

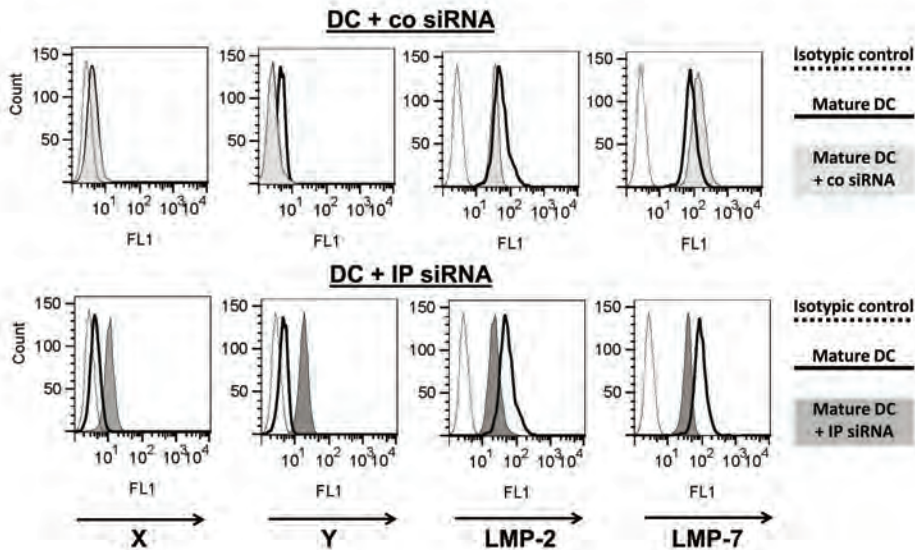
Supplemental Figure Legends

Figure S1. Intracellular Proteasome Subunit Expression Analysis. Cryopreserved monocytes from from a study subject were electroporated with either no siRNAs (Mature DC), control siRNAs (co siRNA) or siRNAs targeting the iP subunits (IP siRNA), differentiated into immature DCs in culture and then matured. Intracellular expression of proteasome subunits Y (β 1), X (β 5), LMP2 (β 1i), and LMP7 (β 5i) was then assessed using flow cytometry, as previously described (7). This analysis confirms that mature DCs derived from monocytes transfected with siRNAs targeting the iP subunits not only express lower levels of iP, but also express the cP.

Figure S2. Kinetics of T cell Responses to Vaccination as Detected by IFN- γ Elispot for Additional Subjects from Study Arms A and B. Subjects were vaccinated with TAA RNA-transfected DCs derived from monocytes that were not transfected with siRNA (Study Arm A, No siRNA) or that were transfected with control siRNA (Study Arm B, cosiRNA). The number of TAA-specific IFN- γ secreting CD8⁺ and CD4⁺ T cells for each of the serially collected PBMC samples is graphed over time. Data represent mean \pm SEM of duplicate measurements.

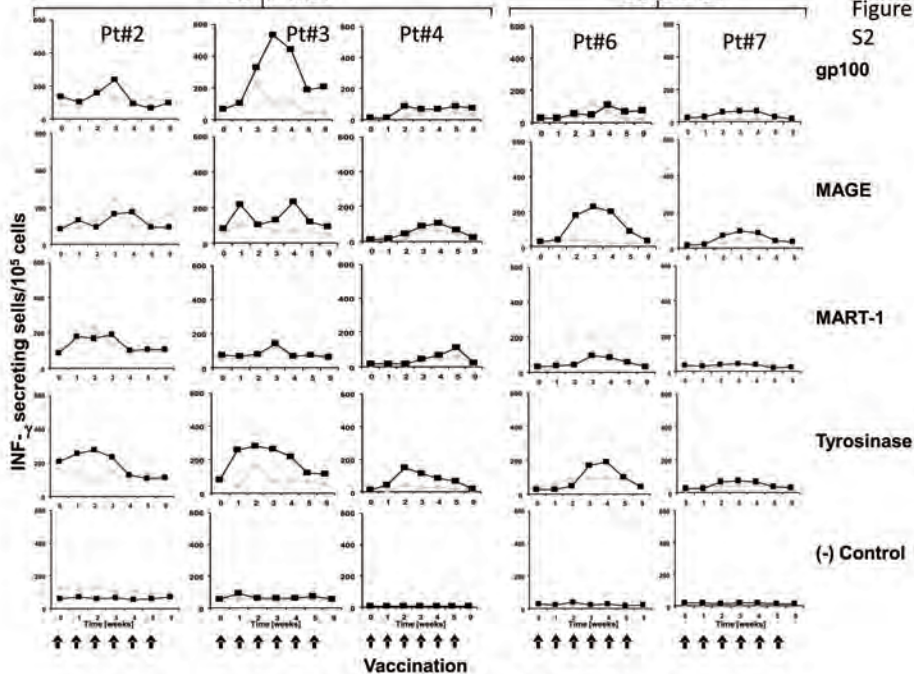
Figure S3. Kinetics of T cell Responses to Vaccination as Detected by IFN- γ Elispot for Additional Subjects from Study Arm C. Subjects were vaccinated with TAA RNA-transfected DCs derived from monocytes that were transfected with siRNA targeting the iP (Study Arm C, iPsiRNA). The number of TAA-specific IFN- γ secreting CD8⁺ and CD4⁺ T cells for each of the serially collected PBMC samples is graphed over time. Data represent mean \pm SEM of duplicate measurements.

Figure S1.



No siRNA

cosiRNA

Figure
S2

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Protocol Title:

ACTIVE IMMUNOTHERAPY OF MELANOMA WITH AUTOLOGOUS DENDRITIC CELLS TRANSFECTED WITH TUMOR ANTIGEN RNA AND SMALL INHIBITORY RNAs TO ALTER PROTEASOMAL ANTIGEN PROCESSING

Study Investigators:

Principal Investigator: Scott K. Pruitt, M.D., Ph.D.

Collaborators: Jens O. Dannull, Ph.D.
Douglas S. Tyler, M.D.
Maria Angelica Selim, M.D.
John H. Sampson, M.D., Ph.D.
Gary E. Archer, Ph.D.

Laboratory Technician: Robert J. Schmittling, B.S.

Study Coordinator*: Nancy Pickett, R.N.

***Study Coordinator's Office:**

Box 3966, Haynes House, Room 239

Duke University Medical Center

Durham, North Carolina, 27710

Office: (919) 684-3726

Fax: (919) 684-6044

Internet: picke010@mc.duke.edu

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APPENDIX E: Consent Form

1.0 PROTOCOL SUMMARY

Protocol Title:

ACTIVE IMMUNOTHERAPY OF MELANOMA WITH AUTOLOGOUS DENDRITIC CELLS TRANSFECTED WITH TUMOR ANTIGEN RNA AND SMALL INHIBITORY RNAs TO ALTER PROTEASOMAL ANTIGEN PROCESSING

Purpose and Rationale: Dendritic cell (DC)-based strategies are increasingly being employed for the immunotherapy of cancer. When DC are loaded with tumor antigens in the form of tumor cell lysates or tumor RNA (1, 2), antigen processing to generate immunogenic tumor antigen-derived peptides is critical for the induction of anti-tumor cytotoxic T cell (CTL) responses. It is the proteasome, the multi-subunit complex responsible for degrading ubiquitinated proteins, that generates these peptides that are presented in the context of HLA class I.

The maturation state of DC is also critical for stimulating anti-tumor CTL responses during DC-based immunotherapy and for inducing immune responses against a variety of pathogenic agents (3). Immature DC, in contrast, can induce immunologic tolerance, a state undesirable in a host responding to infection or tumor immunotherapy (4). The enhanced immunostimulatory properties of mature DC are due to both the increased expression of costimulatory molecules (5) as well as the secretion of cytokines such as IL-12 (6).

DC maturation and peptide epitope generation by the proteasome are not two isolated processes, but are closely linked, as DC maturation also alters the constituents of the proteasome. Specifically, constitutive β subunits X ($\beta 5$), Y ($\beta 1$), and Z ($\beta 2$) are replaced by the inducible β subunits LMP7 ($\beta 5i$), LMP2 ($\beta 1i$), and MECL1 ($\beta 2i$), respectively (7). The resultant “immunoproteasome” produces a different repertoire of peptides that is presented by class I molecules on the surface of the mature DC, when compared with the peptide repertoire produced by the constitutive proteasomes of immature DC and normal cells that have not been exposed to inflammatory mediators (8).

The induction of the immunoproteasome may be one mechanism to control the development of autoimmunity (9). At the site of an infection, for example, inflammatory mediators, such as IFN- γ , released by cells of the innate immune system (10), will induce the immunoproteasome in nearby cells, both altering the repertoire of presented self-peptides and enhancing presentation of pathogen-derived peptides. DC that have taken up foreign antigens at the site of infection and have been induced to mature by the same inflammatory mediators will migrate to regional lymph nodes and present a similar repertoire of peptides, inducing CTL specific for peptides generated by the immunoproteasome. These CTL will then migrate to the site of infection, killing infected cells exposed to inflammatory mediators, but not recognizing non-infected normal cells, whose presented repertoire of peptides will have been generated by the constitutive proteasome, not the immunoproteasome. This would limit both damage to normal tissues and the induction of autoimmunity.

Since many of the antigens overexpressed by tumors are self antigens, such a mechanism to limit autoimmunity may limit the effectiveness of DC-based cancer immunotherapy. When DC are loaded with tumor antigens, in the form of tumor cell lysates, tumor proteins or DNA/RNA encoding tumor associated antigens (TAA), and then induced to mature for use as an anti-tumor vaccine, the constitutive proteasome of the DC converts exclusively to the immunoproteasome (11). These mature DC will therefore potentially stimulate a CTL response against the repertoire of peptides produced by the immunoproteasome, and not the constitutive proteasome. These CTL would not optimally recognize tumor cells that, due to an absence of IFN- γ exposure (12) or through

mutations that prevent immunoproteasome induction (13), generate peptides for HLA class I presentation utilizing the constitutive proteasome.

In vitro, we have demonstrated that conversion of the proteasome of the mature human monocyte-derived DC from the immunoproteasome to the constitutive proteasome, by transfection with siRNAs targeting the inducible immunoproteasome subunits, alters the repertoire of melanoma TAA-derived peptides presented by mature DC transfected with RNA encoding four defined melanoma TAAs. We have also found that TAA RNA-transfected mature DC altered to express the constitutive proteasome using siRNA transfection, but with full costimulatory function associated with the induction of maturation, stimulate an enhanced anti-tumor CTL response in an *in vitro* human melanoma immunotherapy model. This preclinical data forms the basis for our proposed study.

Specific Hypothesis to Be Tested: In subjects with metastatic melanoma, we hypothesize that immunization with mature autologous DC derived from isolated monocytes and then transfected with a combination of four melanoma TAA-encoding RNAs will be safe and non-toxic. Secondly, we hypothesize that immunization with autologous TAA RNA-transfected DC derived from isolated monocytes that have been transfected with control siRNA will also be safe and non-toxic. Finally, we hypothesize that immunization with mature autologous TAA RNA-transfected DC derived from monocytes transfected with siRNAs to inhibit expression of the inducible immunoproteasome β subunits LMP2, LMP7, and MECL1 will also be safe and non-toxic. Secondly, we hypothesize that TAA RNA-transfected DC derived from monocytes transfected with siRNAs targeting the three inducible immunoproteasome β subunits will induce enhanced anti-melanoma immune responses in vaccinated subjects.

Type of Study: This is an open label Phase I safety and toxicity study.

Previous Work in Humans: In a previous trial, investigators at Duke have shown that vaccination with DC transfected with RNA encoding the TAA telomerase (hTERT) is a safe and effective strategy to elicit potentially therapeutic T cell responses in patients with metastatic prostate cancer(14). In that study, eight subjects received six weekly interdermal injections of 1×10^7 autologous DC transfected with RNA encoding hTERT and then induced to mature for 20 hours with a cytokine cocktail consisting of TNF- α , IL-1 β , IL-6, and PGE₂, with no major treatment related toxicities. These subjects with prostate cancer demonstrated post-vaccination induction of anti-hTERT immune responses and clinical responses, as assessed by decreased serum prostate-specific antigen levels.

Study Objectives and Design: The objective of this trial is to determine the safety and obtain preliminary data on the efficacy of vaccination of human subjects with melanoma TAA RNA-transfected mature DC derived from untreated isolated monocytes (Study Arm A), isolated monocytes transfected with non-targeting control siRNA (Study Arm B), and then from isolated monocytes transfected with specific siRNAs targeted to alter the proteasome (Study Arm C). A similar DC dose schedule as proposed for this trial has been evaluated in two previous trials at Duke using RNA-transfected DC and has demonstrated immunological activity without evidence of toxicity (2, 14, 15).

The primary objectives of this trial:

To evaluate the safety of intradermal injections of cultured, melanoma TAA RNA-transfected mature autologous DC, first derived from isolated monocytes (Study Arm A), then derived from monocytes transfected with control siRNA (Study Arm B), and then derived from monocytes transfected with siRNA targeting immunoproteasome subunits LMP2, LMP7, and MECL1 (Study Arm C). Initially, 3 patients in Study Arm A will receive a total of six intradermal (ID) injections using 1×10^7 cells at each cycle, which are administered every week. Safety and toxicity of vaccination will be assessed. If one or two of these three subjects experience toxicity or adverse events, up to a total of 6 subjects will be enrolled in this Study Arm. If none of these initial three subjects experience toxicity or adverse events, up to three additional subjects may be enrolled in this Study Arm (A) at the discretion of the Principal Investigator. Assuming no dose limiting toxicities or adverse events are encountered with the initial three subjects in Study Arm A, a new cohort of 3 subjects will be enrolled in Study Arm B and receive six ID injections using 1×10^7 DC given every week. If one or two of these three subjects experience toxicity or adverse events, up to a total of 6 subjects will be enrolled in this Study Arm. Assuming no dose limiting toxicities of adverse events are encountered, a third cohort of 6 subjects will be enrolled in Study Arm C using the same dosing schedule. Accrual will continue as long as the safety criteria are met.

The secondary objective of this trial:

To analyze the induction of melanoma TAA-specific T cells from pre- and post-therapy PBMC samples among subjects receiving siRNA and TAA RNA-transfected DC derived from monocytes in each Study Arm.

Eligible subjects will consist of patients with metastatic melanoma. Subjects undergoing surgery for their metastatic melanoma as part of their standard clinical care will have melanoma tissue as well as a small (3 mm diameter) sample of normal skin collected at the time of this surgery. Patients not undergoing surgery and with easily accessible melanoma metastatic lesions will undergo biopsy of such a lesion and collection of a small sample of normal skin in clinic for establishment of an autologous melanoma and fibroblast cell lines. Subjects will then undergo leukapheresis and CD14⁺ monocytes will be isolated using magnetic separation. For the final three subjects in Study Arm C, monocytes will be isolated by elutriation. For Study Arm A, DC will be generated *in vitro*, from untreated monocytes, induced to mature, and then transfected with RNA encoding a mixture of four melanoma TAAs (MART, Tyrosinase, gp100, and MAGE-3). Initially, 3 subjects will be vaccinated with such DC, receiving a total of six intradermal (ID) injections using 1×10^7 cells at each cycle, which are administered every week. Safety and toxicity of vaccination will be assessed. If toxicity is observed in one or two of the first three subjects in this Study Arm, up to an additional 3 subjects will be enrolled in Study Arm A. If none of these initial three subjects experience toxicity or adverse events, up to three additional subjects may be enrolled in this Study Arm (A) at the discretion of the Principal Investigator. Assuming no dose limiting toxicity or adverse events are encountered; a second cohort of 3 subjects will be enrolled to be vaccinated with melanoma TAA RNA-transfected mature DC derived from monocytes transfected with control siRNA, receiving a total of six intradermal (ID) injections using 1×10^7 cells at each cycle, which are administered every week (Study Arm B). Safety and toxicity of vaccination will be assessed. If toxicity is observed in one or two of the first three subjects in this Study Arm, up to an additional 3 subjects will be enrolled in Study Arm B. Assuming no dose limiting toxicity or adverse events are encountered; a third cohort of 6 subjects will be enrolled to receive melanoma TAA RNA-transfected mature DC derived from monocytes transfected with siRNAs targeting immunoproteasome subunits LMP2,

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LMP7, and MECL1, with six ID injections of 1×10^7 DC given every week (Study Arm C). Accrual will continue as long as the safety criteria are met.

Subject Population: Subjects with a histologically confirmed diagnosis of malignant melanoma and documented metastatic disease (nodal or distant metastases), Stage any T, N+ or M+, and who meet all eligibility requirements will be candidates for this trial. Subjects must wait 6 weeks after completion of any radiation therapy or chemotherapy and 8 weeks after completion of other immunotherapy prior to study participation. They must not have a history of autoimmune disease, serious concurrent chronic or acute illness, other pulmonary disease, active hepatitis, serologic evidence for HIV or Hepatitis (B and C), previously treated or new central nervous system (CNS) metastases, or be receiving systemic corticosteroid or immunosuppressive therapy. Subjects with documented severe allergies to eggs or soy will also be excluded. All subjects must be 18 years of age or older, not pregnant or nursing, and have a Karnofsky performance status $\geq 70\%$.

Study Procedures: Subjects will be screened to assure that they fulfill all eligibility criteria. For patients undergoing surgical resection as part of their standard clinical treatment of melanoma, metastatic melanoma tissue as well as a small (3 mm diameter) sample of normal skin will be collected at the time of surgery. Those subjects not undergoing surgery but with easily accessible deposits of metastatic melanoma (lymph node or dermal metastatic lesions) will undergo excisional biopsy of one of these lesions, under local anesthesia in the clinic, for tissue collection and analysis. A small sample of normal skin will also be collected from the edge of the biopsy incision to establish an autologous fibroblast cell line. For those subjects not undergoing surgery and without such easily accessible metastatic deposits, tissue analysis will be performed on the patient's prior pathology specimens. Study subjects will then undergo leukapheresis for collection of peripheral blood mononuclear cells (PBMC). Purified monocytes that are untreated (Study Arm A), transfected with non-targeting control siRNA (Study Arm B), or transfected with siRNA targeting the immunoproteasome subunits LMP2, LMP7, and MECL1 (Study Arm C) will be cultured with the cytokines GM-CSF and IL-4 to produce immature DC. Immature DC will then be induced to mature with a cytokine cocktail (IL-6, TNF- α , IL-1 β , and PGE₂) for 48 hours in culture. These mature DC will then be transfected with a combination of RNAs encoding melanoma TAAs MART, tyrosinase, and gp100, and MAGE-3. One to two hours following RNA transfection, the DC will be cryopreserved and stored in liquid nitrogen until administration. Each DC preparation will be characterized to assure the appropriate mature DC phenotype and absence of bacterial, fungal, and mycoplasma contamination. If inadequate numbers of dendritic cells are generated or if the dendritic cells that are generated fail to meet batch release criteria for any reason, the subject will be asked to undergo repeat leukapheresis (and pre-leukapheresis blood testing) to generate additional dendritic cells. Such a repeat leukapheresis will not be performed less than one month after the previous leukapheresis.

On the day the subject returns to receive his/her injection, the autologous DC will be thawed, reconstituted, counted, and upon confirmation of DC viability, a negative gram stain of the DC, and an endotoxin level below the threshold of 5 E.U./kg, then dispensed for administration.

The first 3-6 subjects (Study Arm A) will receive six ID injections given every week. If no dose limiting toxicities (Grade ≥ 3 toxicity affecting any system or Grade ≥ 2 Allergy/Immunology toxicity) or adverse events are encountered, a second cohort of 3-6 subjects will be enrolled into Study Arm B using the same dosing schedule. If no dose limiting toxicities (Grade ≥ 3 toxicity affecting any system or Grade ≥ 2 Allergy/Immunology toxicity) or adverse events are encountered in Study Arm B, a third cohort of 6 subjects will be enrolled into Study Arm C using the same

dosing schedule. Accrual to the study will continue until every treatment position in each study arm is evaluated, or dose-limiting toxicity (DLT) is observed (Grade ≥ 3 toxicity affecting any system with greater stringency applied to Allergy/Immunology adverse events where a Grade ≥ 2 toxicity will be considered unacceptable). All subjects will be evaluated for toxicity and immunological response to therapy. Blood will be drawn immediately prior to each immunization and at two week intervals for the first month, and then monthly thereafter to obtain cells for immunologic monitoring. Two days following the 4th vaccination, a 6mm punch biopsy of the skin at the site of the most recent vaccine administration will be collected under local anesthesia. T cells will be extracted from this skin sample for immune monitoring. After the full course of 6 weekly vaccinations, if sufficient cells are available, subjects will be vaccinated at monthly intervals until the cells are used up. Follow-up visits will occur every two weeks for the first month following the initial 6 vaccinations, then every month for two months, until anti-melanoma immune responses return to baseline levels, or until all of the available DC vaccine is utilized. All patients will be followed clinically in the Duke University Medical Center Melanoma Clinic, with clinic visits every 3 months for the remainder of the first year, then every 6 months for 1 year, then once per year, as is standard for patients with melanoma followed at Duke. Blood will be drawn for immunologic assessment at these clinic visits only in those patients with anti-melanoma immune responses that remain above baseline and are within two years of vaccination.

Statistical Methods: This is a Phase I safety and toxicity trial. The first 3 subjects will receive six weekly intradermal injections of melanoma TAA RNA-transfected autologous DC derived from untransfected monocytes (Study Arm A). All side effects, adverse events, and clinical laboratory information will be recorded. We anticipate that there will be no significant toxicity observed in these three Study Arm A participants, but up to three additional subjects may be enrolled into this Study Arm if any dose-limiting toxicity is observed. If none of these initial three subjects experience toxicity or adverse events, up to three additional subjects may be enrolled in this Study Arm (A) at the discretion of the Principal Investigator. The next 3 subjects will receive six weekly intradermal injections of melanoma TAA RNA-transfected autologous DC derived from monocytes cotransfected with non-targeting control siRNA (Study Arm B). All side effects, adverse events, and clinical laboratory information will be recorded. We again anticipate that there will be no significant toxicity observed in these three Study Arm B participants, but up to three additional subjects may be enrolled into this Study Arm if any dose-limiting toxicity is observed. The next 6 subjects will receive six weekly intradermal injections of melanoma TAA RNA-transfected mature autologous DC derived from monocytes transfected with siRNA targeting immunoproteasome subunits LMP2, LMP7, and MECL1 (Study Arm C). All side effects, adverse events, and clinical laboratory information will be recorded. All subjects in each Study Arm will also be evaluated for specific immune responses against the four melanoma TAAs. For the purposes of evaluating the induction of anti-melanoma immune responses, results from Study Arm A and B participants may be pooled for comparisons to results from Study Arm C.

Duration of Study: It is anticipated that it will take 18-24 months to complete the enrollment of 12-18 subjects. Follow-up blood sampling at biweekly intervals for the first month, then once per month for two months, then every three months for the next 9 months, then every 6 months for the next year, will continue until anti-melanoma TAA-specific immune responses return to baseline, or until two years have elapsed. All patients will continue to be followed clinically every 3 months for the first year, every 6 months for following one year, then once per year, for a minimal total clinical follow up of 5 years.

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Method Used to Recruit Subjects: Subjects with metastatic melanoma will be identified from among patients receiving care at the Duke University Medical Center Melanoma Clinic.

Preparatory Research: Subjects will be identified in the Duke University Medical Center Melanoma Clinic. The clinical trial coordinator will review the medical records of potential subjects who are identified to determine if they meet the inclusion and do not fulfill any of the exclusion criteria. Protected health information is needed for this assessment. A brief description of the protected health information for which use or access has been determined to be necessary: medical record number, name, date of birth, sex, race, review date, diagnosis, pathology reports, radiological reports, recent laboratory results, and history and physical examination. The protected health information will be used solely for this review, is necessary for the purposes of this review, will not leave Duke Property, will be kept locked in a filing cabinet behind a locked office door and/or in a Duke University computer with password protection.

Potential Risks: Risks of biopsy of a metastatic lesion and normal skin under local anesthesia include bleeding, infection, and scarring. Risks of leukapheresis include infection, bleeding, hypotension and hypocalcemic tetani. Intradermal injections of cellular products, such as DC, carry the potential side effects of anaphylaxis with respiratory failure and death, infection, fever, and skin necrosis, but these side effects have not been observed in previous DC-based trials performed at Duke University Medical Center. The DC are cultured in sterile antibiotic-free and serum-free medium, are analyzed for contamination with microorganisms, and will be discarded if contamination is detected. It is still possible that undetected infectious agents could be administered with the DC and cause fever, chills, hypotension, sepsis, or death. The induction of severe autoimmunity-related side effects is possible, but has thus far not been shown in other DC-based cancer immunotherapy clinical trials.

Potential Benefits: It is unknown whether there will be an improvement in survival for those subjects receiving DC-based immunotherapy as part of this study.

Compensation for Subjects: There is no compensation to the subjects for their participation in this study. There is no provision for free medical care or compensation in the event of physical injury, adverse effects, or death resulting from participation in this study.

Responsibility of Subjects for Costs: The costs of a biopsy done solely for the purposes of this study, the leukapheresis, culture and injection of the RNA-transfected DC, and blood work associated solely with the study are provided free of charge. The subject and their insurance company will be responsible for the cost of all medical expenses relating to or arising from these procedures, including the treatment of the malignancy.

Sources of Research Funds: This proposal is being submitted to the National Institutes of Health, National Cancer Institute for funding. Until NIH funding is received, Departmental, Divisional, and philanthropic funding will be utilized.

Procedures to Insure Confidentiality: Study records that identify the subject will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, the study participants will not be

identified by name, social security number, address, telephone number, or any other direct personal identifier in study records disclosed outside of Duke University Health System (DUHS). For records disclosed outside of DUHS, a unique code number will be assigned. Study records may be reviewed in order to meet federal or state regulations. Reviewers may include representatives from the Food and Drug Administration, National Institutes of Health and the Duke University Health System Institutional Review Board. Research records will be retained for at least six years or until after the study is completed, whichever is longer. At that time either the research information not already in the subject's medical record will be destroyed or information identifying the subject will be removed from the study results at DUHS. Any research information included in the medical record will be kept indefinitely.

2.0 STUDY OBJECTIVES

The objective of this trial is to determine the safety (primary endpoint) and obtain preliminary data on the efficacy of administration of melanoma tumor associated antigen (TAA) RNA-transfected DC derived from isolated monocytes (Study Arm A), derived from monocytes transfected with non-targeting control siRNA (Study Arm B) or derived from monocytes transfected with siRNAs targeting the inducible immunoproteasome subunits LMP2, LMP7 and MECL1 (Study Arm C).

The primary objectives of this study are

- a. To evaluate the safety of intradermal injections of cultured, melanoma TAA RNA-transfected and fully matured autologous DC derived from isolated monocytes applied at a dose of 1×10^7 cells per cycle for a total of six weekly cycles (Study Arm A).
- b. To evaluate the safety of intradermal injections of cultured, melanoma TAA RNA-transfected and fully matured autologous DC derived from monocytes transfected with control siRNA applied at a dose of 1×10^7 cells per cycle for a total of six weekly cycles (Study Arm B).
- c. To evaluate the safety of intradermal injections of cultured, melanoma TAA RNA-transfected and fully matured autologous DC derived from monocytes transfected with siRNAs targeting immunoproteasome subunits LMP2, LMP7, and MECL1 applied at a dose of 1×10^7 cells per cycle for a total of six weekly cycles (Study Arm C).

The secondary objectives of this study are

- d. To analyze the induction of melanoma TAA-specific T cells from pre- and post-vaccination PBMC samples among subjects receiving cultured, melanoma TAA RNA-transfected, and fully matured autologous DC in each Study Arm.
- e. To monitor eventual clinical responses as evidenced by clinical response criteria and calculate progression-free survival in subject responding to vaccination. Determine overall survival for all subjects at one and two year follow-up.

3.0 BACKGROUND AND SIGNIFICANCE

3.1 Epidemiology and Treatment of Melanoma

Currently, there are over 62,000 subjects diagnosed with malignant melanoma in the US each year, with over 7900 annual deaths from the disease. The incidence of melanoma is also increasing faster than any other malignancy. The mainstay of treatment is surgical excision of the primary skin

lesion, with regional lymph node dissection of involved nodal basins (16). For those subjects with nodal metastasis, the only available FDA-approved systemic treatment is high dose IFN- α 2b. Unfortunately, treatment with IFN- α is poorly tolerated and associated with severe side effects(17). In addition, recent analyses of IFN- α 2b trial data suggests little or no survival benefit for treated melanoma subjects (18). Thus, in the absence of a clearly effective treatment for metastatic melanoma, immunotherapy offers an alternative strategy. In a variety of clinical immunotherapy trials, immune responses against melanoma antigens have been induced. Rosenberg and associates treated subjects with a variety of malignancies, including metastatic melanoma, with injections of *ex vivo* expanded tumor infiltrating lymphocytes (TILs) and noted clinical responses in several subjects (19). Using peptide antigen-loaded DC, Nestle, et al, vaccinated melanoma subjects using intranodal injection and noted both objective immunological immune responses as well as subjective clinical improvement in some subjects (20). A variety of additional immunotherapy trials for the treatment of melanoma are currently in progress.

3.2 Rationale for Using Dendritic Cell Vaccines

DC-based strategies for active immunotherapy are increasingly being evaluated for the possible treatment of cancer. DC are the most potent antigen presenting cells of the immune system and possess the unique ability to induce T lymphocytes, which can generate a cytotoxic response against a variety of antigens including those expressed by tumor cells. It has been shown that DC can be cultured from monocyte precursors found in peripheral blood, thereby allowing for the availability of large numbers of these cells for active immunotherapy (21). Significant immunologic and clinical responses have been observed in a number of DC-based vaccination protocols such as (a) injection of blood derived DC loaded with lymphoma idiotype (22), (b) vaccination with monocyte-derived DC loaded with a mix of melanoma peptides or tumor lysate (20), or with MAGE-3 peptide in subjects with terminal stage melanoma (23), (c) injection of DC pulsed with prostate specific membrane antigen peptide (24), (d) injection of allogeneic DC fused with autologous tumor that showed clinical responses in subjects with renal cancer (25) and (e) administration of autologous DC pulsed with prostatic alkaline phosphatase protein in subjects with prostate carcinoma (26). These early trials support the concept that DC may play an important role in the immune system as the most potent antigen presenting cells, and further provide a rationale for clinical applications of DC-based therapies in the treatment of subjects with advanced cancers.

3.3 Rationale for Targeting MART, Tyrosinase, gp100, and MAGE-3

We have previously evaluated the expression of MART, tyrosinase, gp100, and MAGE-3 in 31 patient-derived melanoma cell lines using immunohistochemical staining. Of these melanoma cell lines, 77% expressed MART, 77% expressed tyrosinase, 77% expressed gp100, and 84% expressed MAGE-3. Importantly, each of these 31 patient-derived melanoma cell lines expressed at least one of these four melanoma tumor associated antigens (TAA). HLA-A2-restricted immunogenic TAA-derived peptides have been described for MART, gp100, tyrosinase and MAGE-3 (27). In addition, immunogenic peptides recognized in the context of additional non-A2 HLA haplotypes have also been described for all four of these melanoma TAAs. Because of this documented immunogenicity of these TAA as well as the high frequency of expression of these TAAs in melanoma, we conclude that DC transfected with RNA encoding these four TAAs are appropriate for vaccination in subjects with metastatic melanoma as part of this trial.

3.4 Rationale for Evaluating the Effect of Non-Targeting Control siRNA Transfection of DC

siRNAs are short double stranded segments of RNA, approximately 21 nucleotides in length, that bind to and promote the degradation of sequence-specific target RNAs within the cytoplasm. While siRNAs were initially thought to avoid activation of RNA-sensing pathways because of their short length, recent studies have demonstrated that siRNAs can have non-specific effects on the immune system (28). Judge and associates showed that injection of a variety of gene-specific and control siRNAs into mice led to increased serum levels of IFN- α , TNF- α , and IL-6 (29). These same siRNAs that were immunostimulatory in the mouse *in vivo* also stimulated secretion of these same cytokines by human PBMC *in vitro*. siRNAs have also been shown to stimulate secretion of both IL-6 and TNF- α by human monocytes, the source of our DC precursors for this proposed study (30). Similarly, Kariko, et al, have shown that siRNA transfection of immature human monocyte-derived DC with siRNA induces secretion of both type I interferons and TNF- α and enhances surface expression of HLA-DR, changes all consistent with the induction of DC maturation (31). In that study, the effects induced by the siRNAs were sequence independent. Thus, because siRNA transfection alone, regardless of sequence specificity, could induce additional changes in TAA RNA-cotransfected DC, we will establish the safety of administering TAA RNA-transfected mature DC derived from monocytes transfected with non-targeting control siRNA (Study Arm B) before we evaluate the safety of administering TAA RNA-transfected mature DC derived from monocytes transfected with specific siRNAs targeting the inducible immunoproteasome subunits (Study Arm C).

3.5 Rationale for Altering the DC Proteasome

When DC are loaded with tumor antigens in the form of tumor cell lysates or tumor RNA (1, 2), antigen processing to generate immunogenic tumor antigen-derived peptides is critical for the induction of anti-tumor cytotoxic T cell (CTL) responses. It is the proteasome, the multi-subunit complex responsible for degrading ubiquitinated proteins, that generates these peptides that are presented in the context of HLA class I.

The maturation state of DC is also critical for stimulating anti-tumor CTL responses during DC-based immunotherapy and for inducing immune responses against a variety of pathogenic agents (3). Immature DC, in contrast, can induce immunologic tolerance, a state undesirable in a host responding to infection or tumor immunotherapy (4). The enhanced immunostimulatory properties of mature DC are due to both the increased expression of costimulatory molecules (5) as well as the secretion of cytokines such as IL-12 (6).

DC maturation and peptide epitope generation by the proteasome are not two isolated processes, but are closely linked, as DC maturation also alters the constituents of the proteasome. Specifically, constitutive β subunits X ($\beta 5$), Y ($\beta 1$), and Z ($\beta 2$) are replaced by the inducible β subunits LMP7 ($\beta 5i$), LMP2 ($\beta 1i$), and MECL1 ($\beta 2i$), respectively (7). The resultant “immunoproteasome” produces a different repertoire of peptides that is presented by class I molecules on the surface of the mature DC, when compared with the peptide repertoire produced by the constitutive proteasomes of immature DC and normal cells that have not been exposed to inflammatory mediators (8).

The induction of the immunoproteasome may be one mechanism to control the development of autoimmunity (9). At the site of an infection, for example, inflammatory mediators, such as IFN- γ , released by cells of the innate immune system (10), will induce the immunoproteasome in nearby cells, both altering the repertoire of presented self-peptides and enhancing presentation of pathogen-derived peptides. DC that have taken up foreign antigens at the site of infection and have been induced to mature by the same inflammatory mediators will migrate to regional lymph nodes and present a similar repertoire of peptides, inducing CTL specific for peptides generated by the

immunoproteasome. These CTL will then migrate to the site of infection, killing infected cells exposed to inflammatory mediators, but not recognizing non-infected normal cells, whose presented repertoire of peptides will have been generated by the constitutive proteasome, not the immunoproteasome. This would limit both damage to normal tissues and the induction of autoimmunity.

However, since many of the antigens overexpressed by tumors are self antigens, such a mechanism to limit autoimmunity may limit the effectiveness of DC-based cancer immunotherapy. When DC are loaded with tumor antigens, in the form of tumor cell lysates, tumor proteins or DNA/RNA encoding tumor associated antigens (TAA), and induced to mature for use as an anti-tumor vaccine, the constitutive proteasome of the DC converts exclusively to the immunoproteasome (11). These mature DC will therefore potentially stimulate a CTL response against the repertoire of peptides produced by the immunoproteasome, and not the constitutive proteasome. These CTL might not optimally recognize tumor cells that, due to an absence of IFN- γ exposure (12) or through mutations that prevent immunoproteasome induction (13), would generate peptides for HLA class I presentation utilizing the constitutive proteasome. A more effective and clinically relevant anti-tumor immune response therefore might be induced by mature TAA-loaded DC if these cells expressed the constitutive proteasome rather than the immunoproteasome.

To effect this change, we will down-modulate expression of the inducible immunoproteasome subunits by deriving DC from monocytes transfected with siRNAs targeting LMP2, LMP7, and MECL1. Preclinical support for this approach is discussed below (Section 3.6).

3.6 Preclinical Studies

In our preclinical studies, we hypothesized that by converting the proteasome of the mature DC from the immunoproteasome to the constitutive proteasome, we would alter the repertoire of tumor antigenic peptides presented by the mature DC. We expected that such tumor antigen-loaded mature DC, with enhanced reduced immunoproteasome activity, as well as concomitantly increased constitutive proteasome activity, but full costimulatory function associated with the induction of maturation, would then stimulate an enhanced anti-tumor CTL response in an *in vitro* human melanoma immunotherapy model.

3.6.1 Altered Subunit Composition of the Proteasome of the Mature DC after siRNA transfection

We evaluated two methods for proteasome alteration. The first, overexpression of the constitutive proteasome subunits X, Y, and Z using RNA transfection, did alter the subunit composition of the proteasome, but did not alter the presentation of peptides by the mature DC (data not shown). The second method we evaluated was transfection of DC with siRNAs targeting the inducible immunoproteasome subunits LMP2, LMP7, and MECL1. As shown in Figure 1, when DC were transfected with a mixture of siRNAs targeting these inducible immunoproteasome subunits and then induced to mature using a standard cytokine cocktail, intracellular expression of constitutive proteasome subunits X and Y was enhanced while the intracellular levels of the corresponding immunoproteasome subunits LMP7 and LMP2 were reduced.

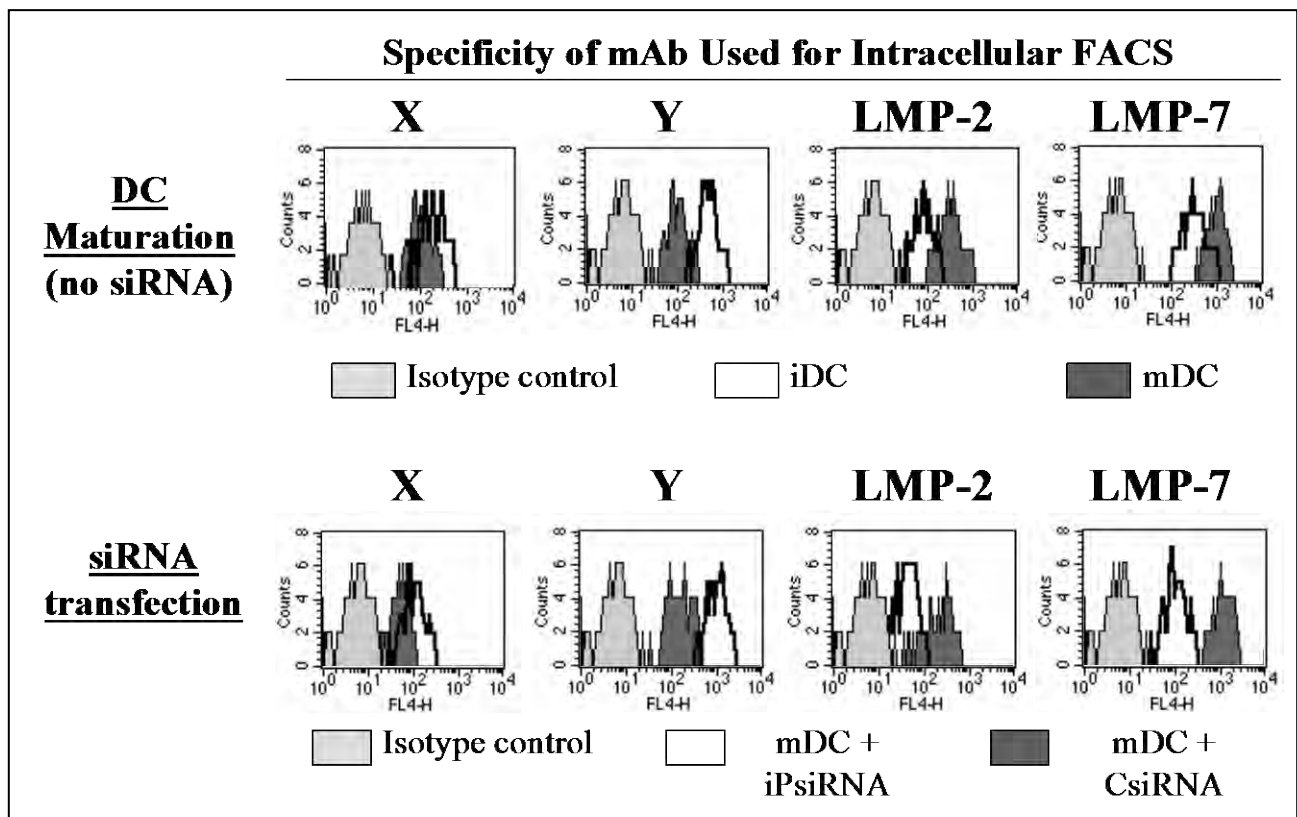


Figure 1. Intracellular expression of cP subunits X and Y and iP subunits LMP2 and LMP7 was assessed using specific mAbs, as well as isotype matched control mAbs, and an APC-conjugated secondary antibody, detected as FL4. In the upper panel, immature DC and mature DC (induced to mature using a cytokine cocktail for 24 h in culture) were analyzed, while in the bottom panel, the DC analyzed were transfected with either CsiRNA or iPsiRNA and induced to mature for 24 h using the cytokine cocktail. Histograms generated during flow cytometric analysis are displayed.

3.6.2 Immunologic recognition of DC with altered proteasomes using a CTL clone specific for a peptide generated exclusively by the constitutive proteasome and not the immunoproteasome.

For these experiments, we utilized the CTL clone 381/384 specific for a peptide generated from the ubiquitously expressed RU1 self protein exclusively through processing by the constitutive proteasome, but not by the immunoproteasome, and restricted by the HLA-B51 allele (this clone was generously provided by Van den Eynde, Ludwig Cancer Institute, Brussels, Belgium) (32). DC were generated from HLA-B51+ PBMC and transfected siRNAs targeting the inducible immunoproteasome subunits. Maturation was

induced using cytokine cocktail (TNF- α , IL-6, IL-1 β and PGE₂) and 48 hrs later, DC were washed, incubated with the CTL clone, and release of TNF- α into the supernatant was assessed by ELISA. As shown in Figure 2, an autologous B51+ renal cell cancer cell (RCC) line (exclusively expressing the constitutive proteasome) presents the constitutive proteasome-derived peptide, as indicated by TNF- α secretion by the CTL clone. Immature DC also stimulated TNF- α release, while DC induced to mature using the cytokine cocktail were not recognized, consistent with replacement of constitutive proteasomes by immunoproteasomes during DC maturation. In contrast, transfection with siRNAs targeting the inducible immunoproteasome subunits LMP2, LMP7 and MECL1 (iPsiRNA) restored recognition of mature DC by the constitutive proteasome-derived peptide-specific CTL clone. These results indicate that the proteasome of the mature DC can be altered toward the constitutive proteasome using our proposed strategy of iPsiRNA transfection. This also indicates that such a strategy can clearly alter the identity of peptides presented by the mature DC.

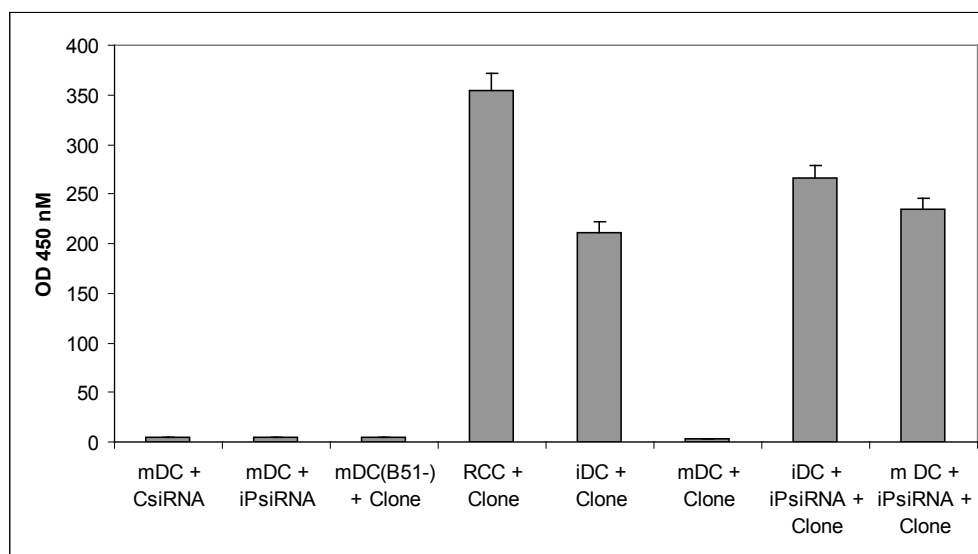


Figure 2. TNF- α secretion by a CTL clone specific for an RU1-derived peptide epitope generated exclusively by the constitutive proteasome and not the immunoproteasome and presented in the context of HLA-B51. B51+ DC were used as targets as either immature (iDC) or mature DC (mDC). Maturation was induced using a cytokine cocktail (TNF- α , IL-6, IL-1 β , PGE₂) for 48 hrs after electroporation of the DC with siRNAs targeting the immunoproteasome subunits (iPsiRNA) or control siRNA (CsiRNA). Tissue culture supernatants were harvested 16 hrs after the addition of the CTL clone and TNF- α concentration was measured by ELISA, expressed as OD450.

3.6.3 Assessment of CTL Induction by DC with Altered Proteasomes.

Based on the results shown above, we next evaluated whether mature DC, transfected with siRNAs targeting the inducible immunoproteasome subunits LMP2, LMP7, and MECL1 could induce antigen-specific CTL with altered specificity, compared with mature DC transfected with control siRNA. For this experiment, DC were again generated from HLA-B51+ PBMC. Immature DC were then either left untreated or electroporated with control or inducible immunoproteasome subunit-specific siRNAs. After the induction of DC maturation using a cytokine cocktail, followed by the addition of a precursor 19 amino acid polypeptide of the RU1 antigen (containing the immunogenic B1-restricted peptide within the polypeptide) and an additional 24 hrs in culture, DC were washed and then used to stimulate autologous T cells. After a single re-stimulation, the cultured T cells were evaluated by IFN- γ ELISPOT. As shown in Figure 3, the mature DC electroporated with siRNAs targeting the immunoproteasome subunits induced the highest number of IFN- γ secreting CTL specific for both autologous B51+ immature DC and a B51+ renal cell carcinoma cell line (RCC), both of which endogenously express the RU1 antigen, and, as demonstrated in Figure 2, present the RU1 peptide generated exclusively by the constitutive proteasome. The two targets not recognized by these CTL (autologous mature DC and RCC incubated for 3 days with IFN- γ) contain the immunoproteasome and therefore do not present this RU1-derived peptide.

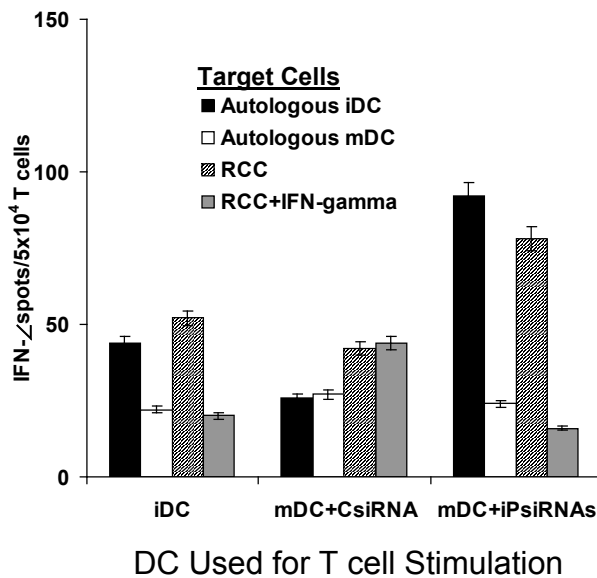


Figure 3. IFN- γ ELISPOT analysis of CTL induced by stimulation of autologous T cells with B51+ DC loaded with RU1 precursor polypeptide after no treatment (immature DC (iDC)), after transfection with control siRNA and the induction of DC maturation (mDC+CsiRNA), or after transfection with siRNA targeting the three inducible immunoproteasome subunits followed by induction of maturation (mDC+iPsiRNA). After one re-stimulation, T cell IFN- γ release in response to the indicated target cells was assessed.

Specific lytic activity of these same CTL populations was also assessed using Europium labeled target cells, as shown in Figure 4. The highest lytic against the RCC renal cancer cell line was found in T cells stimulated with DC that had been transfected with siRNA targeting the three inducible immunoproteasome subunits.

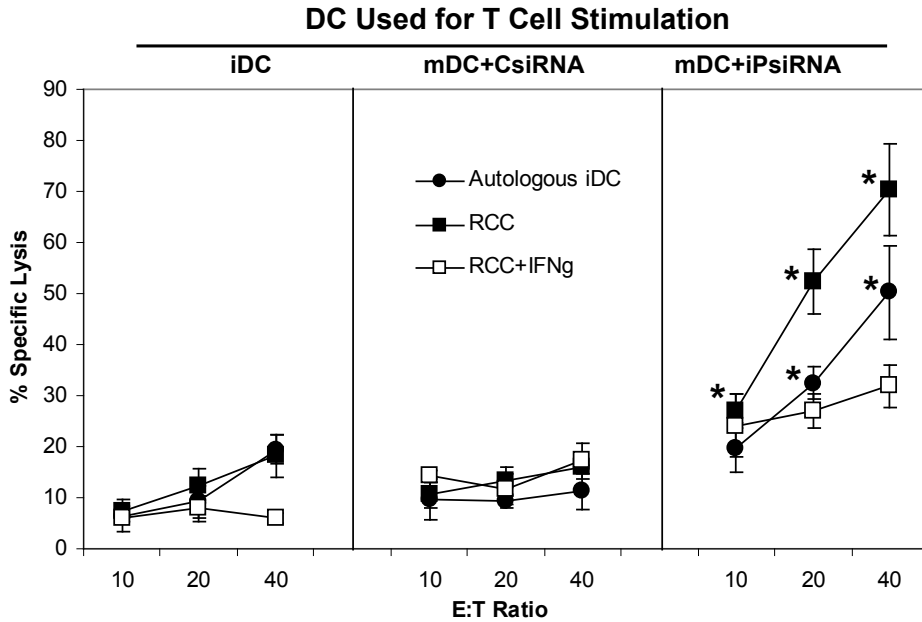


Figure 4. DC transfected with siRNA targeting the immunoproteasome induce CTL with enhanced lytic activity. Specific cytolytic activity of the CTLs generated as described in Figure 3 as assessed by a Europium release assay utilizing either autologous immature DC (autologous iDC), the B51+ renal cell carcinoma cell line (RCC), or RCC pre-incubated for 3 days with IFN- γ (RCC+IFN- γ) as targets. Lytic activity is plotted as mean % specific lysis \pm SD, with * indicating P<0.05 for the comparison between lytic activity of T cells induced by CsiRNA transfected stimulator DC vs iPsiRNA transfected stimulator DC

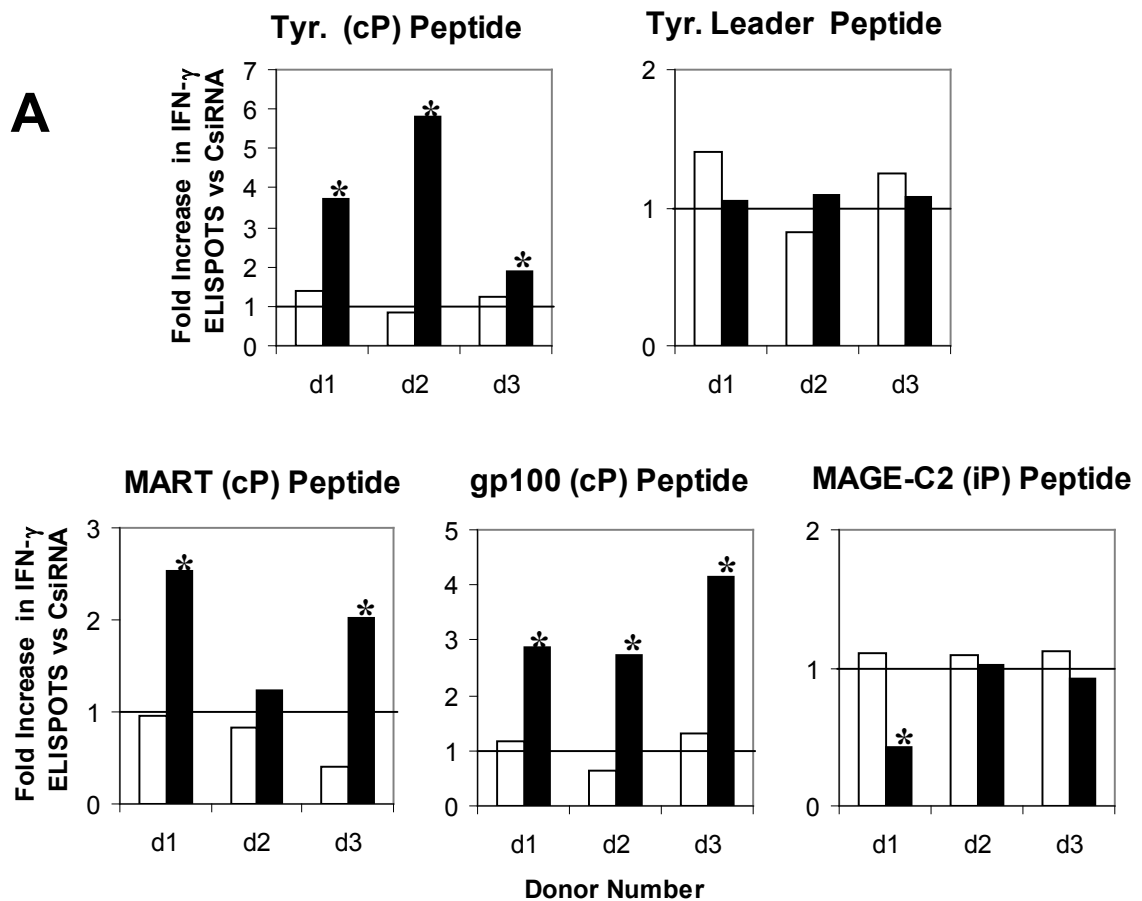
Although RU-1 peptide specificity of these CTL was not specifically evaluated in this experiment, these results, in conjunction with Figure 2, strongly suggest that mature DC transfected with iPsiRNA to alter the proteasome from the immunoproteasome to the constitutive proteasome effectively induce an antigen-specific immune response, in this case against a peptide that is exclusively generated by the constitutive proteasome and not by the immunoproteasome, with increased IFN- γ secretion and lytic activity against tumor cells.

3.6.4 Induction of TAA-Specific Immune Responses by DC is Enhanced by Immunoproteasome Down-Modulation.

To determine if iP down-modulation using iPsiRNA altered the ability of TAA RNA-transfected DC to stimulate TAA-specific immunity, immature DC were generated from three donors who were HLA-A2 positive. After transfection with CsiRNA or iPsiRNA and the induction of maturation, these DC were transfected with RNA encoding TAAs MART, tyrosinase, gp100, or MAGE-C2, then used to stimulate autologous T cells *in vitro*. After two re-stimulations with identically prepared DC, INF- γ Elispot assays were performed in response to defined HLA-A2-restricted peptides. As shown in Figure 5A, significantly higher numbers of IFN- γ -secreting CTL specific for the cP-generated MART peptide were seen in two of the three donors when stimulator DC were transfected with iPsiRNA. For TAA's gp100 and tyrosinase, responses specific for the cP-generated gp100 and tyrosinase peptides in all three donors were significantly increased when stimulator DC were transfected with iPsiRNA. The frequencies of CTL specific for the tyrosinase leader-derived

peptide (1-9) that is not differentially generated by either the cP or the iP were equivalent in all three donors whether stimulator DC had been transfected with either CsiRNA or iPsiRNA. The frequencies of CTL specific for the MAGE-C2 peptide generated exclusively by the iP were unchanged in two of three donors, and significantly reduced in one donor when stimulatory DC were transfected with iPsiRNA, compared with CsiRNA. For all three donors and all four antigenic RNAs, nonspecific IFN- γ background reactivity was not significantly enhanced when TAA RNA-transfected DC stimulators were co-transfected with iPsiRNA.

Immature DC were then generated from the PBMC of six HLA-A2 negative donors, transfected with either CsiRNA or iPsiRNA, induced to mature, then transfected with RNA encoding the melanoma TAAs MART, tyrosinase, gp100, and MAGE-3. These DC were used to stimulate autologous T cells *in vitro*. After a single re-stimulation with identically prepared DC, the resultant CTL were evaluated for antigen-specific effector function by IFN- γ ELISPOT against autologous DC transfected with TAA-encoding RNA. As shown in Figure 5B, for all six donors, iPsiRNA transfected DC induced significantly higher numbers of TAA-specific IFN- γ -secreting T cells, when compared with control siRNA-transfected DC for each TAA. The non-specific background number of IFN- γ Elispots was not significantly increased in any of the six donors for any of the four TAA RNAs when TAA RNA-transfected DC stimulators were cotransfected with iPsiRNA vs CsiRNA, confirming that iPsiRNA transfection did not stimulate non-specific autoimmune responses.



B

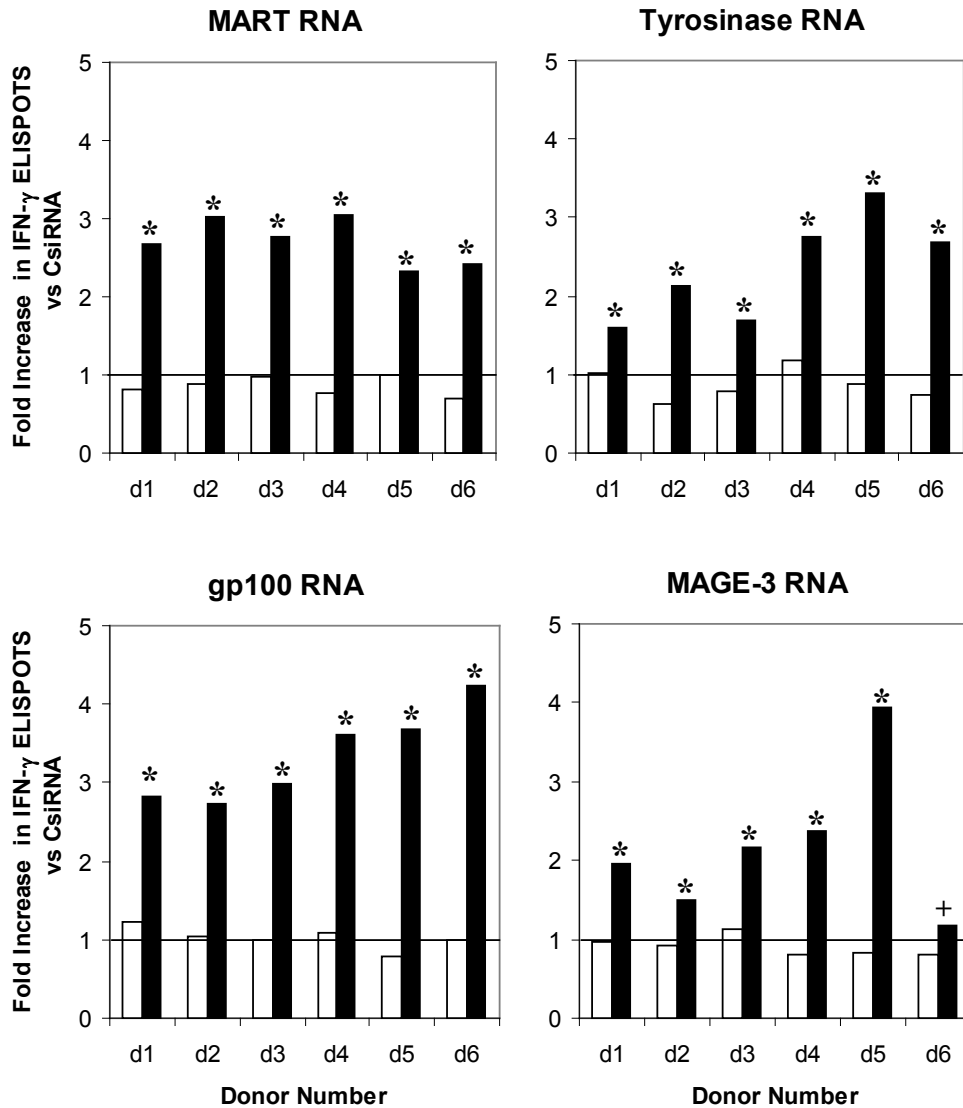


Figure 5. Enhanced Induction of IFN- γ Secreting TAA-Specific T cells When Stimulator DC are Transfected with iPsiRNA. (A) DC generated from three HLA-A2 positive donors were transfected with either CsiRNA or iPsiRNA. After the induction of maturation with CC, these DC were transfected with RNA encoding the indicated TAA and used to stimulate autologous T cells *in vitro*. After a two re-stimulations with identically prepared DC, IFN- γ ELISPOT assays were performed using the indicated defined HLA-A2-restricted peptides generated by the cP, the iP, or independently of the identity of the proteasome (Tyr. Leader). The solid bars indicate the fold increase in the number of peptide-specific Elispots induced by iPsiRNA-transfected DC vs CsiRNA-transfected DC. The open bars indicate the fold increase in non-specific background Elispots against control peptide for T cells induced by iPsiRNA vs CsiRNA-transfected DC. (B) DC generated from six individual HLA-A2 negative donors were transfected with either CsiRNA or iPsiRNA. After the induction of maturation, these DC were transfected with RNA encoding the indicated TAA and used to stimulate autologous T cells *in vitro*. After a single re-stimulation with identically prepared DC, IFN- γ ELISPOT assays were performed using autologous DC transfected with the specific TAA RNA or a negative control RNA as targets. The solid bars indicate the fold increase in the number of TAA-specific Elispots induced by iPsiRNA-transfected DC vs CsiRNA-transfected DC. The open bars indicate the fold increase in non-specific background Elispots against control RNA-transfected autologous DC targets for T cells induced by iPsiRNA vs CsiRNA-transfected DC. For both parts A and B, absolute numbers of IFN- γ Elispots for each depicted ratio for each individual donor were compared by t-test, with *indicating $P < 0.01$ and + indicating $P < 0.05$.

3.6.5. Induction of Immunity Against Melanoma in an Autologous System

To further evaluate the effect of proteasome alteration using iPsiRNA transfection on the ability of DC to stimulate anti-tumor immunity, we developed a fully autologous *in vitro* immunotherapy model. For three patients (640, 641, and 643) with metastatic melanoma previously treated at Duke, autologous melanoma cell lines had been established and PBMC had been cryopreserved after leukapheresis of each patient. First, each of these three melanoma cell lines was assessed for TAA expression using PCR and primer pairs specific for TAAs MART, tyrosinase, gp100, and MAGE-3. The TAA profile for each of these cell lines, as well as HLA-A2 status, is shown in Table 1.

Table 1. HLA-A2 and TAA expression by melanoma cell lines established from three patients with metastatic melanoma.

Melanoma Cell Line	HLA-A2	MART	Tyrosinase	Gp100	MAGE-3
DM640	+	-	+	-	+
DM641	-	+	+	-	-
DM643	-	-	-	-	+

Next, we generated DC using cryopreserved PBMC from each patient. These DC were then electroporated with either control siRNA (CsiRNA) or siRNA targeting the inducible immunoproteasome subunits (iPsiRNA), induced to mature using cytokine cocktail, and then transfected with RNA encoding TAAs MART, Tyrosinase, gp100, and MAGE-3, as determined by the expression profile of the given patient's autologous melanoma cell line. These DC were then used to stimulate autologous T cells *in vitro*. After a single restimulation, these T cells were analyzed for lytic activity against targets that included the autologous melanoma cells.

For patient 640, CTL were induced using DC transfected with RNA encoding both MAGE-3 and Tyrosinase, since the autologous DM640 cell line expressed these two TAAs. As shown in Figure 6A, when the DC stimulators were transfected with iPsiRNA, the MAGE-3 antigen-specific lytic activity against a variety of MAGE-3-expressing targets, including the patient's autologous melanoma DM640, was enhanced, compared with DC transfected with CsiRNA.

The induction of antigen-specific CTL against tyrosinase-expressing target cells by tyrosinase RNA-transfected DC (Figure 6B) was also enhanced when the DC were cotransfected with iPsiRNA compared with CsiRNA. The higher lytic activity against autologous target cells loaded with the tyrosinase peptide exclusively generated by the constitutive proteasome and not by the immunoproteasome when the DC were transfected with iPsiRNA clearly demonstrates that iPsiRNA transfection alters the peptides presented by the TAA-expressing DC, which in this patient results in enhanced CTL lytic activity against autologous melanoma cells.

Importantly, DC transfected with iPsiRNA did not induce CTL activity against autologous DC transfected with control RNA, suggesting that such iPsiRNA-transfected DC do not induce autoimmune CTL responses.

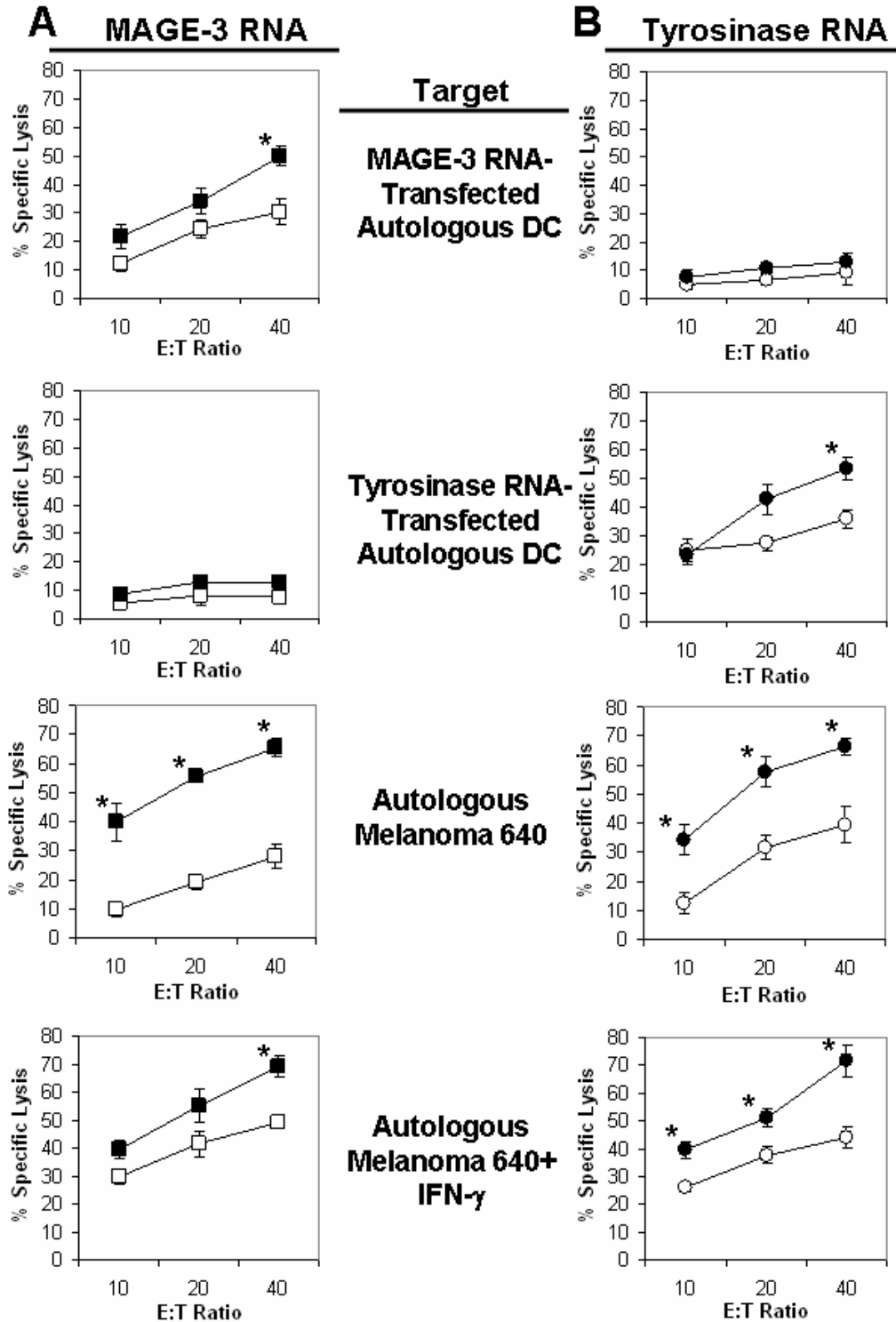


Figure 6. Lytic activity of CTL generated from patient 640. Autologous immature DC were generated from cryopreserved PBMC and then transfected with either CsiRNA (\square/\circ) or iPsiRNA (\blacksquare/\bullet). After the induction of maturation, these DC were transfected with either MAGE-3 RNA (Panel A) or Tyrosinase RNA (Panel B) and then used to stimulate autologous T cells in culture. After one re-stimulation, lytic activity was determined against autologous DC transfected with either MAGE-3 RNA or Tyrosinase RNA, or the autologous melanoma cell line 640 alone or treated with IFN- γ for 3 days. Results are plotted as mean values \pm SD, with * indicating $P < 0.05$ for the comparison between lytic activity of T cells induced by CsiRNA-transfected stimulator DC vs iPsiRNA-transfected stimulator DC measured against the same target.

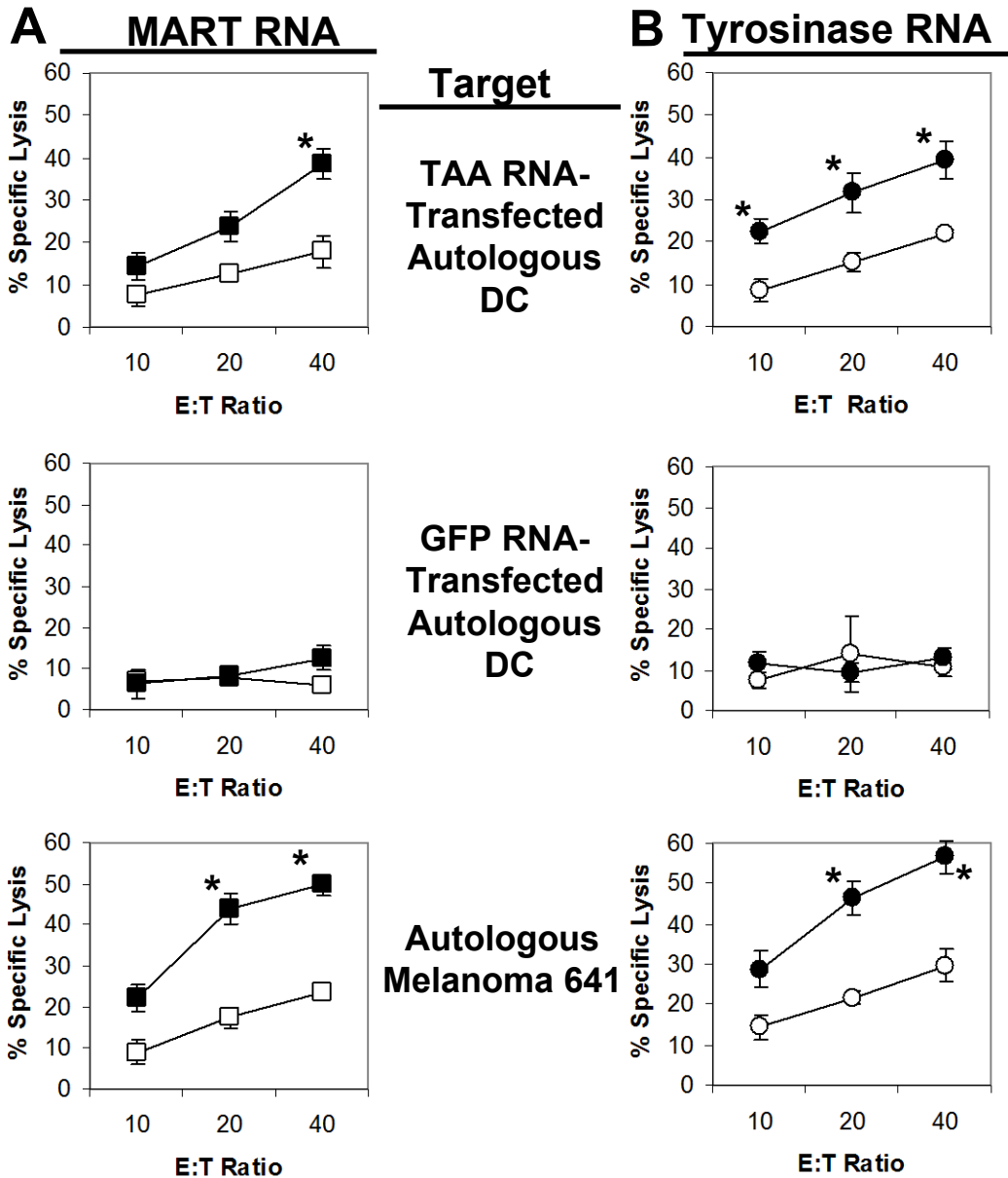


Figure 7. Lytic activity of CTL generated from patient 641. Autologous immature DC were transfected with either CsiRNA (□/○) or iPsiRNA (■/●). After the induction of DC maturation, these DC were transfected with either MART RNA (Panel A) or Tyrosinase RNA (Panel B) and then used to stimulate autologous T cells in culture. After one re-stimulation, lytic activity was determined against autologous DC transfected with TAA RNA (MART RNA for A and Tyrosinase RNA for B) or GFP (control) RNA, and the autologous melanoma cell line 641 derived from this patient. Results are plotted as mean values±SD, with * indicating P<0.05.

For patient 641, CTL were induced using DC transfected with RNA encoding either MART or tyrosinase after transfection with CsiRNA or iPsiRNA and maturation induction. As shown in Figure 7A for CTL induced by MART RNA-transfected DC, alteration of the proteasome using iPsiRNA transfection resulted in the induction of the highest antigen-specific lytic activity against both autologous DC transfected with MART RNA and against autologous DM641 melanoma cells. Higher lytic activity against autologous DC transfected with total cellular RNA extracted from DM641 (RNA extracted from the T2 cell line was used as a negative control) was also observed when CTL were induced by DC cotransfected with iPsiRNA (vs CsiRNA). The same pattern of increased antigen-specific lytic activity was also observed when the DC used for stimulation were cotransfected with tyrosinase RNA (Figure 7B). Importantly, an “autoimmune” response was not observed, as iPsiRNA-transfected DC did not induce increase lytic CTL activity against autologous DC transfected with either GFP control RNA or RNA extracted from control T2 lymphoblastoid cells.

Similar experiments were carried out using DC from patient 643 transfected with RNA encoding MAGE-3 and cotransfected with either CsiRNA or iPsiRNA. As shown in Figure 8A for DC transfected with MAGE-3 RNA, the highest antigen-specific lytic activity against MAGE-3 RNA-transfected autologous DC and against the autologous DM643 melanoma cells was generated when CTL were induced with DC cotransfected with iPsiRNA, as compared with CsiRNA. When the autologous DM643 melanoma cell targets were pre-incubated with IFN- γ for 3 days to induce the immunoproteasome, the cells were more readily lysed by CTL induced by DC cotransfected with CsiRNA (and therefore expressing the immunoproteasome), but were less susceptible to lysis by CTL stimulated by DC transfected with iPsiRNA, a finding clearly consistent with the concept that normally, DC stimulate immunity against peptides generated by the immunoproteasome and not the constitutive proteasome. By inhibiting the immunoproteasome through iPsiRNA transfection of TAA-expressing DC, we have generated a more effective anti-melanoma immune response in our *in vitro* melanoma immunotherapy model, without the apparent induction of autoimmunity.

Although the autologous melanoma cell line DM643 did not express the TAA tyrosinase, we also stimulated CTL using DC from patient 643 that were transfected with tyrosinase RNA in addition to either CsiRNA or iPsiRNA. As shown in Figure 8B, the highest specific lytic activity against autologous DC transfected with tyrosinase RNA was induced by DC transfected with iPsiRNA (vs CsiRNA). Because DM643 does not express tyrosinase, no lytic activity against this autologous melanoma cell line was observed for any of the anti-tyrosinase CTL populations.

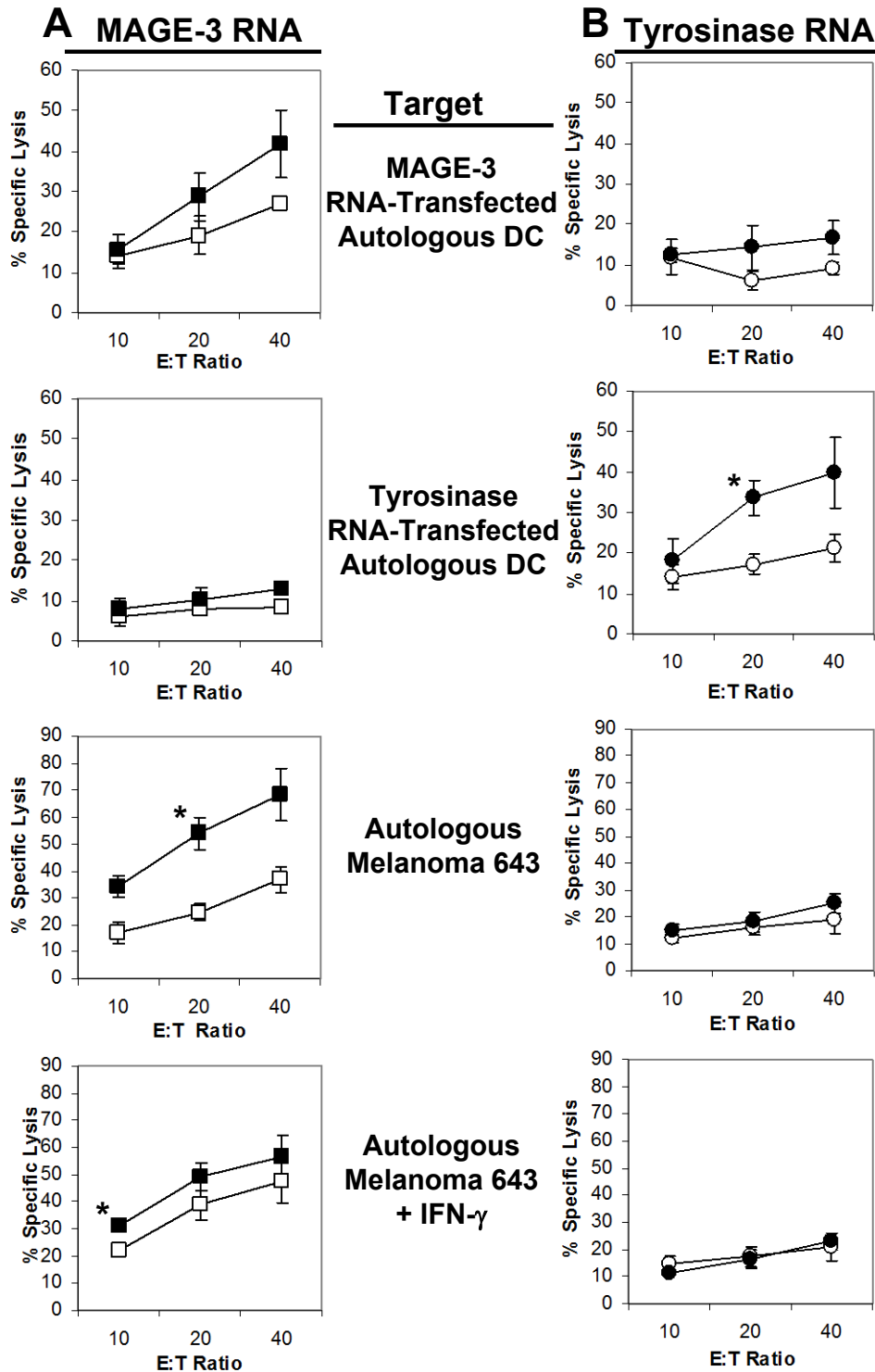


Figure 8. Lytic activity of CTL generated from patient 643. Autologous immature DC were transfected with either CsiRNA (\square/\circ) or iPsiRNA (\blacksquare/\bullet). After the induction of DC maturation, these DC were transfected with either MAGE-3 RNA (Panel A) or Tyrosinase RNA (Panel B) and then used to stimulate autologous T cells in culture. After one re-stimulation, lytic activity was determined against autologous DC transfected with RNA encoding either MAGE-3 or Tyrosinase, and against the autologous melanoma cell line 643 left untreated or pre-incubated with IFN- γ for 3 days in culture. Results are plotted as mean values \pm SD, with * indicating $P < 0.05$.

3.6.6. Generation of Dendritic Cells from siRNA-Transfected Monocytes for Clinical Vaccination

The preclinical studies reviewed above were performed using DC that were generated from adherent monocytes that were first differentiated into immature DC by culture with GM-CSF and IL-4 for 5 days. These immature DC were then transfected with either control siRNA or siRNA targeting all three inducible immunoproteasome subunits using electroporation and then maturation was induced using a cytokine cocktail. Twenty four hours later these DC were transfected with TAA RNA using lipofectamine, and after an additional 24 hours in culture were used to stimulate T cells.

For a number of reasons, such a method for the production of siRNA and TAA RNA-transfected DC would not generate adequate numbers of DC for vaccination, and the DC generated in such a manner would not be suitable for administration to human subjects. First, isolation of large numbers of adherent monocytes necessitates extensive processing of the leukapheresis packs, including density centrifugation over Ficoll, then multiple washings, followed by plastic adherence using multiple tissue culture flasks. After immature DC have been generated by culturing the monocytes in medium supplemented with GM-CSF and IL-4, these DC must be freed from the tissue culture flask surface by either scraping or using dissociation reagents, which reduce DC viability and require additional washing steps, respectively. After siRNA transfection of the immature DC, maturation is induced with the clinically utilized cytokine cocktail, and 24 hours later DC would again be harvested for RNA transfection, with additional DC loss. Transfection with RNA using a lipid reagent is not clinically applicable, and then returning the mature RNA-transfected to tissue culture flasks for an additional 24 hours of culture before being re-harvested from the flasks for use as a vaccine results in the DC having been scraped from the surface of tissue culture flasks three times in a 48 hour period. These manipulations would all serve to increase the chance of contamination of a clinical vaccine DC preparation and decrease DC viability.

We therefore have developed a protocol for the generation of clinical-grade mature RNA-transfected DC that have been transfected with siRNA as monocytes. First, CD14+ monocytes are isolated directly from the leukapheresis cell packs using magnetic bead separation. Next, immediately after isolation, these monocytes are electroporated with siRNA using electroporation before being placed in culture in tissue culture flasks in medium supplemented with IL-4 and GM-CSF to induce differentiation into immature DC. Next, maturation is simply induced by adding the cytokine cocktail to the culture flasks on day 5, without the need to harvest the cells. 48 hours after the induction of maturation, the mature DC are harvest only once, then electroporated with TAA RNA and cryopreserved without being returned to culture. Preclinical studies have been carried out, as outlined below, to validate this approach.

Time course of iPsiRNA effects in DC generated from transfected monocytes.

CD14+ monocytes were isolated from PBMC by magnetic bead separation. These monocytes were immediately (day 0) transfected with siRNA targeting all three inducible immunoproteasome subunits and then place in tissue culture in media supplemented with IL-4 and GM-CSF. On days 3 and 5, and well as on day 7 (2 days after the induction of maturation using the cytokine cocktail), DC were harvested. RNA was extracted from these DC, and after cDNA sythesis, Q-PCR was performed on these samples to assess levels of

LMP2, LMP7 and MECL-1 mRNA. As shown in Figure 9, mRNA levels for all three inducible immunoproteasome subunits were down-modulated by siRNA transfection on day 3 and day 5. Forty-eight hours after the induction of maturation (day 7), mRNA levels for the inducible immunoproteasome subunits remained reduced in mature DC generate from isolated monocytes transfected with siRNA targeting the inducible immunoproteasome subunits.

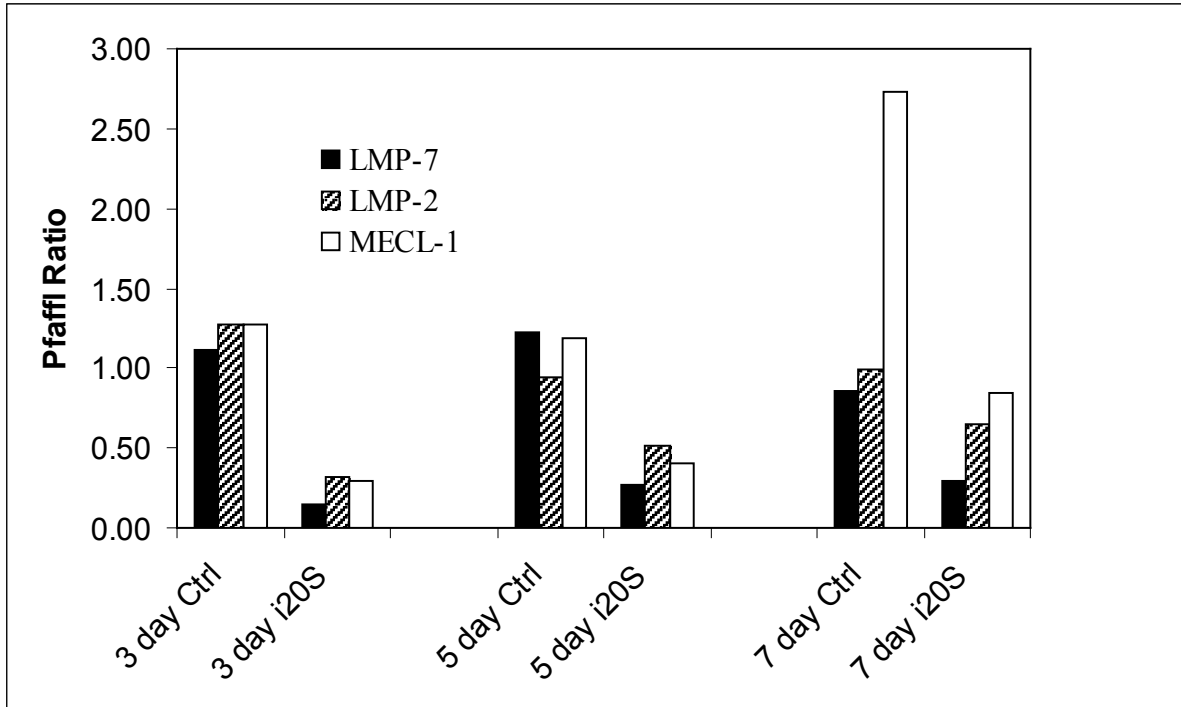


Figure 9. Time course of down-modulation of inducible iP subunits in DC derived from isolated CD14+ monocytes transfected with iPsiRNA. CD14+ monocytes, isolated by magnetic bead separation, were transfected with siRNA targeting the immunoproteasome subunits (i20S) and then differentiated into DC by culture in medium supplemented with IL-4 and GM-CSF. On day 5, DC maturation was induced by adding a cytokine cocktail to the DC medium. DC were harvested on the indicated days. RNA was extracted, and after cDNA synthesis, Q-PCR was performed to determine levels of relative mRNA encoding iP subunits LMP2, LMP7, and MECL-1. The data presented is representative of three separate experiments, each with similar results.

Induction of Anti-Melanoma CTL by TAA RNA-transfected DC generated from siRNA-transfected monocytes.

Having shown that mRNA levels for the inducible immunoproteasome subunits were reduced in day 7 mature DC derived from CD14+ monocytes that were transfected with siRNA targeting these immunoproteasome on day 0, the effect of siRNA transfection of monocytes on proteasome function and antigenic peptide presentation in these DC was assessed. Again, CD14+ monocytes, isolated from PBMC using magnetic bead separation, were electroporated with siRNA targeting the three inducible immunoproteasome subunits, then placed in culture in medium supplemented with IL-4 and GM-CSF. After 5 days in culture, the resultant immature DC were induced to mature using the cytokine cocktail. After

48 hours of maturation, the DC were harvested and electroporated with RNA encoding the melanoma TAA tyrosinase (as well as negative control RNA). These DC were then used to stimulate autologous T cells in culture. After a single re-stimulation 10 days later, the induced T cells were evaluated for anti-tyrosinase immune responses. The CTL activity of these T cell cultures were compared with CTL induced using DC derived from CD14+ monocytes that were electroporated with siRNA as immature DC on day 5 and then induced to mature.

As shown in Figure 10, the CTL induced by stimulation with DC derived from CD14+ monocytes transfected with siRNA immediately after magnetic bead isolation were superior inducers of anti-tyrosinase immunity, when compared with CTL induced by DC transfected with siRNA as immature DC. While similar numbers of cells secreting IFN- γ in response to tyrosinase were induced by either of the RNA transfected DC preparations, the non-specific background number of IFN- γ -secreting T cells was markedly reduced when the DC used to induce the CTL were derived from monocytes transfected with siRNA immediately after isolation.

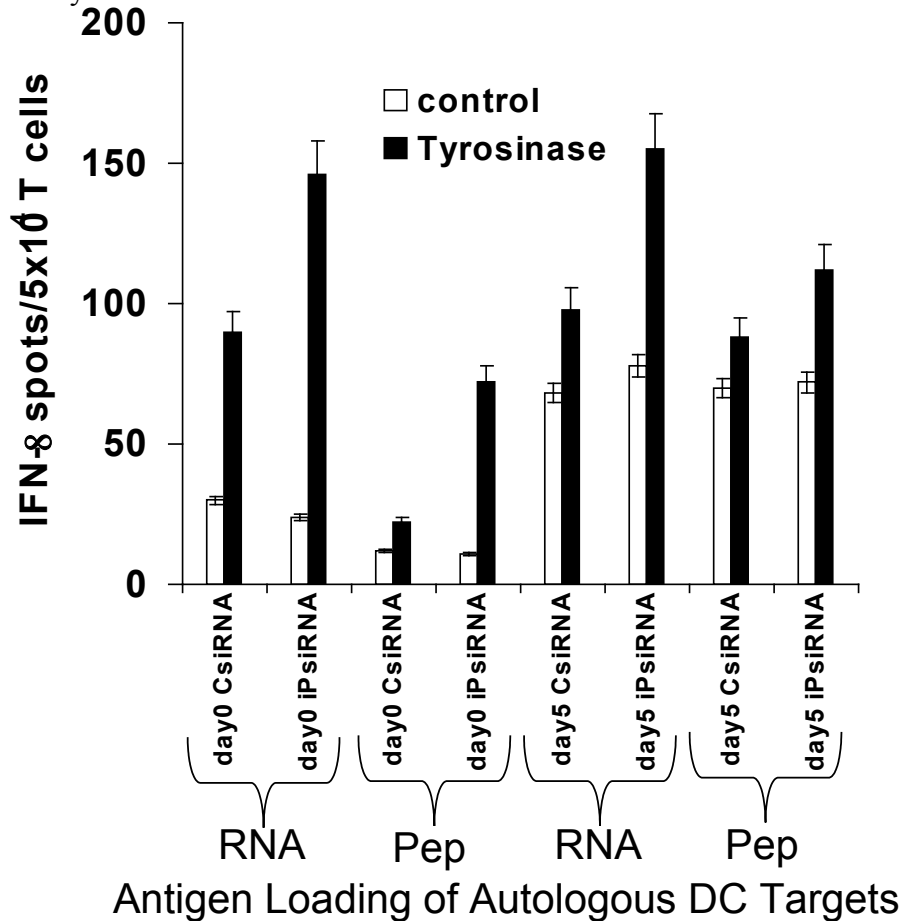


Figure 10. Tyrosinase RNA-transfected DC derived from iPsiRNA-transfected monocytes are superior inducers of tyrosinase-specific CTL activity, when compared to monocyte derived DC transfected with iPsiRNA. Autologous HLA-A2+ T cells were stimulated using tyrosinase RNA-transfected DC that were transfected with control siRNA (CsiRNA) or siRNA targeting the three inducible immunoproteasome subunits (iPsiRNA) either as immature (day 5) DC or as isolated monocytes that were then differentiated into immature DC. All DC were then induced to mature using a cytokine cocktail on day 5 and then used as stimulators 48h later. After a single restimulation, INF- γ ELISPOT analysis was performed using autologous DC stimulators transfected with tyrosinase RNA or loaded with a tyrosinase derived A2-restricted peptide (Pep) previously shown to be generated by constitutive proteasome mediated degradation of tyrosinase.

The lytic activity of these CTL was also assessed using a europium release assay and autologous DC transfected with RNA encoding tyrosinase or gp100 (as a negative control). As shown in Figure 11, the highest antigen-specific lytic activity, with the lowest non-specific activity against control RNA transfected target cells, was induced by tyrosinase RNA-transfected DC that were derived from monocytes transfected (day 0) with siRNA targeting the inducible immunoproteasome subunits (upper right panel).

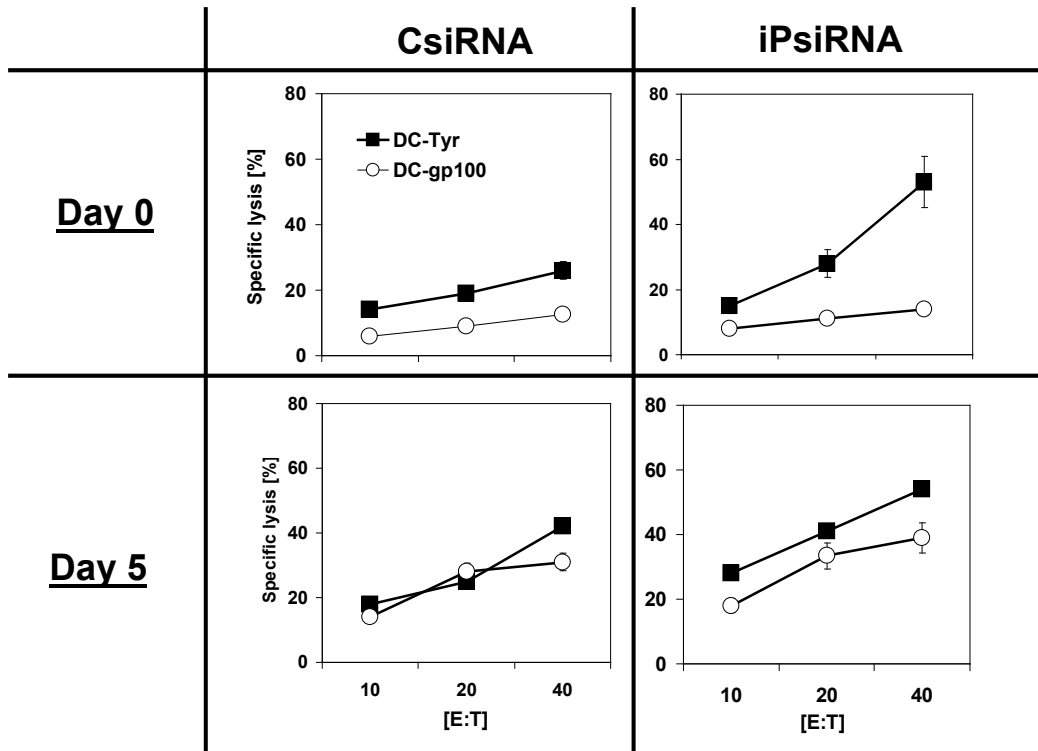


Figure 11. DC derived from iPsiRNA-transfected monocytes induce enhanced anti-tyrosinase lytic activity, compared with monocyte-derived DC transfected with CsiRNA. CD14+ monocytes were isolated and either immediately (day 0) electroporated with siRNA (cCsiRNA vs iPsiRNA), or were not transfected with siRNA but immediately placed in culture (day5). These monocytes were then differentiated into immature DC. On day 5, the DC derived from untransfected DC were harvested and transfected with siRNA (CsiRNA vs iPsiRNA). Maturation was then induced for all DC cultures using the cytokine cocktail. Forty eight hours later, each population of DC was harvested and used to stimulate autologous T cell *in vitro*. After a single re-stimulation, lytic activity was measured by a europium-release assay using autologous DC transfected with RNA encoding Tyrosinase (DC-Tyr) or gp100 (DC-gp100) as targets.

3.6.7 Summary of Preclinical Studies

In summary, our pre-clinical data clearly demonstrate that inhibiting the DC immunoproteasome through transfection with siRNA targeting the inducible immunoproteasome subunits LMP2, LMP7, and MECL1 alters antigen processing and peptide presentation by the mature TAA-loaded DC. DC cotransfected with siRNA targeting the inducible immunoproteasome subunits not only stimulated enhanced antigen-specific immunity against these defined TAA, but most importantly stimulated enhanced lytic activity against melanoma cells in three “patients” in a fully autologous melanoma immunotherapy model. Also of critical importance, no autoimmune CTL activity was induced by iPsiRNA-transfected DC stimulators. In addition, we have developed a method for the manufacture of adequate numbers of TAA RNA-transfected DC for clinical use that are generated from siRNA-transfected isolated monocytes. Despite transfection with siRNA targeting the immunoproteasome at the monocytes stage, functional alteration of DC antigen presentation was clearly present seven days later after these monocytes had been differentiated into iDC, then induced to mature using the cytokine cocktail, and then transfected with TAA-encoding RNA. Therefore, DC produced in this manner will be the product administered to subjects in our proposed clinical trial.

4.0 SUBJECT SELECTION

This study will enroll patients with histologically confirmed metastatic melanoma.

4.1 Criteria for Subject Eligibility

- 4.1.1 Patients with confirmed metastatic melanoma.
- 4.1.2 Karnofsky performance status greater than or equal to 70%.
- 4.1.3 Estimated life expectancy > 6 months.
- 4.1.4 Age \geq 18 years.
- 4.1.5 Adequate hematologic function with:
 - WBC \geq 3000 mm³
 - hemoglobin \geq 9 mg/dl
 - platelets \geq 100,000/mm³
- 4.1.6 Adequate renal and hepatic function with:
 - serum creatinine < 2.5 mg/dl
 - bilirubin < 2.0 mg/dl
 - AST/SGOT < 70 U/L
 - ALT/SGPT < 70 U/L
 - Alkaline Phosphatase \leq 135 U/L
- 4.1.7 Ability to understand and provide signed informed consent that fulfills Institutional Review Board guidelines.

4.1.8 Ability to return to Duke University Medical Center for adequate follow-up as required by this protocol.

4.2 Criteria for Study Exclusion

4.2.1 Subjects undergoing concurrent chemotherapy, radiation therapy, or immunotherapy will be excluded.

4.2.2 The subject has previously irradiated, surgically treated, or newly diagnosed central nervous system (CNS) metastases will be excluded (Pre-enrollment head CT is not required if not indicated by clinical signs or symptoms).

4.2.3 Subjects with a history of autoimmune disease such as, but not restricted to, inflammatory bowel disease, systemic lupus erythematosus, ankylosing spondylitis, scleroderma, or multiple sclerosis will be excluded.

4.2.4 Subjects with serious concurrent chronic or acute illness such as pulmonary (asthma or COPD), cardiac (NYHA class III or IV) or hepatic disease, or other illness considered by the principal investigator to constitute an unwarranted high risk for investigational drug administration will be excluded.

4.2.5 Subjects with medical or psychological impediment to probable compliance with the protocol will be excluded.

4.2.6 Subjects with concurrent second malignancy other than melanoma or non-melanoma skin cancer will be excluded. In the event of prior non-melanoma malignancies treated surgically, the subject must be considered NED (no evidence of disease) for a minimum of 3 years prior to enrollment.

4.2.7 Presence of an active acute or chronic infection, including symptomatic urinary tract infection, HIV (as determined by ELISA and confirmed by Western Blot) or viral hepatitis (as determined by HBsAg and Hepatitis C serology) will lead to subject exclusion.

4.2.8 Subjects receiving steroid therapy (or other immunosuppressive agents such as azathioprine or cyclosporine A) are excluded on the basis of potential immune suppression.

4.2.9 Subjects with inadequate peripheral vein access to undergo leukapheresis will be excluded.

4.2.10 Female subjects with a positive pregnancy test, as well as those who have not previously undergone hysterectomy and/or bilateral oophorectomy and are unwilling to utilize a medically approved form of contraception, from the time of enrollment until 6 weeks after the final immunization, will be excluded.

4.2.11 Male subjects, not previously surgically sterilized, who are unwilling to use a condom with spermicide during any sexual activity occurring over the entire immunization period and for the 6 weeks that immediately follow the final immunization will be excluded.

4.2.12 Subjects with a documented history of severe allergic reaction to beta-lactams, eggs or soy products.

4.3 Accrual

We expect to enroll 1-2 eligible subjects every 2-3 months to this study. Therefore, this study should reach the accrual goal of 12 subjects within approximately 18-36 months. Case report forms will be completed on all subjects who are enrolled on the study.

4.4 Inclusion of Minorities

The numbers in Table 2 reflect the racial and ethnic composition of the subject population with melanoma diagnosed or treated at Duke University between 1998 and 2002. About one third of those subjects presented with metastatic disease. It is well known that Caucasian patients are more susceptible to the development of melanoma. Breslow depth of the primary melanoma is the most accurate predictor of mortality from melanoma. In our clinical trial we will seek to enroll subjects from all ethnic groups, but we anticipate that most of the eligible patients will be Caucasian.

TABLE 2. Racial Distribution of Melanoma Patients Treated At Duke Hospital 1998-2002

Year 1st Contact	Caucasian	Black	American Indian	Asian Indian	Chinese	Other Asian	Other Race	Unknown Race	TOTAL
1998	291	73	9			3			376
1999	307	53	1			3		5	369
2000	271	67	1				2	2	343
2001	286	85	3	2	1			1	378
2002	289	78	1	1		2		1	372

5.0 PRE-STUDY EVALUATION

Study evaluations will be conducted according to the Schedule of Activities (Appendix A)

5.1 History and Physical Exam

- 5.1.1 Inclusion/Exclusion criteria (Eligibility Checklist – Appendix B).
- 5.1.2 Medical history/Review of Systems/Physical Examination
- 5.1.3 Review of any other treatments/medications/biological/blood products the subject is receiving or has received within 30 days.
- 5.1.4 Karnofsky status and weight.

5.2 Review of Pathology Reports

- 5.2.1 Pathology reports pertaining to the analysis of the subject's melanoma, including immunohistochemical studies, will be reviewed for each subject.
- 5.2.2 Immunohistochemical Testing for Expression of TAA. Melanoma specimens from each potential subject will be evaluated for expression of the melanoma TAAs MART, tyrosinase, gp100, and MAGE-3 by immunohistochemical staining using commercially available monoclonal antibodies.

Immunotherapy with DC transfected with siRNA to alter proteasomal processing of tumor antigenic proteins

5.3 Hematological and Biochemical Testing (if not done within 4 weeks)

5.3.1 CBC, differential.

5.3.2 PT, PTT.

5.3.3 OP-15 (Na, K, Cl, CO₂, BUN, Creatinine, Glucose, Total Protein, Albumin, Calcium, Phosphorus, AST, Alkaline Phosphatase, Total Bilirubin, ALT).

5.3.4 Pregnancy test (for pre-menopausal female patients who have not undergone hysterectomy or bilateral oophorectomy)

5.4 Review of Prior Radiological Studies Documenting Metastatic Disease

5.4.1 Any studies documenting metastatic disease, including CT, MRI, PET or bone scans, will be reviewed and repeated if clinically indicated.

5.5 Autoimmune Studies

5.5.1 Anti Nuclear Antibody (ANA) levels, Rheumatoid Factor (RF) levels, Thyroid Stimulating Hormone (TSH) levels, and anti-thyroglobulin thyroid antibody levels will be assessed for all potential subjects.

5.6 Infectious Disease

5.6.1 HIV by ELISA with positives confirmed by Western Blot, Hepatitis B and Hepatitis C serology.

5.7 HLA Typing

5.7.1 Limited HLA typing will be performed for each subject using PBMC collected according to the schedule of activities (Appendix A) using flow cytometry and monoclonal antibodies specific for human HLA-A1, A2 A3, and B40.

5.8 Cellular Immunological Monitoring

Immunological monitoring will be performed using PBMC collected from eligible subjects prior to administering each immunization and during follow-up visits according to the Schedule of Activities (Appendix A). T cells isolated from a biopsy specimen of skin at the vaccination site collected 48 hours following vaccination will also be expanded in culture for immune monitoring purposes. Collected PBMC and culture T cells will be either processed immediately or cryopreserved for analysis as follows:

- 1) Direct assessment of melanoma TAA-specific CD8⁺ T cells through Interferon- γ ELISPOT.
- 2) Cytotoxic activity against HLA-matched melanoma cell targets, HLA-matched target cells loaded with TAA RNA or TAA-derived peptides, and/or autologous melanoma tumor cells for those patients from which primary melanoma cell lines can be established.

5.8.1 Determination of Melanoma TAA-Specific T-cell Frequencies Using the ELISPOT Assay

Vaccine induced T cell frequency will be measured in peripheral blood drawn before immunization, prior to administration of each injection, and post-immunization, according to

the Schedule of Activities (Appendix A) by Interferon- γ ELISPOT. This same assay will also be performed using T cells isolated from the skin at the site of vaccination number 4.

5.8.2 Cytotoxic Activity Assay

PBMC will be obtained prior to the first DC injection, with every injection, and at the time of the repeat leukapheresis, and will be used to monitor induction and of anti-melanoma cytotoxic activity against HLA-matched melanoma cell lines, HLA-matched cells loaded with TAA-derived peptides, or autologous melanoma cells in subjects from whom a stable melanoma cell line can be derived. Obtaining samples following the repeat leukapheresis will be based upon immunologic response. This same assay will also be performed using T cells isolated from the skin at the site of vaccination number 4.

5.8.3 Anti-Melanoma Humoral Immune Responses

Serum will also be collected prior to immunization and at serial time points during and following vaccination. Flow cytometry will be used to detect antibodies that react with the autologous melanoma cell lines.

6.0 STUDY PROCEDURE

6.1 Biopsy of Metastatic Melanoma Deposit

After informed consent is obtained, patients with documented metastatic melanoma will either undergo collection of melanoma tissue and a small (approximately 3 mm diameter) sample of normal skin during standard surgical therapy of their metastatic melanoma or will undergo biopsy of a melanoma metastatic deposit and collection of a small sample of normal skin under local anesthesia in the clinic using standard sterile surgical technique. The type of melanoma deposits to be biopsied in the clinic include subcutaneous and lymph node metastases. A portion of each metastatic melanoma specimen will be transported to the Duke Department of Pathology for standard analysis of formalin-fixed paraffin-embedded tissue. An additional portion of the melanoma metastatic tissue will be preserved in RNA storage solution, while the remainder of the harvested melanoma tissue will be placed in sterile saline. The sample of normal skin will be placed in a separate tube of normal saline. These three specimens will then be transported to the study laboratory for further processing.

6.1.1 Assessment of TAA Expression

The metastatic melanoma tissue will be evaluated for expression of the melanoma TAAs MART, tyrosinase, gp100, and MAGE-3 using immunohistochemical staining. This will be performed in the Immunopathology Laboratory at Duke University Medical Center using formalin-fixed tissue and commercially available monoclonal antibodies. Expression of each TAA will be determined to be positive or negative by a clinically certified dermatopathologist (Dr. Selim or her designee).

6.1.2 RNA Extraction

We will also compare the immunohistochemical TAA expression results with molecular analysis of TAA expression performed in our laboratory. Using the melanoma metastatic tissue preserved in RNA storage solution, standard molecular biological techniques will then be used to extract RNA and synthesize cDNA. PCR will then be used to analyze expression of melanoma TAAs MART, tyrosinase, gp100, and MAGE-3 using gene-specific primer pairs.

By comparing immunohistochemical with molecular TAA-expression results, we hope to validate our molecular TAA expression analysis for use in future melanoma immunotherapy studies.

6.1.3 Establishment of Melanoma and Fibroblast Cell Lines

The metastatic melanoma tissue in sterile saline will be transported to the laboratory and will then be placed in sterile medium in a tissue culture dish and mechanically minced. After several days in culture, melanoma cells and fibroblasts will be isolated using antibody-conjugated magnetic bead separation. Using these isolated cells, both fibroblast and melanoma cell lines will be established and maintained in culture. The sample of normal skin will also be used to establish a normal fibroblast cell line. Fibroblast cell lines will serve as a source of HLA-identical non-melanoma cells for use as a negative autologous control in assessing immune responses to specific melanoma TAAs.

6.2 Leukapheresis

Peripheral blood mononuclear cells (PBMC) will be obtained by leukapheresis at the Duke University Apheresis Unit. This Unit is conveniently located on the second floor of the Morris Building of Duke South Hospital, one above the Melanoma Clinic. To avoid hypocalcemic reactions during the procedure due to the use of citrate anticoagulant during leukapheresis, subjects will be given oral calcium carbonate supplements as needed. A Cobe™ cell separator is used to perform the leukapheresis for 2-4 hours via needle access to bilateral peripheral veins. The machines are equipped with automatic warning devices to detect blood loss or clotting in the machine, and automatic shut off to prevent the possibility of air embolism. All subjects will be observed continuously by trained personnel for any adverse effects and to observe breaks in the efferent peripheral tubing circuit that may not be detected by automatic monitoring devices.

Labeling of mononuclear cell packs will include the following information: 8-digit code including the subject's initials and the date and time of collection.

- 1) One label will be immediately affixed to the collection bag(s).
- 2) One label will be immediately affixed to the flow sheet containing the subject's 8-digit study code, the operator's name, the machine used for leukapheresis, and the time leukapheresis began and ended.
- 3) After labeling, mononuclear cell packs will be placed in a specially labeled Igloo cooler with biohazard stickers and will be transported to the cell processing facility by trained personnel along with a copy of the flow sheet, leukapheresis and transport master batch records, and additional labels.

All personnel will be familiar with these standard operating procedures and the universal precautions applicable to treatment of laboratory specimens.

6.3 Manufacturing and Testing DC

The mononuclear cell product will be processed immediately following transport to the cell processing facility. PBMC produced by leukapheresis contain >95% mononuclear cells at densities of 1×10^8 /ml with hematocrit levels of 0-3%, representing less than 3×10^5 - 1.5×10^6 contaminating red blood cells per mm^3 . Monocytes will be isolated directly from the leukapheresis product using anti-CD14 mAb-conjugated magnetic bead separation. For the final three subjects in Study Arm C,

monocytes will be isolated from the leukapheresis product by elutriation. The isolated monocytes will then be either untreated (Study Arm A), transfected with non-targeting control siRNA (Study Arm B), or transfected with siRNA targeting the three inducible immunoproteasome β subunits LMP2, LMP7, and MECL1 (iPsiRNA, Study Arm C), then resuspended in serum-free medium supplemented with GM-CSF and IL-4 and cultured for 5 days. DC maturation will then be induced by the addition of a cytokine cocktail (TNF- α , IL-6, IL-1 β , and PGE₂) to the medium. Forty-eight hours later, DC will be harvested, and after an aliquot of DC in medium is submitted for mycoplasma culture testing, electroporated with a mixture of RNAs encoding the four melanoma TAAs MART, tyrosinase, gp100, and MAGE-3. One to two hours later, these DC will be cryopreserved and stored in liquid nitrogen until administration. A thawed aliquot of DC will be tested for sterility by bacterial and fungal culture. Sterility must be demonstrated and mycoplasma testing must be negative. Finally, according to our batch release criteria, a mature DC phenotype must be confirmed by FACS analysis. Immediately prior to each DC vaccination, the DC vaccine endotoxin level will be measured and must be below the threshold of 5 E.U./kg body weight per vaccination, and a Gram Stain of the DC will be performed, which must be negative before the subject is vaccinated.

6.3.1 Bacterial and Fungal Culture Testing

Two aliquots from a test thaw of each cryopreserved DC vaccine preparation will be submitted to the Duke University Medical Center Clinical Microbiology Laboratory, one for bacterial and the other for fungal culture. DC will be administered to subjects only after these tests are found to be negative.

6.3.2 Mycoplasma Testing

One aliquot of mature DC in the tissue culture medium, collected when the cultured cells are pooled and prior to RNA transfection and cryopreservation, will be submitted to a commercial laboratory for mycoplasma testing. Only DC free of mycoplasma contamination will be administered to study subjects.

6.3.3 Phenotypic Analysis Using Flow cytometry

The remainder of the DC from the test thaw will then be washed and evaluated by flow cytometry. The DC cell preparation will be deemed suitable for injection if it contains $\leq 10\%$ CD3+ (T) cells, \leq CD14+ monocytes, \leq CD19+ (B) cells, and \leq CD56+ (NK) cells. In addition the final preparation of DC must exhibit a mature DC phenotype ($\geq 40\%$ CD25+, $\geq 50\%$ CD83+, $\geq 50\%$ CD86+).

Table 3. mAbs For Phenotypic Evaluation of DC

Tube #	FITC-conjugated IgG1 mAb			PE-conjugated IgG2a mAb		
	Specificity	Clone	Supplier	Specificity	Clone	Supplier
1	Control IgG1	-----	Invitrogen	Control IgG2a	-----	Invitrogen
2	CD19 (B cells)	SJ25-C1	Invitrogen	CD14 (monocytes)	TÜK4	Invitrogen
3	CD83 (Mature DC)	HB15e	Invitrogen	CD3 (T cells)	S4.1	Invitrogen
4	CD86 (Costim)	BU63	Invitrogen	CD56 (NK cells)	MEM-88	Invitrogen
5	CD25 (Mature DC)	CD25-3G10	Invitrogen	HLA-DR	L243	BD

6.3.4 Endotoxin Testing

After thawing, each DC vaccine dose to be administered to a study subject will be washed once in sterile saline supplemented with 1% human serum albumin and then resuspended in sterile saline supplemented with 1% human serum albumin for injection. An aliquot of each final vaccine dose formulation, consisting of both cells and albumin-supplemented saline, will be analyzed for the presence of endotoxin by the Duke Radiopharmacy clinical laboratory. An additional sample will be analyzed in our laboratory using a commercially available limulus lysate testing kit (Cape Cod Associates). Only DC with endotoxin levels below a threshold of 5 E.U./kg per subject vaccine dose will be administered to study subjects.

6.3.5 Gram stain of each final DC vaccine preparation

After thawing, each DC vaccine dose to be administered to a study subject will be washed once in sterile saline supplemented with 1% human serum albumin and then resuspended in sterile saline supplemented with 1% human serum albumin for injection. An aliquot of 10,000 DC will be immediately submitted to the Duke Clinical Microbiology Laboratory for Gram Stain. Only after confirmation of a negative Gram stain will DC be administered to study subjects.

6.3.6 Bacterial and Fungal Culture Testing of Each Vaccine Dose

Aliquots of each DC vaccine dose to be administered to a study subject (dendritic cells resuspended in sterile saline supplemented with 1% human serum albumin) will also be submitted for both bacterial and fungal culture testing by the Duke Clinical Microbiology Laboratory. These testing results will, of course, not be available prior to injection of the given vaccine dose, but will be completed 14-28 days after each dendritic cell vaccine dose is administered.

If any post-administration culture results are positive and the given subject has not received a complete course of six vaccinations, no further vaccinations will be administered until the source of contamination has been identified and corrected. As specified above, each dendritic cell product must meet all batch release criteria, which include negative culture testing for bacteria and fungus, before the first vaccine dose can be administered. Therefore, if a subsequent test of an individual vaccine dose is retrospectively found to be positive for contamination, we will first test the saline and the albumin used for washing and resuspension. Aliquots of these solutions will be submitted for endotoxin measurement, gram staining, and bacterial and fungal culture testing. The biologic containment hood used for thawing and washing the dendritic cell product immediately prior to injection will be thoroughly cleaned, following our SOP.

As with all positive cultures performed by the Duke Clinical Microbiology Laboratory, the identity of the contaminating organism will be determined and antibiotic sensitivities will be evaluated. Any subject found retrospectively to have received a dose of contaminated vaccine will be contacted immediately and questioned with regard to any symptoms of infection. If any signs of infection are present, the subject will be asked to immediately report to Duke University Medical Center for evaluation, while subjects without any symptoms of infection will still be asked to report to the outpatient clinic at Duke for evaluation within the following seven days. Any such subject with signs of infection will be treated empirically with broad spectrum antibiotics until the antibiotic sensitivity results are available, at which time the antibiotic regimen will be changed to specifically target the infectious organism.

6.3.7 Viability Determination

After thawing and washing, the viability of the final DC product will be determined by trypan blue exclusion. Only if the viability is $\geq 70\%$ will the DC be administered to study subjects.

6.3.8 Repeat Leukapheresis and Dendritic Cell Generation

If inadequate numbers of dendritic cells are generated for vaccination, or if the generated dendritic cells fail to meet batch release criteria for any reason, the study subject will be asked to undergo repeat leukapheresis (with pre-leukapheresis blood testing) in order to generate additional dendritic cells for vaccination. Such a repeat leukapheresis will not be performed less than 30 days from the time of the prior leukapheresis. In such a case, if the subject elects not to undergo repeat leukapheresis, no dendritic cells will be injected and his/her participation in the study will be terminated.

6.4 Study Arm Assignment

The Study Arm Dose Schedule is shown in Table 4. The first 3 subjects will receive six weekly intradermal (ID) doses of 1×10^7 TAA RNA-transfected dendritic cells derived from untransfected monocytes (Study Arm A). If one or two of these three subjects experience dose limiting toxicities (Grade ≥ 3 toxicity affecting any system or Grade ≥ 2 Allergy/Immunology toxicity) or adverse events, up to 3 additional subjects will be enrolled into Study Arm A, until more than two subjects experience dose limiting toxicity or adverse events or a total of 6 subjects are enrolled in this Study Arm. If none of these initial three subjects experience toxicity or adverse events, up to three additional subjects may be enrolled in this Study Arm (A) at the discretion of the Principal Investigator. If none of the first 3 subjects, or if 2 or fewer of six subjects experience dose limiting toxicity or adverse events the next cohort of 3 subjects will be enrolled in Study Arm B to receive six injections of 1×10^7 TAA RNA-transfected DC derived from monocytes transfected with non-targeting control siRNA. If one or two of these three subjects experience dose limiting toxicities or adverse events, up to 3 additional subjects will be enrolled into Study Arm B, until more than two subjects experience dose limiting toxicity or adverse events or a total of 6 subjects are enrolled in this Study Arm. If none of the first 3 subjects, or if 2 or fewer of six subjects in Study Arm B experience dose limiting toxicity or adverse events, then the next cohort of 6 subjects will be enrolled in Study Arm C to receive six injections of 1×10^7 TAA RNA-transfected DC derived from monocytes transfected with siRNAs specific for the inducible immunoproteasome subunits LMP2, LMP7, and MECL1. Enrollment into Study Arm C will continue until 6 subjects have completed their course of vaccination or until more than two subjects experience dose limiting toxicity. If in preparation of the vaccine, insufficient dendritic cells are available to perform the required six injections, the subject may choose to receive the available cells, but will not be included in the analysis. These subjects will be replaced up to a maximum of 3 subjects per Study Arm. Subjects who progress on therapy and decide to discontinue participation in the study will also be replaced. Accrual to the study will continue until every treatment position is evaluated, or dose-limiting toxicity (DLT) (Grade ≥ 3 toxicity affecting any system, with greater stringency applied to Allergy/Immunology adverse events where a Grade ≥ 2 toxicity will be considered unacceptable) is observed.

TABLE 4. Study Arm Dose Schedule

DOSE SCHEDULE				
Study Arm		Route/Dose	Injection Volume	Number of Injections
A	TAA RNA-transfected DC derived from untreated monocytes (n=3*)	ID/ 1×10^7	0.2 ml	6

B	TAA RNA-transfected DC derived from monocytes transfected with control siRNA (n=3*)	ID/1x10 ⁷	0.2 ml	6
C	TAA RNA-transfected DC derived from monocytes transfected with siRNA targeting the immunoproteasome subunits (n=6)	ID/1x10 ⁷	0.2 ml	6

*if any significant toxicity or adverse events are experienced two or fewer of the first 3 subjects, a maximum of 3 additional subjects may be enrolled in this Study Arm,

6.5 Determination of Safety

Based on a similar DC-based trial in patients with metastatic prostate cancer who were vaccinated with RNA-transfected dendritic cells using the same dose and dosage schedule as proposed in this trial, toxicity is expected to be low (14). It is also expected that sufficient cells will be obtainable to perform all injections and the dose schedule will be both safe and feasible to administer. Safety and efficacy of administration will be monitored. Safety (e.g., no dose limiting toxicity) is defined generally as 2 or less of 6 patients experiencing Grade ≥ 3 toxicity affecting any system, with greater stringency applied to Allergy/Immunology adverse events where a Grade ≥ 2 toxicity will be considered unacceptable. Toxicities considered by the Principal Investigator to be cancer related, i.e. progression of disease, and unrelated to the investigational drug, will not be included when determining dose limiting toxicities. Feasibility is defined as the ability to obtain adequate numbers of cells at leukapheresis to administer all study injections at the specified dose level.

The first 3 subjects will receive six weekly intradermal (ID) doses of 1x10⁷ TAA RNA-transfected dendritic cells derived from untransfected monocytes (Study Arm A). If more than two of these three subjects experience dose limiting toxicities (Grade ≥ 3 toxicity using the NIH scale affecting any system or Grade ≥ 2 Allergy/Immunology toxicity), no further subjects will be enrolled until further discussions with the FDA as well as the Duke IRB. If one or two of these three subjects experience dose limiting toxicities or adverse events, additional subjects will be enrolled into Study Arm A until more than two subjects have experienced dose limiting toxicity or adverse events or a total of 6 subjects have been enrolled in this Study Arm. If none of these initial three subjects experience toxicity or adverse events, up to three additional subjects may be enrolled in this Study Arm (A) at the discretion of the Principal Investigator.

If none of the first 3 subjects, or if 2 or fewer of six subjects in Study Arm A experience dose limiting toxicity or adverse events, the next cohort of 3 subjects will be enrolled in Study Arm B to receive six injections of 1x10⁷ TAA RNA-transfected DC derived from monocytes transfected with non-targeting control siRNA. If more than two of these three subjects experience dose limiting toxicities, no further subjects will be enrolled until further discussions with the FDA as well as the Duke IRB. If one or two of these three subjects experience dose limiting toxicities or adverse events, additional subjects will be enrolled into Study Arm B until more than two subjects experience dose limiting toxicity or adverse events or a total of 6 subjects are enrolled in this Study Arm.

If none of the first 3 subjects, or if 2 or fewer of six subjects in Study Arm B experience dose limiting toxicity or adverse events, then the next cohort of 6 subjects will be enrolled in Study Arm C to receive six injections of 1x10⁷ TAA RNA-transfected DC derived from monocytes transfected with siRNAs specific for the inducible immunoproteasome subunits LMP2, LMP7, and MECL1.

Subjects will continue to be enrolled in this Study Arm until a total of 6 subjects have been enrolled or until more than 2 subjects in this Study Arm experience toxicity.

6.5.1 Safety Observation Period

For study Arm A, in which subjects will be immunized with TAA RNA-transfected dendritic cells derived from untransfected monocytes, after the initial subject has begun vaccination, each subsequent subject will not begin vaccination until the preceding subject has received at least three vaccine doses and no acute severe toxicity has been observed.

The toxicity of vaccination with RNA-transfected DC derived from monocytes transfected with siRNA is unknown. Therefore, for Study Arm B, in which subjects will be immunized with TAA RNA-transfected dendritic cells derived from non-targeting control siRNA-transfected monocytes, after the initial subject has begun vaccination, each subsequent subject will not begin vaccination until the preceding subject has received a full course of six weekly vaccine doses and no acute severe toxicity has been observed for a period of at least two weeks following the sixth vaccination.

Similarly, for Study Arm C, in which subjects will be immunized with TAA RNA-transfected dendritic cells derived from monocytes transfected with siRNAs targeting the immunoproteasome, after the initial subject in this study arm has begun vaccination, each subsequent subject will not begin vaccination until the preceding subject has received a full course of six weekly vaccine doses and no acute severe toxicity has been observed for a period of at least two weeks following the sixth vaccination.

6.6 Administration of DC

Cultured DC will be manufactured, transfected with siRNA and TAA RNA, induced to mature, characterized and stored in the DUMC Immunotherapy Cell Processing Facility according to standard operating procedures. Cryopreserved transfected mature DC will be thawed and then administered via ID routes on study weeks 0, 1, 2, 3, 4, and 5 for a total of 6 complete injections. Each dose will be administered on an outpatient basis in the Duke General Clinical Research Unit or Melanoma Clinic. If additional DC are available for a given subject, they will be administered on a monthly basis until no further DC vaccine remains.

Subjects will report to the Duke University General Clinical Research Unit or Melanoma Clinic on the day of the injection. A study physician or the study nurse will perform an interim history and physical examination. A study physician or designee will deliver the ID injection using a 1ml syringe equipped with a 25-gauge needle. In order to optimize immunizations, injection sites will be rotated between the upper arms and upper thighs. Each site will be marked. Vital signs will be monitored prior to the injection and at 15 and 30 minutes after the ID injection to ascertain that the injections are tolerated without acute toxicity. Acetaminophen, non-steroidal inflammatory agents or antihistamines may be used as needed for treatment of symptoms.

6.7 Vaccine Injection Site Skin Biopsy

Forty-eight hours following the fourth dendritic cell vaccination, a 6 mm punch biopsy of the skin at the vaccine injection site will be performed in the clinic under local anesthesia. In the laboratory, infiltrating T cells will be isolated from this skin and assessed for melanoma tumor-associated antigen specificity using immunologic assays. The biopsy site will be closed with sutures that will be removed at the time of vaccination 6. The detection of melanoma antigen-specific T cells

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within such skin biopsy specimens has been shown by de Vries, et al, to correlate with clinical outcome in melanoma patients treated using other vaccine approaches (33) .

6.8 Repeat Leukapheresis

A second leukapheresis will be performed 2 weeks following the last injection to obtain large numbers of cells for extensive immunologic testing, as described in section 5.8. In the event the subject cannot undergo or refuses a second leukapheresis, 100 ml of peripheral blood will be obtained and used for immunologic testing.

7.0 STUDY SUBJECT EVALUATION

7.1 Evaluation During and After DC Administration

An interval history with physical examination, review of systems, performance status, patient weight, and review of concomitant medications will be performed prior to each DC injection. All peripheral blood samples will be drawn before DC administration, according to the Schedule of Activities (Appendix A), with the volume of blood to be draw at each visit listed in the Blood Draw Requirements (Appendix C). Following administration of RNA-transfected DC, subjects will be observed for 30 minutes to assure the absence of any acute toxicity.

7.1.1 Acute Management Plan for Allergic Reactions

If a subject exhibits any signs of allergic reaction to a vaccine dose, such as acute onset of hives, erythema, or edema at the injection site, benadryl (25 mg) will immediately be administered subcutaneously by injection. An intravenous catheter will also be inserted. If allergic symptoms worsen (shortness of breath develops, decreased blood pressure) an intravenous saline infusion will be initiated and intravenous steroids (solumedrol) will be administered. For the treatment of hypotension, subcutaneous epinephrine injections will be administered, as needed. At this point, the code team will also be notified in case of airway edema and the possible need for intubation, and the subject will be either transferred to the Duke Emergency Department or directly admitted to Duke University Medical Center.

7.2 Hematological and Biochemical Assessment

Complete blood count and chemistries including: Na, K, Cl, CO₂, BUN, Creatinine, Glucose, Total Protein, Albumin, Calcium, Phosphorus, AST, Alkaline Phosphatase, Total Bilirubin, and ALT will be completed at every visit during the vaccination phase. Hematological and biochemical assessment will be repeated at the time of the second leukapheresis, and will be repeated according to the Schedule of Activities. Prothrombin time (PT) and partial thromboplastin time (PTT) will be completed every other visit during the vaccination phase and repeated according to Schedule of Activities.

7.2.1 Tumor Markers

Serum LDH will be obtained every visit during the vaccination phase, at the time of the second leukapheresis, and repeated as clinically indicated and according to the Schedule of Activities.

7.2.2 Quantative PCR Analysis of Melanoma Markers in Whole Blood

10 ml of blood samples will be collected serially for qPCR analysis. After removal of RBC, RNA will be extracted from the mononuclear cells and stored at -80°C. cDNA synthesized

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from this RNA will be used as a template for quantitative PCR assessment of each of the four melanoma TAAs used for vaccination as well as additional tumor cell markers.

7.3 Radiological and Other Assessment

All disease considered measurable at the time of the radiograph review at study entry will be re-evaluated with CT, MRI, PET scanning, or bone imaging following the final DC injection, according to assigned dose schedule and as clinically indicated for one year or until removed from study.

7.4 Autoimmune Studies

ANA, RF, TSH, and anti-thyroglobulin thyroid antibody levels will be reassessed at the time of the second leukapheresis.

7.5 Immunological Monitoring Studies

As described in the Schedule of Activities (Appendix A), subjects will have blood samples drawn prior to receiving each DC injection for immunologic analysis. T cells will also be isolated from a biopsy of the skin at the vaccine injection site collected 48 hours after DC vaccine injection number 4 and will be used for immunologic analysis. Obtaining samples for immunological monitoring after the second leukapheresis will be based upon immunologic response.

7.6 Evaluation for Premature Withdrawals

Study evaluations should be conducted according to the Schedule of Activities presented in Appendix A. For subjects who receive treatment and are prematurely withdrawn from the study, the investigator will determine the degree of follow-up based on the subject's condition and reason for withdrawal.

7.7 Follow-up Evaluation

After the repeat leukapheresis for immune analysis, follow-up, consisting of biochemical and hematologic assessment, LDH level, review of systems, Karnofsky performance status, and review of concomitant medications will be completed according to Schedule of Activities every two weeks for one month, then monthly for the following two months. Long-term standard clinical follow-up, consisting of LDH measurement and physical examination will continue every three months for one year, then every 6 months for one year, then yearly thereafter, as is standard for patients with melanoma treated at Duke University Medical Center. Study evaluations will be conducted according to the Schedule of Activities presented in Appendix A. For subjects who receive treatment and are prematurely withdrawn from the study, the investigator will determine the degree of follow-up based on the subject's condition and/or reason for withdrawal.

7.8 Management of Concurrent Events

7.8.1 Concomitant Medications

7.8.1.1. The following medications will exclude subjects from this protocol:

7.5.1.1(a) Concomitant chemotherapeutic agents.

7.4.1.1(b) Subjects requiring therapy with corticosteroids or other immunosuppressive agents.

7.4.1.1(c) Other forms of immunotherapy.

- 7.8.1.2. After meeting the inclusion criteria, all other medications deemed appropriate for the subject by the investigator may be administered. All medications and changes in medication during study participation will be recorded.

7.9 Risks and Toxicities Related to DC Administration

- 7.9.1 Toxicity will be graded according the NCI Common Toxicity Criteria, Version 3, as described in Appendix D. This protocol will be considered successful if no unacceptable toxicity (Grade ≥ 2 Allergy/Immunology toxicity or Grade ≥ 3 affecting any other system) occurs.
- 7.9.2 Safety variables will include grading of the incidence and severity of pulmonary toxicity, hematological parameters, blood chemistries and urine analysis. Vital signs and subjective symptoms will be recorded using a standardized grading system.
- 7.9.3 Possible immediate side effects from DC injection may include allergic reactions such as fever, hives, or rash. Other possible side effects include skin necrosis or pulmonary compromise although, to our knowledge, these have not been reported. The subjects will be carefully monitored for potential induction of autoimmunity as a result of DC-based immunotherapy by obtaining anti-nuclear antibody, rheumatoid factor, TSH and anti-thyroglobulin antibody tests, in addition to clinical adverse events that may signal an autoimmune disease. Grading of autoimmune toxicity according the NCI toxicity criteria can be found in Appendix D.

8.0 STUDY ENDPOINTS AND ANALYSIS

8.1 Study Endpoints

This is a phase 1 safety study of active immunotherapy using mature DC transfected with siRNA and TAA RNA in subjects with metastatic melanoma. A minimum of three and a maximum of eighteen subjects will be enrolled on this trial. Every enrolled subject who receives the assigned number of injections for treatment will be evaluated for toxicity.

8.1.1 To determine the short and long term toxicities associated with administration of mature DC transfected with siRNA and TAA RNA in subjects with metastatic melanoma.

- a. The incidence, type, and severity of adverse events will be recorded during all phases of treatment and follow-up according to the Schedule of Activities shown in Appendix A.
- b. For Study Arms A and B, after the first subject in the given study arm is vaccinated, each subsequent subject in that study arm will not begin vaccination until the preceding subject has received at least three vaccine doses and no acute significant toxicity has been observed. For Study Arm C, after the first subject is vaccinated, each subsequent subject will not begin vaccination until at least two weeks have elapsed since the preceding subject has either completed the vaccination schedule or has been removed from the study, and no acute significant toxicity has been observed. Enrollment of subjects into Study Arm B will only occur if no dose-limiting toxicity is observed in Study Arm A, while enrollment of subjects into Study Arm C will only occur if no dose-limiting toxicity is observed in Study Arm B.

8.1.2 To determine the cellular immune response to intradermal injection of mature DC transfected with siRNA and TAA RNA.

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The following parameters will be measured according to the Schedule of Activities shown in Appendix A.

- a. The frequencies of melanoma TAA-specific CD8 T cells in peripheral blood.
- b. The functional activity of the induced T cells to kill HLA-matched and/or autologous TAA expressing DC or autologous melanoma targets (if available).

8.1.3 To assess any clinical responses of subjects with metastatic melanoma after DC administration.

- a. As this is a safety and toxicity study, the primary response to be measured is any evidence of toxicity in the subjects. In addition, potential clinical response will be determined by performing a history, physical exam, by monitoring serum LDH levels, and by reviewing any available radiographic studies completed prior to and following treatment, including bone scans, chest radiographs, PET scans, and MRI/CT scans.
- b. The following clinical response criteria will be used:

LDH Response

In almost all patients with progressive metastatic melanoma, serum LDH are elevated (>240 U/L) and continue to rise with progression of disease (34). We will therefore measure serum levels of LDH prior to each immunization, at the time of repeat leukapheresis, and during follow-up, according to the Schedule of Activities (Appendix A).

Objective Response

Complete response (CR): Disappearance of all clinical evidence of tumor on examination, X-ray, CT, etc., and/or biochemical evaluation, for a minimum of 4 weeks.

Partial response (PR): Greater than 50% decrease on physical examination or radiography (including CT, MRI, Ultrasound) of the summed products of the perpendicular diameters of all measured lesions maintained for a minimum of 4 weeks.

Minor response (MR): A 25%-49% decrease in the summed products of the perpendicular diameters of measured lesions maintained for a minimum of 4 weeks.

Stable disease (STAB): Less than 25% decrease in tumor size for at least 3 months, including no new lesions on imaging studies or examination.

Progression (PROG): Greater than 25% increase of tumor marker serum levels. Greater than 25% increase in the summed products of the perpendicular diameters of measured lesions or appearance of new lesions, or appearance of a new lesion on imaging studies.

Duration of objective response is measured from the first observation of response until progression as defined above.

8.2 Statistical Considerations

The primary endpoint of this study is safety of treatment with mature DC transfected with siRNA and TAA RNA. For Study Arm A and B, a Dose Schedule will be considered safe if the initial three subjects in the given Study Arm do not experience grade ≥ 2 Allergy/Immunology toxicity or Grade ≥ 3 toxicity affecting any other system or if six subjects in the given Study Arm are treated and two or fewer subjects experience Grade ≥ 2 Allergy/Immunology toxicity or Grade ≥ 3 toxicity affecting any other system. For Study Arm C, the Dose Schedule will be considered safe if six subjects in this Study Arm are treated and two or fewer subjects experience Grade ≥ 2 Allergy/Immunology toxicity or Grade ≥ 3 toxicity affecting any other system. Toxicities considered by the Principal Investigator to be cancer related, i.e. progression of disease, and unrelated to the investigational drug will not be included when determining dose limiting toxicities and

recommended dose level. Subjects who have completed the entire treatment schedule will be considered appropriate for evaluation of toxicity. A grade ≥ 3 toxicity affecting any system will be considered undesirable or dose-limiting. If more than two of 6 subjects in a dose level experience unacceptable toxicity, the dose schedule will be considered unsafe. The probability of rejecting the study dose level after 6 subjects is tabulated below as a function of the true probability of unacceptable toxicity.

True Probability of Unacceptable Toxicity	Probability of Concluding that the Treatment is Unsafe (i.e. 3 or more of 6 subjects with DLT)
0.1	0.114
0.2	0.345
0.3	0.580
0.33	0.649
0.4	0.767
0.5	0.891

We strongly anticipate that vaccination with TAA RNA-transfected mature autologous DC derived from control siRNA-transfected monocytes (Study Arm B) will be safe and non-toxic and will be comparable to vaccination with TAA RNA-transfected mature autologous DC derived from untreated monocytes (Study Arm A). For this reason, we plan to initially enroll only three subjects into each of these Study Arms and for the purposes of immunologic comparisons will pool the results from subjects in these two “control” Study Arms. Thus, we will compare the immunologic responses noted in subjects in Study Arm C, vaccinated with TAA RNA-transfected mature autologous DC derived from monocytes transfected with siRNA targeting the immunoproteasome, with the responses in subjects in the combined Study Arms A and B.

8.3 Statistical Analysis of Study Results

Toxicity due to this treatment is expected to be low. A description of each of the following study endpoints will be made at the conclusion of subject accrual and one year of follow-up:

1. The number of subjects experiencing Grades 1, 2, 3, and 4 toxicities, the nature of these events, and the timing of their occurrence.
2. The presence and magnitude of detectable T cell frequency and immune activity specific for melanoma TAAs. For the purposes of these comparisons, immunologic response data from participants in Study Arms A and B will be pooled for comparisons with data from Study Arm C.
3. The serum LDH levels at baseline and during follow-up.
4. The number of subjects achieving complete or minor clinical remissions, no response, or stable disease.

The results of these evaluations will guide the development of subsequent phase 2 trials.

9.0 REASONS FOR STUDY DISCONTINUATION

Subjects may be removed from the study for the following reasons:

- 9.1 Unacceptable toxicity (Grade ≥ 3 toxicity affecting any system with greater stringency applied to Allergy/Immunology adverse events where a Grade ≥ 2 toxicity will be considered unacceptable).

9.2 Subject voluntarily decides to withdraw.

9.3 Subject non-compliance with the study protocol.

9.4 Intercurrent disease, which would affect the assessment of the subject's clinical status to a significant degree.

10.0 STUDY CONDUCT, ETHICAL AND REGULATORY CONSIDERATIONS

10.1 Institutional Review Board (IRB)

This phase I study must be approved by the IRB as defined by the CFR Title 21, volume 5, section 312. IRB approval and the informed consent form for this study must be given in writing. The IRB must also approve any significant changes to the protocol as well as a change of principal investigator. Records of all study review and approval documents must be kept on file by the investigator and are subject to inspection during or after completion of the study. Adverse experiences as defined in Section 10.3.2 must be reported to the IRB. The IRB will receive notification of the completion of the study and final report following study completion or termination. The investigator must maintain an accurate and complete record of all submissions made to the IRB.

10.2 IRB Reporting

According to Standing Operating Procedure, the following will be reported to the IRB:

10.2.1 Annual Progress Report: Due prior to the initial IRB approval anniversary date. Includes number of subjects enrolled by race and gender (past year and accumulative), number of premature withdrawals and reason for withdrawals, description of adverse events, protocol and informed consent changes, preliminary results, if any, and results of audits performed.

10.2.2 Safety Report: According to DUMC IRB Adverse Event Reporting Guidelines, only adverse events that the PI determines to be serious, unanticipated, and related or possibly related to the research must be reported to the IRB. The PI will review each adverse event in terms of its relationship to the study and address possible changes in the risk-benefit ratio that necessitate changes in the protocol and/or consent form. The DUHS Adverse Event (AE) Report form will be completed to determine reportable events.

10.2.3 Amendments: Any significant change in the study design or treatment plan will be approved by the IRB chairman prior to implementation. This report includes rationale and details of the amendment, the revised protocol version, and the informed consent with changes highlighted.

10.2.4 Final Report: When the research project is completed, the investigator will promptly notify the IRB and file a final report. This report includes accumulative number of subjects enrolled by race and gender, number of premature withdrawals and reason for withdrawals, summary of adverse events, results of audits performed, and study findings.

10.3 Adverse Events

In compliance with the Federal Registry, 21 CFR 312.32, effective April 1, 2001, we will use the following definitions and criteria for adverse event reporting.

10.3.1 Definitions

Associated with the use of the drug: There is a reasonable possibility that the experience may have been caused by the drug.

Disability: A substantial disruption of a person's ability to conduct normal life functions.

Life-threatening adverse drug experience: Any adverse drug experience that places the subject or subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

Serious adverse drug experience: 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, 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 appropriate medical judgment, they may jeopardize the subject or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result inpatient hospitalization, or the development of drug dependency or drug abuse.

Unexpected adverse drug experience: Any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure; or, if an investigator brochure is not required or available, the specificity or severity of which is not consistent with the risk information described in the general investigational plan or specified elsewhere in the current application. For example, under this definition, hepatic necrosis would be unexpected (by virtue of greater severity) if the investigator brochure only referred to elevated hepatic enzymes or hepatitis. Similarly, cerebral thromboembolism and cerebral vasculitis would be unexpected (by virtue of greater specificity) if the investigator brochure only listed cerebral vascular accidents. "Unexpected," as used in this definition, refers to an adverse drug experience that has not been previously observed (e.g., included in the investigator brochure) rather than from the perspective of such experience not being anticipated from the pharmacological properties of the pharmaceutical product.

10.3.2 Adverse Event Reporting

The Safety Requirements for Reporting Adverse Events (21 CFR 312.32) will be adhered to fully. Until reporting of any serious or unexpected events is completed and discussions with the FDA and IRB are also satisfactorily completed, no further vaccinations will be administered. All other less serious events will be included in the annual report to the FDA.

10.4 Informed Consent

The investigating physician will explain the study's goals, risks, potential discomforts, and limitations to each prospective subject. In accordance with guidelines in the Federal Register, 45 CFR 46.116, all subjects are required to sign a statement of informed consent. This phase 1 study involves research, which presents risk, but holds the prospect of direct benefit to the individual subject. The consent form is attached to this protocol (Appendix E). The investigator will report to the IRB, the FDA, and OBA changes in the research protocol and all unanticipated problems involving risks to human subjects and others, and no changes will be implemented in the research activity without IRB approval.

10.5 Documentation and Monitoring

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Data will be collected for all subjects enrolled. Accurate completion of the case report forms for all subjects is the responsibility of the investigator.

10.5.1 Case Report Form (CRF)

The CRF is used to record study data and is an integral part of the study and subsequent reports. Therefore, all reports must be legible and complete. All forms should be filled out using a black ballpoint pen. Errors should be lined out but not obliterated and the correction inserted, initialed, and dated by the study coordinator. A CRF must be completed and signed by the principal investigator for each subject enrolled in the study who has completed the initial leukapheresis. For subjects who are removed from the study, the reason for removal must be noted on the final report by the investigator for each subject. CRF must be kept current to reflect subject status at each phase during the course of the study. Subjects are not to be identified on CRF by name; appropriate coded identification and subject initials must be used. Because of the potential for errors, inaccuracies, and illegibility in transcribing data onto CRF, copies of laboratory and other test results will be kept on file with subject's CRF or clinical chart. CRF and copies of test results will be available at all times for inspection.

10.5.2 Maintenance of Study Documentation

It is the responsibility of the investigator and the study staff to maintain a comprehensive and centralized filing system of all relevant study documentation. Such documentation includes:

- a. CRF/Subject Files - which must be kept legible, accurate, and up-to-date, substantiates the data entered on the case report forms for all required test and evaluation procedures
- b. Signed Informed Consent - verifies that the subject has signed an informed consent to enter the study.
- c. Subject Exclusion Record - which should reflect the reason any subject was screened and found ineligible for the study.
- d. Regulatory Documents - including protocol, FDA Form 1572, CVs, IRB correspondence, IRB approval/renewals, and IRB approved consent form.
- e. Adverse Event Forms - which should explain any serious or unexpected adverse experiences.

All study documentation pertaining to the conduct of the study must be kept on file by the investigator for a minimum of six years or until after the study is completed, whichever is longer. The investigator and staff are expected to cooperate and provide all relevant study documentation upon request for review.

10.6 Departure from the Protocol

There should be no departure from the protocol if at all possible. If an emergency occurs that requires departure from this protocol, the physician in attendance in such emergency will, if circumstances and time permit, contact the principal investigator immediately by telephone. Such contacts with the principal investigator will be made to permit a decision as to whether or not the subject will be continued on the study. Such departures need to be clearly documented and reported to the IRB by the principal investigator.

10.7 Subject Privacy

All subject data will be identified only by subject identification number and subject initials to protect the subject's privacy. The data will be blinded accordingly in all data analysis. However, in compliance with federal guidelines regarding the monitoring of clinical studies, it is required that the investigator permit review of that portion of the subject's medical record that is directly related

to the study. This will include all relevant study documentation including medical history, to verify eligibility, laboratory test results to verify transcription accuracy, X-ray reports, admission, discharge summaries for hospital/outpatient admissions while the subject is on-study, and reports for deaths occurring during the study. As part of the required content of informed consent, the subject must be informed that the investigator and regulatory agencies may review his medical chart. Should access to the medical record require a separate waiver or authorization, it is the investigator's responsibility to obtain such permission from the subject in writing before the subject is entered into the study.

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APPENDIX A: Schedule of Activities

	Screening	Biopsy of Melanoma Metastasis	Leukapheresis	Vaccination Period							Follow-up			Follow-up Based on Anti-Melanoma Immune Response				
				-6	-5	-4	0	1	2	3	4	5	2	1	2	3	6	9
Study Week																		
Follow-up (weeks after last vaccination)																		
Follow-up (months after last vaccination)																		
Review of Eligibility Criteria	X																	
History & Physical	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Review of Systems	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Review of Medications	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
KPS	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Weight	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Consent	X																	
Review of Pathology & Immunohistochemistry	X																	
Review of Radiologic Studies	X																	
Infectious Disease (HIV, Hep BsAg, Hep C)	X																	
Autoimmune Studies (ANA, RF, TSH, Anti-thyroglobulin Ab)	X																	
Hematologic Analysis (CBC with diff, Platelets)	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Biochemical Analysis (OP 15)	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Coagulation Analysis (PT, PTT)	X																	
Serum Pregnancy Test (Women of childbearing potential)	X																	
Diphtheria Antibody Titer	X																	
Limited HLA Typing	X																	
LDH Level	X				X	X	X	X	X	X	X	X	X	X	X	X	X	X
Study Procedures																		
Biopsy of Melanoma Metastasis		X ¹																
Leukapheresis			X											X ²				
DC Vaccination					X ³	X ³	X ³	X ³	X ³	X ³	X ³	X ³	X ³	X ³	X ⁴	X ⁴	X ⁴	X ⁴
Biopsy of Skin at Site of Vaccination																		
Immunological Monitoring																		
Assessment of Anti-melanoma Immune Responses																		
					X	X	X	X	X	X	X	X	X	X	X	X	X	X

¹ Initial biopsy of melanoma metastasis for immunohistochemical TAA expression analysis, cell culture and RNA extraction
² Optional repeat leukapheresis for immunologic monitoring vs collection of 100 ml of peripheral blood
³ 10 million RNA transfected DC administered by intradermal injection
⁴ Additional monthly DC Vaccination based on DC availability and lack of adverse events during primary vaccination series
⁵ 6mm punch biopsy of skin at site of 4th vaccination under local anesthesia

ACTIVE IMMUNOTHERAPY OF MELANOMA WITH AUTOLOGOUS DENDRITIC CELLS TRANSFECTED WITH TUMOR ANTIGEN RNA AND SMALL INHIBITORY RNAs TO ALTER PROTEASOMAL ANTIGEN PROCESSING

Principal Investigator: Scott K. Pruitt, M.D., Ph.D.

ELIGIBILITY CHECKLIST

INCLUSION CRITERIA. All must be checked “Yes” for study inclusion.	Yes	No
1. The subject has histologically confirmed melanoma, Stage any T, N+ or M+, with nodal or distant metastasis (determined by fine needle aspiration cytology or biopsy of clinically involved lymph nodes or metastatic lesions).	<input type="radio"/>	<input type="radio"/>
2. The subject has a Karnofsky performance status greater than or equal to 70%.	<input type="radio"/>	<input type="radio"/>
3. The subject is 18 years of age or older.	<input type="radio"/>	<input type="radio"/>
4. The subject has adequate hematologic function with WBC \geq 3000 mm ³ Hemoglobin \geq 9 mg/dl Platelets \geq 100,000/mm ³	<input type="radio"/> <input type="radio"/> <input type="radio"/>	<input type="radio"/> <input type="radio"/> <input type="radio"/>
5. The subject has adequate renal and hepatic function with: Serum Creatinine < 2.5 mg/dl Bilirubin < 2.0 mg/dl AST/SGOT < 70 U/L ALT/SGPT < 70 U/L Alkaline Phosphatase \leq 135 U/L	<input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	<input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>
6. The subject has the ability to understand and provide signed informed consent that fulfills Institutional Review Board guidelines.	<input type="radio"/>	<input type="radio"/>
7. The subject has the ability to return to the investigating site for adequate follow-up as required by the protocol.	<input type="radio"/>	<input type="radio"/>

ELIGIBILITY CHECKLIST (continued)

EXCLUSION CRITERIA. All must be checked "No" for study inclusion.	Yes	No
1. The subject is receiving concurrent chemotherapy, radiation therapy, or immunotherapy. (Subjects must wait 6 weeks after completion of radiation therapy or chemotherapy and 8 weeks after completion of any immunotherapy prior to initiation of treatment.)	<input type="radio"/>	<input type="radio"/>
2. The subject has previously irradiated, surgically treated, or new central nervous system (CNS) metastases. (Pre-enrollment head CT is not required if not indicated by clinical signs or symptoms.)	<input type="radio"/>	<input type="radio"/>
3. The subject has a history of autoimmune disease such as, but not restricted to, inflammatory bowel disease, systemic lupus erythematosus, ankylosing spondylitis, scleroderma, or multiple sclerosis.	<input type="radio"/>	<input type="radio"/>
4. The subject has a serious intercurrent chronic or acute illness such as pulmonary (asthma or COPD), cardiac (NYHA class III or IV) or hepatic disease, or other illness considered by the P.I. to constitute an unwarranted high risk for investigational drug treatment.	<input type="radio"/>	<input type="radio"/>
5. The subject has a medical or psychological impediment to probable compliance with the protocol.	<input type="radio"/>	<input type="radio"/>
6. The subject has a concurrent second malignancy other than non-melanoma skin cancer or <i>in-situ</i> or thin (Breslow depth <1 mm) melanoma. (In the event of prior malignancies treated surgically, the subject must be considered no evidence of disease (NED) for a minimum of 3 years prior to enrollment.)	<input type="radio"/>	<input type="radio"/>
7. The subject has an active acute or chronic infection, including symptomatic urinary tract infection, HIV (as determined by ELISA and confirmed by Western Blot) or viral hepatitis (as determined by HBsAg and Hepatitis C serology).	<input type="radio"/>	<input type="radio"/>
8. The subject is receiving systemic steroid therapy or other immunosuppressive agents such as azathioprine or cyclosporine A. Steroid therapy must be discontinued 6 weeks prior to enrollment.	<input type="radio"/>	<input type="radio"/>
9. The subject is female with a positive pregnancy test, or is sexually active, has not previously undergone hysterectomy and/or bilateral oophorectomy and is unwilling to utilize a medically approved form of contraception during from the time of enrollment until 6 weeks after the final immunization.	<input type="radio"/>	<input type="radio"/>
10. The subject is male, has not previously surgically sterilized, and is unwilling to use a condom with spermicide during any sexual activity occurring over the entire immunization period and for the 6 weeks that immediately follow the final immunization.	<input type="radio"/>	<input type="radio"/>
11. The subject has inadequate peripheral vein access to undergo leukapheresis.	<input type="radio"/>	<input type="radio"/>
12. The subject has a documented history of a severe allergic reaction to beta-lactams, eggs or soy.	<input type="radio"/>	<input type="radio"/>

Study Coordinator:
Nancy J, Pickett, RN
Phone: (919) 684-3726
Fax: (919) 684-6044

APPENDIX C: Blood Draw Requirements (ml)

	Screening	Leukapheresis	Vaccination Period					Follow-up			Follow-up Based on Anti-Melanoma Immune Response				
			0	1	2	3	4	5	1	2		3	6	9	12
Study Week	-6	-4	0	1	2	3	4	5							
Follow-up (weeks after last vaccination)									2						
Follow-up (months after last vaccination)										1	2	3	6	9	12
Volume of Blood to be Drawn (ml)															
Infectious Disease (HIV, Hep BsAg, Hep C)	10														
Autoimmune Studies (ANA, RF, TSH, Anti-thyroglobulin Ab)	10								10						
Hematologic Analysis (CBC with diff, Platelets)	3		3	3	3	3	3	3	3	3					
Biochemical Analysis (OP 15)	3		3	3	3	3	3	3	3	3					
Coagulation Analysis (PT, PTT)	5			5		5		5		5					
Serum Pregnancy Test (Women of childbearing potential)	3														
Limited HLA Typing	3														
Serum Collection			3							3	3				3
LDH Level	3		3	3	3	3	3	3	3	3					3
Blood for RNA Extraction			10	10	10	10	10	10	10	10					10
Immunological Monitoring			120	60	60	60	60	60	60	60	100 ^a	60	60 ^b	60 ^b	60 ^b
Total Blood Draw Per Visit	40		142	79	84	79	84	79	84	79	37	63	24		16
Total Blood Draw for Entire Study			711^c												

^a Collection of 100 ml of peripheral blood if study subject does not undergo repeat leukapheresis

^b Blood collected for immunologic monitoring only if anti-melanoma immune response above baseline based on most recent assessment

^c Additional blood drawn based on whether subject undergoes repeat leukapheresis and on persistence of anti-melanoma immune response



Consent To Participate In A Research Study
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ANTIGEN PROCESSING

You are being asked to take part in this research study because you have metastatic melanoma (melanoma that has spread from the skin to other parts of you body). This study is designed to test the safety of a new type of melanoma vaccine. Research studies include only people who choose to take part. Please read this consent form carefully and take your time making your decision. As your study doctor or study staff discusses this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. We encourage you to talk with your family and friends before you decide to take part in this research study. The nature of the study, risks, inconveniences, discomforts, and other important information about the study are listed below.

Please tell the study doctor or study staff if you are taking part in another research study.

Dr. Scott K. Pruitt will conduct the study and it is funded by the Melanoma Research Fund, a philanthropic organization composed primarily of patients treated at Duke for melanoma and their family members.

WHO WILL BE MY DOCTOR ON THIS STUDY?

If you decide to participate, Dr. Scott K. Pruitt will be your doctor for the study and will be in contact with your regular health care provider throughout the time that you are in the study and afterwards, if needed.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to test the safety of a new investigational cancer vaccine strategy. This vaccine is designed to boost the immune system against the melanoma cells within your body. It is believed that the body's immune system can attack tumor cells and kill them. In most patients with advanced cancer, including metastatic melanoma; however, the immune system is not able to adequately destroy the cancer.

This study is "investigational" because the study vaccine is being tested in research studies and is not approved by the U.S. Food and Drug Administration (FDA).



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HOW MANY PEOPLE WILL TAKE PART IN THIS STUDY?

Up to 18 people will take part in this study at Duke University Medical Center.

WHAT IS INVOLVED IN THE STUDY?

If you are interested in participating in this study and you are found eligible, you will first be asked to undergo a biopsy (surgical removal) of a portion of your metastatic melanoma either during surgical treatment for your melanoma or by making an incision in your skin under local anesthesia (numbing medicine) in the clinic. A small sample (approximately 3 millimeters in diameter) of normal skin will also be collected that time using the same incision. You will then undergo leukapheresis, a laboratory procedure in which white blood cells are separated from a sample of blood. Monocytes, a type of immune cell, will then be purified from the collected white blood cells. These monocytes will then either be left untreated, or will be mixed with small inhibitory RNAs (siRNAs), tiny strings of nucleic acids which can block production of specific proteins inside of cells. The siRNAs to be tested in this study are either control siRNA that does not block production of a specific protein or a mixture of siRNAs that are designed to block production of three specific proteins of the immunoproteasome (a group of proteins that chops up unneeded proteins within the cell). These monocytes will then be used to generate dendritic cells (immune cells), which will then be mixed with melanoma protein RNAs, nucleic acids which make proteins which are manufactured inside melanoma cells. You will then receive six weekly injections of these dendritic cells under your skin.

If you agree to be in this study, you will be asked to sign this consent form. You will have the following tests and procedures to make sure that you are eligible:

- ***Physical examination and medical history***
- ***Vital signs***
- ***Blood tests***

You will have approximately 40 ml of blood (about 3 tablespoons) drawn to perform general lab tests such as blood count and chemistries to determine whether or not you will qualify for this study.



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As part of this protocol, you will be tested for Hepatitis and HIV (human immunodeficiency virus, which is the virus that causes the acquired immunodeficiency syndrome [AIDS]). You will be notified of the results of the testing, and counseled as to the meaning of the results, whether they are positive or negative. If the test indicates that you are infected with HIV or Hepatitis, you will receive additional counseling about the significance of your care and possible risks to other people. We are required to report all positive HIV and Hepatitis results to the North Carolina State Board of Health. The test results will be kept confidential to the extent permissible under the law. If you do not want to be tested for HIV and Hepatitis, then you should not agree to participate in this study.

Biopsy of Deposit of Metastatic Melanoma

If you are eligible for the study and agree to participate, you will be asked to have a biopsy of a metastatic melanoma site (surgical removal of the melanoma deposit). The size of the removed melanoma tissue will vary, depending upon the size of the melanoma tissue deposit. A small sample of normal skin (approximate size = ●) will also be collected at the same time from the edge of the skin incision. If you are scheduled for surgery, the biopsy will be done during the scheduled surgical procedure. If you are not scheduled for surgery, you will be asked to have the biopsy done in the clinic. A biopsy, which requires the use of a local numbing medicine injected into the skin and an incision (knife cut) in the skin approximately 1-2 inches in length, may cause some discomfort. The numbing medicine will be given by a small injection into the skin at the site of the skin incision. To speed healing, several stitches (also known as sutures) may be placed, which will be removed 5 - 10 days later. During the healing process, you will be asked to keep the biopsy site clean and dry. Only 10-15 minutes will be required for the biopsy procedure. After the numbing medicine wears off, there will be some soreness at the biopsy site that will last for a few hours. A small scar will develop at the site of the biopsy. A portion of the collected melanoma tissue will be sent for routine evaluation by the Department of Pathology. The remainder of the melanoma tissue, as well as the sample of normal skin, will be transported to the research laboratory for further processing.



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Leukapheresis

If you are eligible and agree to participate in the study, you will have white blood cells collected from your blood by a procedure called leukapheresis. Leukapheresis is a standard medical procedure by which large numbers of selected blood products including white blood cells can be obtained. Leukapheresis requires insertion of a large needle into both arms. You will lie in a bed for 2-4 hours while blood is collected, much like in a standard blood donation. The main difference is that only the white blood cells are collected while the red blood cells are returned to you. A medication (called citrate) is added to the blood while in the machine to keep the blood from clotting in the tubing.

The cells that are collected will be brought to a laboratory where cells called monocytes will be isolated. These monocytes will then either be untreated or will be mixed with a type of RNA (nucleic acid) called small inhibitory RNA (siRNA) which can block production of specific proteins within cells. The type of siRNA that will be mixed with your dendritic cells will be either a control siRNA that is designed to not block any proteins, or a mixture of siRNAs designed to block three proteins that are part of the immunoproteasome, a group of proteins that chops up unneeded proteins inside of cells. These monocytes will then be grown in an incubator to produce the dendritic cells (immune cells). The dendritic cells will then be mixed with another type of RNA which directs the production of melanoma proteins. These dendritic cells mixed with RNA, and made from either untreated or siRNA treated monocytes, will then be frozen for later use. A small sample will be thawed and tested for evidence of bacterial and fungal infection. If they are infected, you will not be able to receive any injections of the dendritic cells; however, this is unlikely.

It is possible that after the initial leukapheresis and cell processing, there will not be enough dendritic cells for you to be vaccinated or the dendritic cells that have been generated are not suitable for injection. If such a situation occurs, you will be asked to undergo a repeat leukapheresis (with pre-leukapheresis blood testing) to generate new dendritic cells for vaccination. In such a case, if you decide not to undergo repeat leukapheresis, you will not be vaccinated and your participation in this study will end.



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Dendritic Cell Vaccination

Once the testing of the dendritic cells is completed, you will return to the clinic every week for a total of 6 visits to receive an injection of your dendritic cells. Each visit will take approximately 1 hour. The injections will be given under your skin, and the sites will be rotated between upper arms and thighs. You will be asked to remain at the Melanoma Clinic for 30 minutes after each injection to be certain you have no reactions. If additional vaccine cells are available, you may choose to receive additional vaccinations at monthly intervals until your vaccine cells have all been used.

Monitoring and Blood Draws

You will be examined on the days you receive your dendritic cell vaccination. You will have approximately 80 ml (5 tablespoons) of blood drawn prior to each injection, with an additional 70 ml (4 and ½ tablespoons) being drawn at the time of your initial vaccination. Two weeks after your final dendritic cell vaccination, you will be asked to undergo repeat leukapheresis, or have 100 ml (about 7 tablespoons) of blood drawn. In addition, you will have 24 ml (less than 2 tablespoon) of blood drawn at this visit to monitor general labs tests such as blood counts and blood chemistries. You will also be asked to return after another 2 weeks for evaluation and have 60 ml (4 tablespoons) of blood drawn. Based on immune cell levels, you may be asked to have 60 ml (4 tablespoons) of blood drawn every month over the following 2 months, then every 3 months until immune cell levels have returned to normal or 2 years have elapsed since your final immunization. If possible, blood draws will be done at the same time as your routine follow-up visits.

Injection Site Biopsy

You will be asked to return to the clinic 48 hours following your fourth dendritic cell vaccination to undergo a punch biopsy of the skin at your most recent vaccine injection site. After cleaning the skin with antiseptic and anesthetizing the skin with an injection of numbing local anesthetic, a 6 mm (approximate size = ●) punch of skin will be removed. To speed healing, several stitches (also known as sutures) will be placed, which will be removed 12 days later, at the time of your 6th vaccination. During the healing process, you will be asked to keep the biopsy site clean and dry. Only 10-15 minutes will be required for the biopsy



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procedure. After the numbing medicine wears off, there will be some soreness at the biopsy site that will last for a few hours. A small scar will develop at the site of the biopsy. Immune cells will be extracted from this skin for monitoring your immune system's response to the vaccine.

Repeat Leukapheresis

Two weeks after receiving your 6th injection, you will be asked to undergo a repeat leukapheresis or have 100 ml (7 tablespoons) blood drawn with your routine laboratory studies to obtain an adequate number of cells for us to perform further immunologic studies. This blood will not be used to generate dendritic cells for your vaccination but will be used to determine if your body has produced immune cells that can recognize and kill melanoma cells.

HOW LONG WILL I BE IN THIS STUDY?

The period between collection of cells (leukapheresis) and your first dendritic cell vaccination is approximately 6 weeks. The initial vaccination period will last 6 weeks, with a total of six vaccinations given once per week. You will return for follow up evaluations 2 weeks and 4 weeks after the final injection, and then one month and two months later for evaluation in clinic. You will then be followed in the clinic every three months for the remainder of the first year after the vaccination, as is standard for melanoma patients seen and treated at Duke. You will then return for visits every 6 months for the next year. At that point (2 years after vaccination), if you desire to continue with your care in the Duke Melanoma Clinic, you will continue with yearly clinic visits and routine laboratory testing until a minimum of 5 years has elapsed.

If sufficient numbers of your dendritic cells are available after the full course of 6 vaccinations is completed, you will be vaccinated with these cells at monthly intervals until they are used up. As with the initial course of vaccinations, you will remain under observation in the clinic for 30 minutes following each injection.

You can choose to stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to your doctor first.



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WHAT ARE THE RISKS OF THE STUDY?

As a result of your participation in this study, you are at risk for the following side effects. You should discuss these with the study doctor and your regular health care provider if you choose.

Biopsy of a metastatic melanoma deposit and collection of a small sample of normal skin in clinic, and a punch biopsy of the skin at the vaccination site – These minor surgical procedures, generally non-hazardous procedures that normally require 10-15 minutes, may cause some, all or none of the side-effects listed below:

More likely

- *After the anesthesia wears off, there will be some soreness at the biopsy site that will last for a few hours.*
- *A small scar will develop at the site of the biopsy.*

Less Likely

- reaction to anesthetic (numbing medicine) - While the local numbing medicine xylocaine is almost entirely free from allergic properties (such as causing hives), an allergic reaction is possible, and you will not be given xylocaine if you have a history of such a reaction.
- excessive bleeding
- bruising
- infection - Infection rarely occurs and is largely prevented by the use of an aseptic (or sterile) biopsy technique and proper wound care. If infection does occur, you will be instructed to keep the wound clean and to apply warm, wet compresses for 15 minutes, two to three times a day, until the infection subsides. If the infection persists, antibiotics may be prescribed, and the biopsy site may need to be opened and packed with gauze dressings.
- excessive scarring

Leukapheresis may cause some, all or none of the side-effects listed below. During the procedure, you are closely monitored for any bad effects.

More likely

- discomfort or a bruise at the site of the needle puncture



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- *chills caused by cooling of the blood when it is contained in the special machine*

Less Likely

- *slight risk of developing a local infection at the needle insertion site*
- light-headedness, fainting, vomiting, and rapid breathing
- tingling and nausea caused by the blood thinning medicine (called citrate)

These last two side effects can be controlled by slowing the rate at which blood is withdrawn, by warm blankets, by changing the amount of the blood thinning medicine, by giving calcium supplements (tablets taken by mouth or liquid calcium given by vein), or by discontinuing the procedure.

Highly Unlikely

- loss of blood, breakdown of the blood, clotting of the blood
- allergic reactions
- accidental addition of air to the blood going back to you
- fluid overload resulting in shortness of breath
- fluid loss resulting in decreased blood pressure

Most side effects can be controlled by discontinuing the leukapheresis procedure and providing appropriate medical care.

- previously unknown side effects
- life-threatening side effects and death

Dendritic Cell Injections may cause some, all or none of the side-effects listed below.

More likely

- soreness and inflammation at the dendritic cell vaccination sites
- Fevers, chills, headache

Less Likely

- allergic reactions with inflammation in such places as your throat or lungs - this could lead to shortness of breath, respiratory failure and death



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- Some other types of cell injections have caused blood clots, but none have been seen with dendritic cell injections.
- Your immune system could be stimulated to attack your own body (called autoimmunity) leading to a low white blood cell count, skin rash, joint swelling, intestinal inflammation, and fluid around the heart and lungs.
- Although the dendritic cell cultures are grown in a sterile environment and are monitored for contamination, it is still possible for an undetected bacteria or fungus to be injected with the dendritic cells. This could cause fever, chills, a drop in blood pressure and/or life threatening infection. These side effects can be serious and may require intensive treatment in the hospital
- There may also be side effects and discomforts that are not yet known.

For Those of Reproductive Potential

Females

Being a part of this study while pregnant may expose the unborn child to significant risks, some of which may be currently unforeseeable. Therefore, pregnant women will be excluded from the study. If you are a woman of childbearing potential, a blood pregnancy test will be done (using 1 teaspoon of blood drawn from a vein by needle-stick), and it must be negative before you can continue in this study. If sexually active, you must agree to use appropriate contraceptive measures for the duration of the study and for three months afterwards. Medically acceptable contraceptives include: (1) surgical sterilization (such as a tubal ligation or hysterectomy), (2) approved hormonal contraceptives (such as birth control pills, patches, implants or injections), (3) barrier methods (such as a condom or diaphragm) used with a spermicide, or (4) an intrauterine device (IUD). Contraceptive measures such as Plan B(TM), sold for emergency use after unprotected sex, are not acceptable methods for routine use. If you do become pregnant during this study or if you have unprotected sex, you must inform your study physician immediately.

Males

Your participation in this research may damage your sperm, which could cause harm to a child that you may father while on this study. Such harm may be currently unforeseeable. If you are sexually active, you must agree to use a medically acceptable form of birth control in order to be in this study and for three months afterward. Medically acceptable contraceptives include: (1) surgical sterilization (such as a vasectomy), or (2) a condom used with a spermicide.



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Contraceptive measures such as Plan B(TM), sold for emergency use after unprotected sex, are not acceptable methods for routine use. You should inform your partner of the potential for harm to an unborn child. She should know that if pregnancy occurs, you will need to report it to the study doctor, and she should promptly notify her doctor.

Risks of Drawing Blood:

Risks associated with drawing blood from your arm include minimal discomfort and/or bruising. Infection, excess bleeding, clotting, or fainting are also possible, although unlikely.

Drug Interactions:

For your safety, you must tell the study doctor or nurse about all the prescription drugs, herbal products, over-the-counter (OTC) drugs, vitamins and natural remedies that you are taking before you start the study and before taking any of these products while you are on the study.

There may be risks, discomforts, drug interactions or side effects that are not yet known.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

If you agree to take part in this study, there may or may not be direct medical benefit to you. We hope the information learned from this study will benefit other patients with metastatic melanoma or other cancers in the future.

WHAT ALTERNATIVES ARE THERE TO PARTICIPATION IN THIS STUDY?

No known effective standard therapy for metastatic melanoma is available to prolong your life. Instead of being in this study, Alternative treatments for your metastatic melanoma could include chemotherapy, interferon-alpha immunotherapy, or radiation either in or outside of another research study. You can also elect to have treatment of your symptoms only. If your cancer becomes



Consent To Participate In A Research Study
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WITH TUMOR ANTIGEN RNA AND SMALL
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ANTIGEN PROCESSING

worse while you are participating in this study, you may choose to continue to participate in this study or choose another form of therapy.

Please talk to your doctor about these and other options.

WILL MY INFORMATION BE KEPT CONFIDENTIAL?

Study records that identify you will be kept confidential as required by law. Federal Privacy Regulations provide safeguards for privacy, security, and authorized access. Except when required by law, you will not be identified by name, social security number, address, telephone number, or any other direct personal identifier in study records disclosed outside of Duke University Health System (DUHS). For records disclosed outside of DUHS, you will be assigned a unique code number. The key to the code will be kept in a locked file in Dr. Pruitt's office.

As part of the study, Dr. Pruitt and his study team will report the results of your study-related laboratory tests and x-rays to those named below. These will include laboratory tests such as your blood counts and tests to measure the function of your liver and kidneys, and x-rays. Some of the blood, urine and x-ray studies would have been done as part of your regular care. Dr. Pruitt will use these test results both to treat you and to complete this research. These test results will be recorded in your medical record and will be reported to the research data office at Duke, as well as to your primary care physician and/or medical oncologist. Results of tests and studies done solely for this research study and not as part of your regular care will also be included in your medical record.

Your records may be reviewed in order to meet federal or state regulations. Reviewers may include representatives from the Food and Drug Administration, and the Duke University Health System Institutional Review Board. If any of these groups review your research record, they may also need to review your entire medical record.

If this information is disclosed to outside reviewers for audit purposes, it may be further disclosed by them and may not be covered by the federal privacy regulations.



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The study results will be retained in your research record for at least six years after the study is completed. At that time either the research information not already in your medical record will be destroyed or information identifying you will be removed from such study results at DUHS. Any research information in your medical record will be kept indefinitely.

While the information and data resulting from this study may be presented at scientific meetings or published in a scientific journal, your identity will not be revealed.

Some information collected about you only for this research study may be kept in a research study record separate from your medical record, and some research information may also be part of your medical record. You will not have access to this research information until the end of the study. However, it will be available to your physicians if needed for your care.

WHAT ARE THE COSTS?

All study-related costs exclusively associated with your being in this study will be paid by funds from the Duke Melanoma Research Fund. The costs of blood collection, melanoma biopsy, leukapheresis, dendritic cell vaccination, and laboratory tests associated solely with this study are provided free of charge. There will be no additional costs to you as a result of being in this study. However, routine medical care for your condition (care you would have received whether or not you were in this study) will be charged to you or your insurance company. You may wish to contact your insurance company to discuss this further.

In order to make sure that tests and studies done solely for research purposes are charged correctly, we will carefully monitor your Duke Hospital and Clinic charges as long as you are participating in this study. These tests and studies are not a part of routine care, and people who are not part of the study do not usually have them performed. Please ask if you would like to know more about which tests and studies are being done solely for research purposes.



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WHAT ABOUT COMPENSATION?

You will not receive any monetary compensation for participating in this research study.

WHAT ABOUT RESEARCH RELATED INJURIES?

Immediate necessary medical care is available at Duke University Medical Center in the event that you are injured as a result of your participation in this research study. However, there is no commitment by Duke University, Duke University Health System, Inc., or your Duke physicians to provide monetary compensation or free medical care to you in the event of a study-related injury.

For questions about the study or research-related injury, contact Dr. Scott K. Pruitt at 919-684-3942 during regular business hours and at 919-684-8111 and ask for Dr. Pruitt to be paged after hours and on weekends and holidays.

WHAT ABOUT MY RIGHTS TO DECLINE PARTICIPATION OR WITHDRAW FROM THE STUDY?

You may choose not to be in the study, or, if you agree to be in the study, you may withdraw from the study at any time. If you withdraw from the study, no new data about you will be collected for study purposes unless the data concern an adverse event (a bad effect) related to the study. If such an adverse event occurs, we may need to review your entire medical record. Your decision not to participate or to withdraw from the study will not involve any penalty or loss of benefits to which you are entitled, and will not affect your access to health care at Duke. If you do decide to withdraw, we ask that you contact Dr. Scott K. Pruitt in writing and let him know that you are withdrawing from the study. His mailing address is Box 3966, DUMC, Durham, NC 27710. We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Your doctor may decide to take you off this study if your condition gets worse, if you have serious side effects, or if your study doctor determines that it is no longer in your best interest to continue. The sponsor or regulatory agencies may



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stop this study at anytime without your consent. If this occurs, you will be notified and your study doctor will discuss other options with you.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, or if you have complaints, concerns or suggestions about the research, contact Dr. Scott K. Pruitt at 919-684-3942 during regular business hours and at 919-684-8111 and ask for Dr. Pruitt to be paged after hours and on weekends and holidays.

For questions about your rights as a research participant, or to discuss problems, concerns or suggestions related to the research, or to obtain information or offer input about the research, contact the Duke University Health System Institutional Review Board (IRB) Office at (919) 668-5111.

STATEMENT OF CONSENT

"The purpose of this study, procedures to be followed, risks and benefits have been explained to me. I have been allowed to ask questions, and my questions have been answered to my satisfaction. I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research. I have read this consent form and agree to be in this study, with the understanding that I may withdraw at any time. I have been told that I will be given a signed copy of this consent form."

Signature of Subject

Date

Signature of Person Obtaining Consent

Date

ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

The purpose of this form is to provide readers of your manuscript with information about your other interests that could influence how they receive and understand your work. The form is designed to be completed electronically and stored electronically. It contains programming that allows appropriate data display. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in four parts.

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Enter your full name. If you are NOT the corresponding author please check the box "no" and a space to enter the name of the corresponding author in the space that appears. Provide the requested manuscript information. Double-check the manuscript number and enter it.

2. The work under consideration for publication.

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Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

ICMJJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) _____ 2. Surname (Last Name) _____ 3. Effective Date (07-August-2008) _____
 Jens _____ Dannull _____ 04-March-2013 _____

4. Are you the corresponding author? Yes No Corresponding Author's Name _____
 Scott Pruitt _____

5. Manuscript Title _____
 Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome _____

6. Manuscript Identifying Number (if you know it) _____
 67544-RG-1 _____

Section 2. The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc...)?

Complete each row by checking "No" or providing the requested information. **If you have more than one relationship click the "Add" button to add a row. Excess rows can be removed by clicking the "X" button.**

The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
						ADD

* This means money that your institution received for your efforts on this study.

** Use this section to provide any needed explanation.

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were present during the 36 months prior to submission.

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Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD

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Hide All Table Rows Checked 'No'

SAVE

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) N. Rebecca	2. Surname (Last Name) Haley	3. Effective Date (07-August-2008) 04-March-2013
4. Are you the corresponding author?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Corresponding Author's Name Scott Pruitt
5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome		
6. Manuscript Identifying Number (if you know it) 67544-RG-1		

Section 2. The Work Under Consideration for Publication

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The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	National Institutes of Health		X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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						ADD

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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
6. Provision of writing assistance, medicines, equipment, or administrative support	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	National Institutes of Health		X
						ADD
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD

* This means money that your institution received for your efforts on this study.

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						ADD
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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Gary 2. Surname (Last Name) Archer 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No Corresponding Author's Name
Scott Pruitt

5. Manuscript Title
Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome

6. Manuscript Identifying Number (if you know it)
67544-RG-1

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The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

ICMJE Form for Disclosure of Potential Conflicts of Interest

The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
						ADD

* This means money that your institution received for your efforts on this study.

** Use this section to provide any needed explanation.

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were present during the 36 months prior to submission.

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Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Payment for lectures including service on speakers bureaus	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
7. Payment for manuscript preparation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

ICMJE Form for Disclosure of Potential Conflicts of Interest

Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD

* This means money that your institution received for your efforts.

** For example, if you report a consultancy above there is no need to report travel related to that consultancy on this line.

Section 4. Other relationships

Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

- No other relationships/conditions/circumstances that present a potential conflict of interest
- Yes, the following relationships/conditions/circumstances are present (explain below):

At the time of manuscript acceptance, journals will ask authors to confirm and, if necessary, update their disclosure statements. On occasion, journals may ask authors to disclose further information about reported relationships.

Hide All Table Rows Checked 'No'

SAVE

ICMJE Form for Disclosure of Potential Conflicts of Interest

Evaluation and Feedback

Please visit <http://www.icmje.org/cgi-bin/feedback> to provide feedback on your experience with completing this form.

ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

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1. Identifying information.

Enter your full name. If you are NOT the corresponding author please check the box "no" and a space to enter the name of the corresponding author in the space that appears. Provide the requested manuscript information. Double-check the manuscript number and enter it.

2. The work under consideration for publication.

This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking "No" means that you did the work without receiving any financial support from any third party -- that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check "Yes". Then complete the appropriate boxes to indicate the type of support and whether the payment went to you, or to your institution, or both.

3. Relevant financial activities outside the submitted work.

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For grants you have received for work outside the submitted work, you should disclose support ONLY from entities that could be perceived to be affected financially by the published work, such as drug companies, or foundations supported by entities that could be perceived to have a financial stake in the outcome. Public funding sources, such as government agencies, charitable foundations or academic institutions, need not be disclosed. For example, if a government agency sponsored a study in which you have been involved and drugs were provided by a pharmaceutical company, you need only list the pharmaceutical company.

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Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Smita 2. Surname (Last Name) Nair 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No Corresponding Author's Name Scott Pruitt

5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome

6. Manuscript Identifying Number (if you know it) 67544-RG-1

Section 2. The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc...)?

Complete each row by checking "No" or providing the requested information. **If you have more than one relationship click the "Add" button to add a row. Excess rows can be removed by clicking the "X" button.**

The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

ICMJE Form for Disclosure of Potential Conflicts of Interest

The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
						ADD

* This means money that your institution received for your efforts on this study.

** Use this section to provide any needed explanation.

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were present during the 36 months prior to submission.

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Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Payment for lectures including service on speakers bureaus	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD

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No other relationships/conditions/circumstances that present a potential conflict of interest

Yes, the following relationships/conditions/circumstances are present (explain below):

S Nair is a co-inventor on a patent that describes the use of dendritic cells transfected with tumor antigen encoding RNA that has been licensed by Argos Therapeutics (Durham, NC) through Duke University. S Nair has no financial interests in Argos Therapeutics and is not compensated by Argos Therapeutics.

ICMJE Form for Disclosure of Potential Conflicts of Interest

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Hide All Table Rows Checked 'No'

SAVE

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Enter your full name. If you are NOT the corresponding author please check the box "no" and a space to enter the name of the corresponding author in the space that appears. Provide the requested manuscript information. Double-check the manuscript number and enter it.

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) David	2. Surname (Last Name) Boczkowski	3. Effective Date (07-August-2008) 04-March-2013
4. Are you the corresponding author?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Corresponding Author's Name Scott Pruitt
5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome		
6. Manuscript Identifying Number (if you know it) 67544-RG-1		

Section 2. The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc...)?

Complete each row by checking "No" or providing the requested information. **If you have more than one relationship click the "Add" button to add a row. Excess rows can be removed by clicking the "X" button.**

The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

ICMJE Form for Disclosure of Potential Conflicts of Interest

The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
						ADD

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Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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						ADD
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						ADD
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						ADD
7. Payment for manuscript preparation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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Relevant financial activities outside the submitted work						
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						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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Hide All Table Rows Checked 'No'

SAVE

Evaluation and Feedback

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Instructions

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) _____
Mark

2. Surname (Last Name) _____
Harper

3. Effective Date (07-August-2008)
04-March-2013

4. Are you the corresponding author? Yes No
Corresponding Author's Name
Scott Pruitt

5. Manuscript Title
Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome

6. Manuscript Identifying Number (if you know it)
67544-RG-1

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The Work Under Consideration for Publication

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1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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The Work Under Consideration for Publication						
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7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
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1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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Hide All Table Rows Checked 'No'

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Nicole	2. Surname (Last Name) De Rosa	3. Effective Date (07-August-2008) 04-March-2013
4. Are you the corresponding author?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Corresponding Author's Name Scott Pruitt
5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome		
6. Manuscript Identifying Number (if you know it) 67544-RG-1		

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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
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						ADD
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						ADD
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						ADD
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ICMJJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Nancy 2. Surname (Last Name) Pickett 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No Corresponding Author's Name Scott Pruitt

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1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
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Scott Pruitt

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Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) James 2. Surname (Last Name) Burchette 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No Corresponding Author's Name
Scott Pruitt

5. Manuscript Title
Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome

6. Manuscript Identifying Number (if you know it)
67544-RG-1

Section 2. The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc...)?

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The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
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Section 3. Relevant financial activities outside the submitted work.

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Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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SAVE

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) M Angelica	2. Surname (Last Name) Selim	3. Effective Date (07-August-2008) 04-March-2013
4. Are you the corresponding author?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Corresponding Author's Name Scott Pruitt
5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome		
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1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
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						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Duane 2. Surname (Last Name) Mltchell 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No Corresponding Author's Name
Scott Pruitt

5. Manuscript Title
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1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
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						ADD
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						ADD
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6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Payment for lectures including service on speakers bureaus	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
7. Payment for manuscript preparation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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Relevant financial activities outside the submitted work						
Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD

* This means money that your institution received for your efforts.

** For example, if you report a consultancy above there is no need to report travel related to that consultancy on this line.

Section 4. Other relationships

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Hide All Table Rows Checked 'No'

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1. Identifying information.

Enter your full name. If you are NOT the corresponding author please check the box "no" and a space to enter the name of the corresponding author in the space that appears. Provide the requested manuscript information. Double-check the manuscript number and enter it.

2. The work under consideration for publication.

This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking "No" means that you did the work without receiving any financial support from any third party -- that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check "Yes". Then complete the appropriate boxes to indicate the type of support and whether the payment went to you, or to your institution, or both.

3. Relevant financial activities outside the submitted work.

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Section 1. Identifying Information

1. Given Name (First Name) Douglas	2. Surname (Last Name) Tyler	3. Effective Date (07-August-2008) 04-March-2013
4. Are you the corresponding author?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Corresponding Author's Name Scott Pruitt
5. Manuscript Title Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome		
6. Manuscript Identifying Number (if you know it) 67544-RG-1		

Section 2. The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc...)?

Complete each row by checking "No" or providing the requested information. **If you have more than one relationship click the "Add" button to add a row. Excess rows can be removed by clicking the "X" button.**

The Work Under Consideration for Publication

Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

ICMJE Form for Disclosure of Potential Conflicts of Interest

The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
						ADD

* This means money that your institution received for your efforts on this study.

** Use this section to provide any needed explanation.

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were present during the 36 months prior to submission.

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Type of Relationship (in alphabetical order)	No	Money Paid to You	Money to Your Institution*	Entity	Comments	
1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	AGTC		X
2. Consultancy	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	Amgen		X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	Scherring-Plough	Clinical Trial Support	X
5. Grants/grants pending	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	BMS	Clinical Trial Support	X
						ADD
6. Payment for lectures including service on speakers bureaus	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>			X

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						ADD
7. Payment for manuscript preparation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	Up to Date		X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
13. Other (err on the side of full disclosure)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
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Section 1. Identifying Information

1. Given Name (First Name) Scott 2. Surname (Last Name) Pruitt 3. Effective Date (07-August-2008) 04-March-2013

4. Are you the corresponding author? Yes No

5. Manuscript Title
Melanoma Immunotherapy Using Mature Dendritic Cells Expressing the Constitutive Proteasome

6. Manuscript Identifying Number (if you know it)
67544-RG-1

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Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
1. Grant	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consulting fee or honorarium	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Support for travel to meetings for the study or other purposes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Payment for writing or reviewing the manuscript	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Provision of writing assistance, medicines, equipment, or administrative support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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The Work Under Consideration for Publication						
Type	No	Money Paid to You	Money to Your Institution*	Name of Entity	Comments**	
7. Other	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			ADD
						X
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1. Board membership	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
2. Consultancy	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
3. Employment	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
4. Expert testimony	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
5. Grants/grants pending	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
6. Payment for lectures including service on speakers bureaus	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
7. Payment for manuscript preparation	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X

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Relevant financial activities outside the submitted work						
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						ADD
8. Patents (planned, pending or issued)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
9. Royalties	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
10. Payment for development of educational presentations	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
11. Stock/stock options	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
12. Travel/accommodations/meeting expenses unrelated to activities listed**	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>			X
						ADD
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