratory values captured which are not included in the above list, but are considered to be pertinent positive clinical signs/symptoms, and laboratory results obtained as part of routine care of patients will be recorded in the UVA Cancer Center database and reported as described in Section 10.6. If there is any doubt on the part of study personnel concerning what constitutes a pertinent positive finding, the PI and sponsor will be consulted.

10.9 Dose-limiting Toxicities

The study will be monitored continuously for treatment-related adverse events.

A DLT is defined as any **unexpected** adverse event that is possibly, probably or definitely related to treatment and meets the following criteria:

- ≥ Grade 3, with the exception of grade 3 injection site reaction with ulceration ≤ 2 cm
- ≥ Grade 1 ocular adverse events as defined below
- ≥ Grade 2 allergic/autoimmune reactions as defined below

Ocular – Any AE coded under one of the following CTCAE v4.03 Adverse Event Terms should be reported under the Ocular adverse event classification:

1) A single treatment-related experience of the following adverse events will be classified as a DLT:

   - Eye Disorders: Night blindness (nyctalopia)
   - Eye Disorders: Papilledema
   - Eye Disordersl: Retinopathy

   Participants will be referred for an ophthalmologic exam if any of these ocular adverse events occur.

2) A prolonged treatment-related experience (e.g., lasting > 5 days) of the following non-severe adverse events will be classified as a DLT:

   - Eye Disorders: Blurred vision
   - Eye Disorders: Flashing lights
   - Eye Disorders: Floaters

   Participants will be referred for an ophthalmologic exam if any of these ocular adverse events occur.

Allergic/Autoimmune – Only AEs coded as Immune System Disorder: Allergic reaction, autoimmune disorder, or anaphylaxis should be reported under the Allergic/Autoimmune adverse event classification. Other AEs coded under Immune System Disorder should be reported under Non-hematologic/Non-metabolic adverse event classification.

10.10 Management of Toxicity

The study will be monitored continuously for treatment-related adverse events. Expected treatment-related toxicities of 6MHP combined with IFA and/or polyICLC, with

or without mCy, will be managed in accord with section 7.5.1, which allows for dose delays, but not dose reductions.

10.11 Data Collection

10.11.1 Endpoint Data

- Endpoint data will be collected using HITC IML data forms, participant-specific binders, and the HITC laboratory database.
- The HITC laboratory database, which has password-restricted access, is stored on the UVA Health System Computing Services secured server.

10.12 Monitoring Plan

10.12.1 The University of Virginia Cancer Center Data and Safety Monitoring Committee (CC DSMC) will provide oversight of the conduct of this study. The CC DSMC will report to the UVA Protocol Review Committee (PRC).

10.12.2 The UVA CC DSMC will review the following:

- All adverse events
- Audit results
- Application of study designed stopping/decision rules
- Whether the study accrual pattern warrants continuation/action
- Protocol violations

10.12.3 The UVA CC DSMC will meet every month for aggregate review of data. Tracking reports of the meetings are available to the PI for review. Issues of immediate concern by the DSMC are brought to the attention of the sponsor (and if appropriate to the PRC and IRB) and a formal response from the sponsor is requested. Per the UVA Cancer Center NIH approved institutional plan, this study will be audited approximately every 6 months. The audit may include direct access to source data/documents.

11.0 STUDY CONDUCT AND ETHICAL CONSIDERATIONS

This study will be conducted in accordance with ICH Good Clinical Practice (GCP) Guidelines and in accord with the ethical principles that originated in the Declaration of Helsinki. In addition, all local laws and regulations will apply. The PI will ensure that staff are trained and carry out the study in accord with the protocol specifications.

11.1 UVA Institutional Review Board for Health Sciences Research

The UVA Institutional Review Board for Health Sciences Research (UVA IRB-HSR) will approve all aspects of this study, including the clinical trial protocol, informed consent documents, and patient materials. Modifications to the protocol or consent form will be reviewed and approved by the UVA IRB-HSR prior to implementation, except when necessary to eliminate apparent immediate hazards to the study participants. The study will undergo continuing IRB review based on the level of risk as assessed by the IRB. This review will take place no less than annually. Reporting to the UVA IRB-HSR will occur as specified in Section 9.6.

11.2 Consent Forms and the Consenting Process

Consent forms will be written in accord with 21 CFR 50 and will be reviewed and approved by the UVA IRB-HSR prior to use. Participants will be given a consent form to review and a member of the study team will be available to answer any questions. Informed consent will be obtained from each participant prior to conducting any study-specific procedures or administering study drug.

11.3 Maintenance of Study Documents

Signed consent forms and other research records will be retained in a confidential manner. Study records will be kept for at least 6 years after completion of the study.

12.0 APPENDICES

Appendix 1: Study Calendar

Appendix 2: AJCC Staging System

Appendix 3: ECOG Performance Status

Appendix 4: New York Heart Association Disease Classification

Appendix 5: RECIST 1.1 Criteria

Appendix 6: Vaccine Lot Release and Stability Testing

Appendix 7: Summary of Changes

Appendix 1: Study Calendar

Studies & Tests	Pre	Active Treatment									Follow-up			
	Day	-6	1	8	15	22	36	50[h]	57	78	85	127	183	225
	Week	-1	0	1	2	3	5	7[h]	8	11	12	18	26	32
Informed consent	X[a]													
Pathology review	X[a]													
CBC with differential	X[b,e]	X[f]	X		X	X	X	X	X		X			
Comprehensive chemistry	X[b]	X[f]	X		X	X	X	X	X		X			
HGBA1C	X[b]													
Urinalysis	X[b]					X			X					
β-HCG	X[c]													
HIV / Hepatitis C	X[d]													
CT chest/abdomen/pelvis or PET-CT	X[b]													
CXR, or other imaging as indicated.											X		X	
Head MRI / CT	X[b]													
History & physical	X[b]	X[f]	X	X	X	X	X		X	X	X	X	X	X
Medication review	X[b]	X	X	X	X	X	X		X	X	X	X	X	X
Toxicity assessment (or baseline)		X	X	X	X	X	X		X	X	X	X	X	X
Designation of potential vaccination sites	X[b]													
Assessment of skin and nodal basins for evidence of disease	X[b]													
Assessment of skin for vitiligo		X				X					X	X	X	X
Assessment of hair and eye color		X				X					X	X	X	X
Visual acuity exam/ color vision		X												
120cc green top tubes		X[g]												
80cc green top tubes			X	X	X	X	X		X	X	X	X	X	X
20cc red top tubes		X	X	X	X	X	X		X	X	X	X	X	X
Anti-nuclear antibody / Rf factor		X							X					
Vaccination			X	X	X		X		X	X				
Skin biopsy at vaccine site				X[i]		X[i]								
Biopsy of sentinel immunized node						X[i]								
Tumor Biopsy[k]		X[j,l]				X[j]								
Participant diary reviewed and/or distributed		X	X	X	X	X	X		X	X	X	X		
Oral cyclophosphamide 50 mg daily x 7 days (**Arms B and D only**)		X		X		X	X	X						

[a] Any point prior to registration
[b] Pre-study within 6 weeks of registration
[c] Within 2 weeks of registration (for childbearing women)
[d] Within 6 months of registration
[e] To include fasting glucose
[f] History & physical, comprehensive chemistry, and CBC with differential scheduled for Day -6 are not required if prestudy assessments were within 10 calendar days of day -6.
[g] Blood for HLA typing is included in the research bloods.
[h] The day 50 study visit will only be required for participants on arms B & D.
[i] Part 1 only.
[j] Part 2 only.
[k] Optional biopsies may occur at the time of recurrence or later as clinically indicated for subjects enrolled in either Part 1 or Part 2 of the study.
[l] Optimally, biopsies will occur within 3 weeks of starting study treatment; however, archival tissue may be used in the pre-study analyses.

Appendix 2: AJCC Staging System

Melanoma TNM Classification

T Classification	Thickness	Ulceration Status
T1	≤ 1.0 mm	a: without ulceration or mitoses
		b: with ulceration or mitoses ≥ 1
T2	1.01 – 2.0 mm	a: without ulceration
		b: with ulceration
T3	2.01 – 4.0 mm	a: without ulceration
		b: with ulceration
T4	> 4.0 mm	a: without ulceration
		b: with ulceration

N Classification	# of Metastatic Nodes	Nodal Metastatic Mass
N1	1 node	a: micrometastasis*
		b: macrometastasis†
N2	2 – 3 nodes	a: micrometastasis*
		b: macrometastasis†
		c: in transit met(s)/satellite(s) without metastatic nodes
N3	4 or more metastatic nodes, or matted nodes, or in transit met(s)/satellites(s) with metastatic node(s)	

M Classification	Site	Serum Lactate Dehydrogenase
M1a	Distant skin, subcutaneous or nodal mets	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastatsis	Elevated

* Micrometastases are diagnosed after sentinel or elective lymphadenectomy.

† Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

Stage Groupings for Cutaneous Melanoma

	Clinical Staging			Pathologic Staging		
	T	**N**	**M**	**T**	**N**	**M**
0	Tis	N0	M0	Tis	N0	M0
IA	T1a	N0	M0	T1a	N0	M0
IB	T1b	N0	M0	T1b	N0	M0
	T2a	N0	M0	T2a	N0	M0
IIA	T2b	N0	M0	T2b	N0	M0
	T3a	N0	M0	T3a	N0	M0
IIB	T3b	N0	M0	T3b	N0	M0
	T4a	N0	M0	T4a	N0	M0
IIC	T4b	N0	M0	T4b	N0	M0
III‡	Any T	N1-3	M0			
IIIA				T1-4a	N1a	M0
				T1-4a	N2a	M0
IIIB				T1-4b	N1a	M0
				T1-4b	N2a	M0
				T1-4a	N1b	M0
				T1-4a	N2b	M0
				T1-4a/b	N2c	M0
IIIC				T1-4b	N1b	M0
				T1-4b	N2b	M0
				Any T	N3	M0
IV	Any T	Any N	Any M1	Any T	Any N	Any M1

* Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

† Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial or complete lymphadenectomy. Pathology stage 0 or stage 1A patients are the exception; they do not require pathologic evaluation of their lymph nodes.

‡ There are no stage III subgroups for clinical staging.

Staging for Mucosal Melanomas
This system is based on the staging of cutaneous melanomas.

Stage IIB: Clinically localized primary melanoma > 4mm thick
Stage III: Lymph node metastases
Stage IV: Distant metastases

Appendix 3: ECOG Performance Status

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

* As published in Am. J. Clin. Oncol.:
Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.

Appendix 4: New York Heart Association Disease Classification

Functional Capacity	Objective Assessment
Class I. Patients with cardiac disease but without resulting limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain.	No objective evidence of cardiovascular disease.
Class II. Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.	Objective evidence of minimal cardiovascular disease
Class III. Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes fatigue, palpitation, dyspnea, or anginal pain.	Objective evidence of moderately severe cardiovascular disease.
Class IV. Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of heart failure or the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.	Objective evidence of severe cardiovascular disease.

* The Criteria Committee of the New York Heart Association. Nomenclature and Criteria for Diagnosis of Diseases of the Heart and Great Vessels. 9th ed. Boston, Mass: Little, Brown & Co; 1994:253-256

Appendix 5: RECIST 1.1 Criteria

Please refer to the following publication for evaluation of clinical response by RECIST 1.1.

E.A. Eisenhauer et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). European Journal of Cancer, 2009, 45: 228-247.

PMID: 19097774

Appendix 6: Vaccine Lot Release and Stability Testing

A. Preparation of the synthetic melanoma and tetanus peptides
All peptides were synthesized under GMP conditions by Multiple Peptide Systems (San Diego, CA).

Peptide preparation and vialing were performed under GMP conditions by Clinalfa (Merck Biosciences AG, Laufelfingen, Switzerland). Documentation relating to the procedures used to prepare and vial the peptides were included in the Chemistry and Manufacturing Section of prior IND application applications (10825 and 12191).

B. Quality Assurance Testing

Prepared peptides were subjected to the following tests:

1. Identity. Identity was confirmed by structural studies. The individual peptides were tested for identity by mass spectrometry (to define molecular mass and amino acid sequence) and HPLC (to confirm purity) in a GMP laboratory (Polypeptide Group).

2. Purity. Purity was assessed before and after vialing the peptide mixtures. Before vialing the peptide mixtures, each synthetic peptide was evaluated for the presence of a single dominant species by high pressure liquid chromatography (HPLC) in a GMP laboratory (Polypeptide Group). Purity of each peptide component exceeded 90%. Variants of the original peptide may have included incomplete products of synthesis, minor degradation products due to oxidation of methionine residues, and dimerization of cysteine-containing peptides. After vialing the peptide mixture, purity was reconfirmed by HPLC in a GMP laboratory (Clinalfa).

3. Trifluoroacetic acid (TFA). The amount of total fluorine in each peptide preparation was less than 0.5% or 5000 ppm as determined by Multiple Peptide Systems.

4. Potency. Peptides are synthesized under GMP conditions and the net peptide content calculated for each. The amounts of each peptide (mcg quantities) added to the vaccine vials are calculated based on the net peptide content of the original stock of lyophilized peptides.

5. Pyrogenicity. Pyrogenicity testing was conducted by Clinalfa in accordance with USP guidelines.

6. General Safety. General safety testing was conducted by Clinalfa in accordance with USP guidelines.

7. Sterility. Sterility testing was conducted by Clinalfa in accordance with USP guidelines.
8. Stability. The peptide preparations were assayed for stability at months 3, 6, 12, 24, and 36 and were shown to be stable. The peptides will continue to be assessed yearly for stability while subjects are on active treatment. The following analyses will be performed to confirm stability.
   a. HPLC: HPLC will be performed to confirm purity. An optical comparison to previous HPLC data will be performed. Ideally, the purity of each peptide component will exceed 90% (94%-98%). Variants of the original peptide may include incomplete products of synthesis, minor degradation products due to oxidation of methionine residues, and dimerization of cysteine-containing peptides. Such minor variants will be tolerated as long as the peptide represents at least 75% of the intended peptide species. Because measures of peptide quantity are subject to variability, a peptide lot will be rejected only if two sequential measures fail to meet the criterion stated above

b. Sterility. One vial of peptide will be submitted to the Clinical Microbiology Laboratory at the University of Virginia or Microbiology Research Associates, Inc. (Acton, MA) for sterility testing.

Appendix 7: Summary of Changes

12-04-14	1) Table of Contents: Updated 2) Section 1.3.2: editorial correction 3) Section 1.8.2: editorial correction 4) Section 6.2: The language regarding the supply of mCy has been reworded to eliminate the requirement to receive mCy by prescription. 5) Section 9.5: editorial correction 6) Section 10.4.3: Separated out the risks and side effects for the vaccine site biopsy and the SIN biopsy. Added numbness as a possible side effects for the SIN biopsy.
02-18-15	1) Updated IRB # in header and removed PRC# 2) Section 1.1: changed "three" to "two" in reference to the number of local adjuvants. 3) Added investigator's statement. 4) Formatting adjusted throughout study document 5) Table of Contents-updated 6) Section 4.6: reference to Appendix 6 replaces "vaccine manual" 7) Section 4.8: "mixing sheets" replace "manual" 8) Section 7.2.1: "mixing sheets" replaces "manual" 9) Section 7.2.4: "vaccine mixing sheets" replace "laboratory manual" 10) Section 10.6.4: AE reporting requirements for reporting to the LICR were added. 11) Appendix 6: Lot release and stability testing of the vaccine were added. 12) Summary of Changes is now Appendix 7.
03-17-15	1) Updated investigator list 2) Updated investigator's statement 3) Formatting and editing changes made throughout the study document 4) Updated Table of contents 5) Section 7.2.1: Additional text added to clarify the contents of the vaccine. 6) Section 7.3.1: Added text to clarify that cyclophosphamide may be taken before or after a vaccine, on days when both vaccine and cyclophosphamide are administered. 7) Section 8.4: Added reflexive testing for HCV and HIV screening, if needed to be consistent with screening criteria. Also added ANA and Rf testing in the text to be consistent with the X-page (Appendix 1) 8) Section 10.4.3 and Table 14: expected toxicities for cyclophosphamide were updated. 9) Section 10.10: removed text related to Cy dose reductions. Dose reductions in Cy will not be permitted per protocol. 10) Appendix 1: added urinalysis testing at days 22 and 57. 11) Appendix 1: added CBC and comp chem at days 15, 36, and 50.
04-17-15	1) Section 6.2 and Section 7.3.1: added capsules as an option for cyclophosphamide 2) Section 7.2.2: Volume of vaccine to be administered has been changed from 1 ml to 2 ml. 3) Editorial changes made throughout document. 4) Updated investigator's list. 5) Updated Table of Contents
07-16-15	1) Updated investigator list: removed Geoffrey Weiss, Christopher Blackwell, and Connor Poland. 2) Updated Table of Contents. 3) Section 3.1.1: Clarified in inclusion criteria that subjects should be clinically free of disease, as originally intended and stated in the protocol synopsis indication. Included the statement indicating that patients with small radiologic or clinical findings of an indeterminate nature may be eligible. 4) Section 3.2.12: Removed exclusion criteria regarding other cancer diagnosis. . 5) Section 3.3: Removed statement indicating randomization would be discussed "no sooner than 7 days prior to the start of treatment" and indicated that subjects should receive study treatment within 3 weeks of registration.

	6) Section 10.4: Clarified risk of infection from vaccine site biopsy is less than 2%. 7) Appendix 1: Updated the study calendar to indicate day 50 visit would only be Yes.required for subjects on arms B and D.
10-26-16	1) Updated investigator list 2) Updated table of contents 3) Updated Investigator's Agreement 4) Protocol Synopsis— • Indication: Added details to describe Part 2 of the study including the addition of a new patient population-Stage IIIB-IV melanoma with one or more tumor deposits accessible for biopsy and/or excision. • Objectives and Endpoints: Added objective 3 to address Part 2 of the study. • Regimen: Specified the study regimen for Part 2. The study regimen includes the 6MHP vaccine with the optimal adjuvant combination identified from Part 1 of the study. • Schema: Added Figure 1B (Part 2). • Biopsies: Clarified that vaccine site biopsies and SIN biopsies will be performed in Part 1 of the study and that tumor biopsies in Part 1 are optional at the time of recurrence or later as clinically indicated. Added a section to describe the tumor biopsies that are required in Part 2. Additional, optional tumor biopsies may also be completed in Part 2 at the time of progression or later as clinically indicated. • Population: Added a summary of the patient population that will be accrued to Part 2. • Increased the approximate accrual goal from 50 to 65 patients. 5) Section 1.9: Added text to the summary section to clarify the purpose of the addition of Part 2 of the study. Added text to specify that immune responses will be evaluated at the tumor site. 6) Section 2.1: Revised to include a study objective for Part 2. 7) Section 3.1.1: Revised to include inclusion criteria to describe the patient population that will be accrued to Part 2 of the study. 8) Section 3.1.4: Revised to specify that the most recent surgical resections or gamma-knife therapy for malignant melanoma must have been completed ≥ 1 week and for Part 1 ≤ 6 months prior to registration. 9) Section 3.1.5: removed "all participants must have" 10) Section 3.1.10: Revised entry criteria to specify that participants in part 1 must have at least 2 intact (undissected) axillary and/or inguinal lymph node basins and that participants in part 2 need to have at least 1 intact (undissected) axillary and/or inguinal lymph node basin. 11) Section 3.2.10: corrected to specify that participants with uncontrolled diabetes are defined as having a HgbA1c > 7.5% 12) Section 3.3: Revised to distinguish the treatment allocation procedures for Parts 1 and 2. Moved text related to the timing of the first study treatment (within 3 weeks of registration) to the first paragraph of this section. 13) Table 5: Added a treatment window for the pre-study biopsies. 14) Section 7.6: Revised protocol to clarify the conditions whereby biopsies are not a basis for discontinuing therapy. 15) Section 7.11: Revised to clarify that vaccine site biopsies and SIN biopsies will be performed in Part 1 of the study and that tumor biopsies in Part 1 are optional at the time of recurrence or later as clinically indicated. Added a section to describe the tumor biopsies that are required in Part 2. Additional, optional tumor biopsies may also be completed in Part 2 at the time of progression or later as clinically indicated. 16) Section 8.9: Added this section to describe the size requirements, sampling, and procedures for the tumor biopsies. 17) Sections 9.1 and 9.2: Revised the statistical overview and study design sections to provide a description of the study objective for Part 2. 18) Section 9.3: Revised to specify that the accrual allocation is used for the determination of the recommended optimal combination in Part 1 only.

	19) Section 9.5.1: Revised to specify that the optimal combination in this section is for Part 1. 20) Section 9.5.2: Added this section to describe the sample size calculation for Part 2. 21) Section 9.6: Revised to specify that the stopping rules in this section pertain to Part 1 only. 22) Section 9.7.2: Revised to distinguish and describe the efficacy endpoints for Parts1 and 2. 23) Section 9.7.3: Revised to include study conclusions for Part 2. 24) Appendix 1: Study Calendar-revised to include tumor biopsies. Revised to specify that vaccine site biopsies and SIN biopsies are only required for Part 1. 25) References: Reference #82 was added..
03-13-17	1) Section 10.9: corrected the DLT definition to specify that grade 3 injection site reactions with ulceration ≤ 2 cm are not DLTs. 2) Updated TOC

Reference List

1. Topalian, S. L., M. I. Gonzales, M. Parkhurst, Y. F. Li, S. Southwood, A. Sette, S. A. Rosenberg, and P. F. Robbins. 1996. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. *J. Exp. Med.* 183: 1965-1971.

2. Kobayashi, H., T. Kokubo, K. Sato, S. Kimura, K. Asano, H. Takahashi, H. Iizuka, N. Miyokawa, and M. Katagiri. 1998. CD4+ T cells from peripheral blood of a melanoma patient recognize peptides derived from nonmutated tyrosinase. *Cancer Res.* 58: 296-301.

3. Zarour, H. M., J. M. Kirkwood, L. S. Kierstead, W. Herr, V. Brusic, C. L. J. Slingluff, J. Sidney, A. Sette, and W. J. Storkus. 2000. Melan-A/MART-1(51-73) represents an immunogenic HLA-DR4-restricted epitope recognized by melanoma-reactive CD4(+) T cells. *Proc. Natl. Acad. Sci. USA* 97: 400-405.

4. Manici, S., T. Sturniolo, M. A. Imro, J. Hammer, F. Sinigaglia, C. Noppen, G. Spagnoli, B. Mazzi, M. Bellone, P. Dellabona, and M. P. Protti. 1999. Melanoma cells present a MAGE-3 epitope to CD4(+) cytotoxic T cells in association with histocompatibility leukocyte antigen DR11. *J. Exp. Med.* 189: 871-876.

5. Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon, and P. van der Bruggen. 1999. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes [see comments]. *J. Exp. Med.* 189: 767-778.

6. Halder, T., G. Pawelec, A. F. Kirkin, J. Zeuthen, H. E. Meyer, L. Kun, and H. Kalbacher. 1997. Isolation of novel HLA-DR restricted potential tumor-associated antigens from the melanoma cell line FM3. *Cancer Res.* 57: 3238-3244.

7. Li, K., M. Adibzadeh, T. Halder, H. Kalbacher, S. Heinzel, C. Muller, J. Zeuthen, and G. Pawelec. 1998. Tumour-specific MHC-class-II-restricted responses after in vitro sensitization to synthetic peptides corresponding to gp100 and Annexin II eluted from melanoma cells. *Cancer Immunol. Immunother.* 47: 32-38.

8. Brahmer, J. R., S. S. Tykodi, L. Q. Chow, W. J. Hwu, S. L. Topalian, P. Hwu, C. G. Drake, L. H. Camacho, J. Kauh, K. Odunsi, H. C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T. M. Salay, S. Alaparthy, J. F. Grosso, A. J. Korman, S. M. Parker, S. Agrawal, S. M. Goldberg, D. M. Pardoll, A. Gupta, and J. M. Wigginton. 2012. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. *N. Engl. J Med.*

9. Topalian, S. L., F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake, D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton, and M. Sznol. 2012. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *N. Engl. J Med.*

10. Rosenberg, S. A., J. C. Yang, R. M. Sherry, U. S. Kammula, M. S. Hughes, G. Q. Phan, D. E. Citrin, N. P. Restifo, P. F. Robbins, J. R. Wunderlich, K. E. Morton, C. M. Laurencot, S. M.

Steinberg, D. E. White, and M. E. Dudley. 2011. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin. Cancer Res.* 17: 4550-4557.

11. Higano, C. S., P. F. Schellhammmer, E. J. Small, P. A. Burch, Nemunaitis J, L. Yuh, N. Provost, and M. W. Frohlich. 2009. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer* 115: 3670-3679.

12. Schwartzentruber, D. J., D. H. Lawson, J. M. Richards, R. M. Conry, D. M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, K. L. Kendra, R. L. White, R. Gonzalez, T. M. Kuzel, B. Curti, P. D. Leming, E. D. Whitman, J. Balkissoon, D. S. Reintgen, H. Kaufman, F. M. Marincola, M. J. Merino, S. A. Rosenberg, P. Choyke, D. Vena, and P. Hwu. 2011. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N. Engl. J Med.* 364: 2119-2127.

13. Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410: 1107-1111.

14. Kayaga, J., B. E. Souberbielle, N. Sheikh, W. J. Morrow, T. Scott-Taylor, R. Vile, H. Chong, and A. G. Dalgleish. 1999. Anti-tumour activity against B16-F10 melanoma with a GM-CSF secreting allogeneic tumour cell vaccine. *Gene Therapy* 6: 1475-1481.

15. Hunder, N. N., H. Wallen, J. Cao, D. W. Hendricks, J. Z. Reilly, R. Rodmyre, A. Jungbluth, S. Gnjatic, J. A. Thompson, and C. Yee. 2008. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N. Engl. J Med.* %19;358: 2698-2703.

16. Kahn, M., H. Sugawara, P. McGowan, K. Okuno, S. Nagoya, K. E. Hellstrom, and P. Greenberg. 1991. CD4+ T cell clones specific for the human p97 melanoma-associated antigen can eradicate pulmonary metastases from a murine tumor expressing the p97 antigen. *Journal of Immunology* 146: 3235-3241.

17. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of CD4+ T-cells in the antitumor immune response. *J. Exp. Med.* 188: 2357-2368.

18. Matsui, S., J. D. Ahlers, A. O. Vortmeyer, M. Terabe, T. Tsukui, D. P. Carbone, L. A. Liotta, and J. A. Berzofsky. 1999. A model for CD8+ CTL tumor immunosurveillance and regulation of tumor escape by CD4 T cells through an effect on quality of CTL. *Journal of Immunology* 163: 184-193.

19. Woodson, E. M., K. A. Chianese-Bullock, C. J. Wiernasz, E. A. Bissonette, W. W. Grosh, P. Y. Neese, P. K. Merrill, D. L. Barnd, G. R. Petroni, and C. L. Slingluff, Jr. 2004. Assessment of the toxicities of systemic low-dose interleukin-2 administered in conjunction with a melanoma peptide vaccine. *Journal of Immunotherapy* 27: 380-388.

20. Slingluff, C. L., Jr., S. Lee, F. Zhao, K. A. Chianese-Bullock, W. C. Olson, L. H. Butterfield, T. L. Whiteside, P. D. Leming, and J. M. Kirkwood. 2013. A randomized phase II trial of multi-epitope vaccination with melanoma peptides for cytotoxic T-cells and helper T-cells for patients with metastatic melanoma (E1602). *Clin. Cancer Res.* 19: 4228-4238.

21. Slingluff, C. L., Jr., G. R. Petroni, K. A. Chianese-Bullock, M. E. Smolkin, M. I. Ross, N. B. Haas, M. von Mehren, and W. W. Grosh. 2011. A randomized multicenter trial of the effects of

melanoma-associated helper peptides and cyclophosphamide on the immunogenicity of a multipeptide melanoma vaccine. *J. Clin. Oncol.* 29: 2924-2932.

22. Slingluff, C. L., Jr., G. R. Petroni, W. Olson, A. R. Czarkowski, W. W. Grosh, M. Smolkin, K. A. Chianese-Bullock, Neese P.Y., D. H. Deacon, C. J. Nail, P. Merrill, R. Fink, and P. K. Rehm. 2008. Helper T cell responses and clinical activity of a melanoma vaccine with multiple peptides from MAGE and melanocytic differentiation antigens. *J. Clin. Oncol.* 26: 4973-4980.

23. Hu, Y., M. E. Smolkin, E. J. White, G. R. Petroni, Neese P.Y., and C. L. Slingluff, Jr. 2014. Inflammatory Adverse Events are Associated with Disease-Free Survival after Vaccine Therapy among Patients with Melanoma. *Annals of Surgical Oncology* in press.

24. Hu, Y., G. R. Petroni, W. C. Olson, A. Czarkowski, M. E. Smolkin, W. W. Grosh, K. A. Chianese-Bullock, and C. L. Slingluff, Jr. 2014. Immunologic hierarchy, class II MHC promiscuity, and epitope spreading of a melanoma helper peptide vaccine. *Cancer Immunol. Immunother.*

25. Touloukian, C. E., W. W. Leitner, S. L. Topalian, Y. F. Li, P. F. Robbins, S. A. Rosenberg, and N. P. Restifo. 2000. Identification of a MHC class II-restricted human gp100 epitope using DR4-IE transgenic mice. *Journal of Immunology* 164: 3535-3542.

26. Herr, W., J. Schneider, A. W. Lohse, K. H. Meyer zum Buschenfelde, and T. Wolfel. 1996. Detection and quantification of blood-derived CD8+ T lymphocytes secreting tumor necrosis factor alpha in response to HLA-A2.1-binding melanoma and viral peptide antigens. *J. Immunol. Methods* 191: 131-42.

27. Scheibenbogen, C., K. H. Lee, S. Mayer, S. Stevanovic, U. Moebius, W. Herr, H. G. Rammensee, and U. Keilholz. 1997. A sensitive ELISPOT assay for detection of CD8+ T lymphocytes specific for HLA class I-binding peptide epitopes derived from influenza proteins in the blood of healthy donors and melanoma patients. *Clin. Cancer Res.* 3: 221-226.

28. Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael. 1997. Rapid effector function in CD8+ memory T cells. *J. Exp. Med.* 186: 859-865.

29. Tanchot, C., S. Guillaume, J. Delon, C. Bourgeois, A. Franzke, A. Sarukhan, A. Trautmann, and B. Rocha. 1998. Modifications of CD8+ T cell function during in vivo memory or tolerance induction. *Immunity* 8: 581-590.

30. Reynolds, S. R., R. Oratz, R. L. Shapiro, P. Hao, Z. Yun, M. Fotino, S. Vukmanovic, and J. C. Bystryn. 1997. Stimulation of CD8+ T cell responses to MAGE-3 and Melan A/MART-1 by immunization to a polyvalent melanoma vaccine. *Int. J. Cancer* 72: 972-976.

31. Slingluff, C. L., Jr., G. Yamshchikov, P. Neese, H. Galavotti, S. Eastham, V. H. Engelhard, D. Kittlesen, D. Deacon, S. Hibbitts, W. W. Grosh, G. Petroni, R. Cohen, C. Wiernasz, J. W. Patterson, B. P. Conway, and W. G. Ross. 2001. Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. *Clin. Cancer Res.* 7: 3012-3024.

32. Scalzo, A. A., S. L. Elliott, J. Cox, J. Gardner, D. J. Moss, and A. Suhrbier. 1995. Induction of protective cytotoxic T cells to murine cytomegalovirus by using a nonapeptide and a human-compatible adjuvant (Montanide ISA 720). *Journal of Virology* 69: 1306-1309.

33. Fernandez, I. M., A. Snijders, B. J. Benaissa-Trouw, M. Harmsen, H. Snippe, and C. A. Kraaijeveld. 1993. Influence of epitope polarity and adjuvants on the immunogenicity and

efficacy of a synthetic peptide vaccine against Semliki Forest virus. *Journal of Virology* 67: 5843-8.

34. Slingluff, C. L., Jr., G. R. Petroni, M. E. Smolkin, K. A. Chianese-Bullock, K. Smith, C. Murphy, N. Galeassi, P. Y. Neese, W. W. Grosh, C. J. Nail, M. I. Ross, N. B. Haas, M. E. Boisvert, and J. M. Kirkwood. 2010. Immunogenicity for $CD8^+$ and $CD4^+$ T cells of two formulations of an incomplete Freund's adjuvant for multipeptide melanoma vaccines. *Journal of Immunotherapy* 33: 630-638.

35. Slingluff, C. L., Jr., G. R. Petroni, W. C. Olson, M. E. Smolkin, M. I. Ross, N. B. Haas, W. W. Grosh, M. E. Boisvert, J. M. Kirkwood, and K. A. Chianese-Bullock. 2009. Effect of GM-CSF on circulating $CD8^+$ and $CD4^+$ T cell responses to a multipeptide melanoma vaccine: Outcome of a multicenter randomized trial. *Clin. Cancer Res.* 15: 7036-7044.

36. Slingluff, C. L., Jr., G. R. Petroni, K. A. Chianese-Bullock, M. E. Smolkin, S. Hibbitts, C. Murphy, N. Johansen, W. W. Grosh, G. V. Yamshchikov, Neese P.Y., J. W. Patterson, R. Fink, and P. K. Rehm. 2007. Immunologic and clinical outcomes of a randomized phase II trial of two multipeptide vaccines for melanoma in the adjuvant setting. *Clin. Cancer Res.* 13: 6386-6395.

37. Hailemichael, Y., Z. Dai, N. Jaffarzad, Y. Ye, M. A. Medina, X. F. Huang, S. M. Dorta-Estremera, N. R. Greeley, G. Nitti, W. Peng, C. Liu, Y. Lou, Z. Wang, W. Ma, B. Rabinovich, K. S. Schluns, R. E. Davis, P. Hwu, and W. W. Overwijk. 2013. Persistent antigen at vaccination sites induces tumor-specific CD8(+) T cell sequestration, dysfunction and deletion. *Nat. Med.* 19: 465-472.

38. Salerno, E. P., S. M. Shea, W. C. Olson, G. R. Petroni, M. E. Smolkin, C. McSkimming, K. A. Chianese-Bullock, and C. L. Slingluff, Jr. 2013. Activation, dysfunction and retention of T cells in vaccine sites after injection of incomplete Freund's adjuvant, with or without peptide. *Cancer Immunol. Immunother.* 62: 1149-1159.

39. Slingluff, C. L. J., S. J. Lee, K. A. Chianese-Bullock, W. C. Olson, L. H. Butterfield, P. D. Leming, and J. M. Kirkwood. 2010. First report of a randomized phase II trial of multi-epitope vaccination with melanoma peptides for cytotoxic T cells and helper T cells in patients with metastatic melanoma: An Eastern Cooperative Oncology Group Study (E1602)., 28 ed. Abstract 8508.

40. Faries, M. B., E. C. Hsueh, X. Ye, M. Hoban, and D. L. Morton. 2009. Effect of granulocyte/macrophage colony-stimulating factor on vaccination with an allogeneic whole-cell melanoma vaccine. *Clin Cancer Res.* 15: 7029-7035.

41. Hodi, F. S., S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbe, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos, and W. J. Urba. 2010. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. *N. Engl. J Med.* 363: 711-723.

42. Korn, E. L., P. Y. Liu, S. J. Lee, J. A. Chapman, D. Niedzwiecki, V. J. Suman, J. Moon, V. K. Sondak, M. B. Atkins, E. A. Eisenhauer, W. Parulekar, S. N. Markovic, S. Saxman, and J. M. Kirkwood. 2009. Meta-analysis of phase II cooperative group trials in metastatic stage IV melanoma to determine progression-free and overall survival benchmarks for future phase II trials. *J Clin Oncol* 26: 527-534.

43. Kawakami, Y., and S. A. Rosenberg. 1997. Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy. [Review] [103 refs]. *International Reviews of Immunology* 14: 173-192.

44. Lore, K., M. R. Betts, J. M. Brenchley, J. Kuruppu, S. Khojasteh, S. Perfetto, M. Roederer, R. A. Seder, and R. A. Koup. 2003. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J Immunol.* 171: 4320-4328.

45. Perrot, I., F. Deauvieau, C. Massacrier, N. Hughes, P. Garrone, I. Durand, O. Demaria, N. Viaud, L. Gauthier, M. Blery, N. Bonnefoy-Berard, Y. Morel, J. Tschopp, L. Alexopoulou, G. Trinchieri, C. Paturel, and C. Caux. 2010. TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA. *J Immunol.* 185: 2080-2088.

46. Sabbatini, P., T. Tsuji, L. Ferran, E. Ritter, C. Sedrak, K. Tuballes, A. A. Jungbluth, G. Ritter, C. Aghajanian, K. Bell-McGuinn, M. L. Hensley, J. Konner, W. Tew, D. R. Spriggs, E. W. Hoffman, R. Venhaus, L. Pan, A. M. Salazar, C. M. Diefenbach, L. J. Old, and S. Gnjatic. 2012. Phase I Trial of Overlapping Long Peptides from a Tumor Self-Antigen and Poly-ICLC Shows Rapid Induction of Integrated Immune Response in Ovarian Cancer Patients. *Clin. Cancer Res.* 18: 6497-6508.

47. Sabbatini, P., T. Tsuji, L. Ferran, E. Ritter, C. Sedrak, K. Tuballes, A. A. Jungbluth, G. Ritter, C. Aghajanian, K. Bell-McGuinn, M. L. Hensley, J. Konner, W. Tew, D. R. Spriggs, E. W. Hoffman, R. Venhaus, L. Pan, A. M. Salazar, C. M. Diefenbach, L. J. Old, and S. Gnjatic. 2012. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. *Clin. Cancer Res.* 18: 6497-6508.

48. Berd, D., H. C. Maguire, Jr., and M. J. Mastrangelo. 1986. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. *Cancer Res.* 46: 2572-2577.

49. Proietti, E., G. Greco, B. Garrone, S. Baccarini, C. Mauri, M. Venditti, D. Carlei, and F. Belardelli. 1998. Importance of Cyclophosphamide-induced Bystander Effect on T Cells for a Successful Tumor Eradication in Response to Adoptive Immunotherapy in Mice.[Miscellaneous Article]. *J. Clin. Invest.* 101: 429-441.

50. Machiels, J. P., R. T. Reilly, L. A. Emens, A. M. Ercolini, R. Y. Lei, D. Weintraub, F. I. Okoye, and E. M. Jaffee. 2001. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. *Cancer Res.* 61: 3689-3697.

51. Berd, D., H. C. Maguire, Jr., and M. J. Mastrangelo. 1984. Potentiation of human cell-mediated and humoral immunity by low-dose cyclophosphamide. *Cancer Res.* 44: 5439-5443.

52. Sahasrabudhe, D. M., J. B. deKernion, J. E. Pontes, D. M. Ryan, R. W. O'Donnell, D. M. Marquis, G. S. Mudholkar, and C. S. McCune. 1986. Specific immunotherapy with suppressor function inhibition for metastatic renal cell carcinoma. *J. Biol. Response Mod.* 5: 581-594.

53. Matar, P., V. R. Rozados, S. I. Gervasoni, and G. O. Scharovsky. 2002. Th2/Th1 switch induced by a single low dose of cyclophosphamide in a rat metastatic lymphoma model. *Cancer Immunol. Immunother.* 50: 588-596.

54. Matar, P., V. R. Rozados, A. D. Gonzalez, D. G. Dlugovitzky, R. D. Bonfil, and O. G. Scharovsky. 2000. Mechanism of antimetastatic immunopotentiation by low-dose cyclophosphamide. *Eur. J. Cancer* 36: 1060-1066.

55. Schiavoni, G., F. Mattei, P. T. Di, S. M. Santini, L. Bracci, F. Belardelli, and E. Proietti. 2000. Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. *Blood* 95: 2024-2030.

56. Awwad, M., and R. J. North. 1988. Cyclophosphamide (Cy)-facilitated adoptive immunotherapy of a Cy-resistant tumour. Evidence that Cy permits the expression of adoptive T-cell mediated immunity by removing suppressor T cells rather than by reducing tumour burden. *Immunology* 65: 87-92.

57. Berd, D., and M. J. Mastrangelo. 1988. Effect of low dose cyclophosphamide on the immune system of cancer patients: depletion of CD4+, 2H4+ suppressor-inducer T-cells. *Cancer Res.* 48: 1671-1675.

58. Hoon, D. S., L. J. Foshag, A. S. Nizze, R. Bohman, and D. l. Morton. 1990. Suppressor cell activity in a randomized trial of patients receiving active specific immunotherapy with melanoma cell vaccine and low dosages of cyclophosphamide. *Cancer Res.* 50: 5358-5364.

59. North, R. J. 1982. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J. Exp. Med.* 155: 1063-1074.

60. Loeffler, M., J. A. Kruger, and R. A. Reisfeld. 2005. Immunostimulatory effects of low-dose cyclophosphamide are controlled by inducible nitric oxide synthase. *Cancer Res.* 65: 5027-5030.

61. Lutsiak, M. E., R. T. Semnani, P. R. De, S. V. Kashmiri, J. Schlom, and H. Sabzevari. 2005. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood.* 105: 2862-2868.

62. Tanaka, H., H. Matsushima, N. Mizumoto, and A. Takashima. 2009. Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. *Cancer Res.* 69: 6978-6986.

63. Salem, M. L., A. N. Kadima, S. A. El-Naggar, M. P. Rubinstein, Y. Chen, W. E. Gillanders, and D. J. Cole. 2007. Defining the ability of cyclophosphamide preconditioning to enhance the antigen-specific CD8+ T-cell response to peptide vaccination: creation of a beneficial host microenvironment involving type I IFNs and myeloid cells. *J. Immunother.* 30: 40-53.

64. Ercolini, A. M., B. H. Ladle, E. A. Manning, L. W. Pfannenstiel, T. D. Armstrong, J. P. Machiels, J. G. Bieler, L. A. Emens, R. T. Reilly, and E. M. Jaffee. 2005. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J. Exp. Med.* 201: 1591-1602.

65. Liu, J. Y., Y. Wu, X. S. Zhang, J. L. Yang, H. L. Li, Y. Q. Mao, Y. Wang, X. Cheng, Y. Q. Li, J. C. Xia, M. Masucci, and Y. X. Zeng. 2007. Single administration of low dose cyclophosphamide augments the antitumor effect of dendritic cell vaccine. *Cancer Immunol. Immunother.* 56: 1597-1604.

66. Ghiringhelli, F., N. Larmonier, E. Schmitt, A. Parcellier, D. Cathelin, C. Garrido, B. Chauffert, E. Solary, B. Bonnotte, and F. Martin. 2004. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur. J. Immunol.* 34: 336-344.

67. Berd, D., H. C. Maguire, Jr., and M. J. Mastrangelo. 1984. Impairment of concanavalin A-inducible suppressor activity following administration of cyclophosphamide to patients with advanced cancer. *Cancer Res.* 44: 1275-1280.

68. Berd, D., H. C. Maguire, Jr., and M. J. Mastrangelo. 1986. Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. *Cancer Res.* 46: 2572-2577.

69. Sahasrabudhe, D. M., J. B. deKernion, J. E. Pontes, D. M. Ryan, R. W. O'Donnell, D. M. Marquis, G. S. Mudholkar, and C. S. McCune. 1986. Specific immunotherapy with suppressor function inhibition for metastatic renal cell carcinoma. *J. Biol. Response Mod.* 5: 581-594.

70. Emens, L. A., J. M. Asquith, J. M. Leatherman, B. J. Kobrin, S. Petrik, M. Laiko, J. Levi, M. M. Daphtary, B. Biedrzycki, A. C. Wolff, V. Stearns, M. L. Disis, X. Ye, S. Piantadosi, J. H. Fetting, N. E. Davidson, and E. M. Jaffee. 2009. Timed sequential treatment with cyclophosphamide, doxorubicin, and an allogeneic granulocyte-macrophage colony-stimulating factor-secreting breast tumor vaccine: a chemotherapy dose-ranging factorial study of safety and immune activation. *J Clin Oncol.* 27: 5911-5918.

71. Berd, D., H. C. Maguire, Jr., and M. J. Mastrangelo. 1984. Potentiation of human cell-mediated and humoral immunity by low-dose cyclophosphamide. *Cancer Res.* 44: 5439-5443.

72. Audia, S., A. Nicolas, D. Cathelin, N. Larmonier, C. Ferrand, P. Foucher, A. Fanton, E. Bergoin, M. Maynadie, L. Arnould, A. Bateman, B. Lorcerie, E. Solary, B. Chauffert, and B. Bonnotte. 2007. Increase of CD4+ CD25+ regulatory T cells in the peripheral blood of patients with metastatic carcinoma: a Phase I clinical trial using cyclophosphamide and immunotherapy to eliminate CD4+ CD25+ T lymphocytes. *Clin. Exp. Immunol.* 150: 523-530.

73. Peretz, Y., D. Sauve, D. Gagnon, S. A. Khan, G. Levesque, N. Saha, G. Croteau, V. Hebert, K. Savard, B. Y. Diab, J.-F. Poulin, C. Landry, M. Karkada, and M. Mansour. 2014. Immune profiling of patients vaccinated with the survivin targeted therapeutic vaccine DPX-Survivac demonstrates durable polyfunctional CD4+ and CD8+ T cells in ovarian cancer patients ., abstract CT411 ed.

74. Mross, K., and S. Steinbild. 2012. Metronomic anti-cancer therapy - an ongoing treatment option for advanced cancer patients. *Journal of Cancer Therapeutics and Research*.

75. Levy, H., and A. Salazar. 1992. Interferon inducers. In *Interferon: Principles and Medical Applications*. S. Baron, D. Copenhaver, F. Dianzani, and et al., eds. Galveston University, University of Texas Medical Branch, Galveston, TX. 65-76.

76. Lapi, F., A. Kezouh, S. Suissa, and P. Ernst. 2013. The use of inhaled corticosteroids and the risk of adrenal insufficiency. *Eur. Respir. J.* 42: 79-86.

77. Ernst, P., and S. Suissa. 2012. Systemic effects of inhaled corticosteroids. *Curr. Opin. Pulm. Med.* 18: 85-89.

78. Wages, N. A., M. R. Conaway, and J. O'Quigley. 2011. Continual reassessment method for partial ordering. *Biometrics.* 67: 1555-1563.

79. Wages, N. A., M. R. Conaway, and J. O'Quigley. 2011. Dose-finding design for multi-drug combinations. *Clin. Trials.* 8: 380-389.

80. Lee, S. M., and Y. K. Cheung. 2009. Model calibration in the continual reassessment method. *Clin. Trials.* 6: 227-238.

81. O'Quigley, J. 1992. Estimating the probability of toxicity at the recommended dose following a phase I clinical trial in cancer. *Biometrics* 48: 853-862.

82. Mauldin, I.S., Wages, N.A., Stowman, A.M., Wang, E., Smolkin, M.E., Olson, W.C., Deacon, D.H., Smith, K.T., Galeassi, N.V., Chianese-Bullock, K.A., Dengel, L.T., Marincola, F.M., Petroni, G.R., Mullins, D.W., Slingluff, C.L., Jr. Intratumoral interferon-gamma increases chemokine production but fails to increase T cell infiltration of human melanoma metastases. *Cancer Immunol Immunother*. 65: 1189-1199.