Activation of pDCs at the Tumor and Vaccine Site with a TLR Agonist

Abstract

Although cancer vaccines offer significant promise, clinical effectiveness has yet to be realized. Effective vaccines may require inclusion of basic elements of the immune response which allow successful elimination of pathogens. It is now clear that strong adaptive immune responses are preceded by a potent innate immune response, triggered by pathogen-associated molecular patterns (PAMPs) that are recognized by immune cells expressing Toll-like receptors (TLRs). In the absence of TLR signaling, release of inflammatory cytokines by innate immune cells is sub-optimal, likely explaining why tumors are poor at eliciting antigen-specific adaptive immunity. Harnessing and adapting the mechanisms used by pathogens to induce effective specific immunity represents a very promising approach to improving antigen-specific antitumor immune responses. The plasmacytoid dendritic cell, the primary producer of type I interferons in the body, is a central mediator of inflammation in response to TLR activation and coordinates immune cell interactions which lead to a potent adaptive immune response. This inflammatory response is important not only to trigger strong T-cell priming, but also to induce inflammation at the target site which leads to enhanced T-cell migration and effector function. In our murine models, we have found that plasmacytoid dendritic (pDC) cells can lead to enhanced antigen-specific immune responses, partially through synergy with myeloid dendritic cells (mDC). In addition, we and others have found that pDC can be activated directly in vivo through specific toll-like receptor (TLR) ligands. In this proposal, we will test these concepts in melanoma patients and will utilize a vaccine in combination with a TLR agonist capable of activating both pDC and mDC in order to model the synergy observed in our murine system. We will measure T-cell priming in patients immunized in the presence or absence of TLR activation. Subsequent to T-cell priming, we will administer the TLR agonist at the tumor site in order to induce inflammation at sites of cutaneous metastases and will test the ability of this intervention to activate pDC at the tumor site and enhance T-cell migration into the tumor and T-cell effector function. Our goal is to identify principles which may be generalized towards improving cancer vaccines against other common cancers.

1.0 Objectives

1.1 Primary Objective

To compare the ability of vaccines in combination with TLR stimulation at the site of vaccine (R848; Resiquimod) to vaccines alone in the ability to enhance the generation of circulating antigen-specific T-cells (T-cell priming).

1.2 Secondary Objectives

A. To evaluate the ability of **locally administered** TLR agonist (R848 gel) to activate innate immune cells at the gp100 (g209-2M) **vaccine site**.

B. To evaluate the ability of R848 gel, administered at the **tumor site,** to:

i. Induce inflammation and upregulation of adhesion molecules on tumor vasculature

ii. Enhance T-cell infiltration into tumor

iii. Generate T-cells against additional tumor antigens, not present in the vaccine (i.e., antigen spreading).

iv. Mediate clinical response with measure of objective tumor response rate and progression free survival.

C. To assess the association between clinical response and laboratory parameters of T-cell priming, T-cell migration to tumor, and inflammation at the vaccine and tumor sites.

2.0 Background Drug Information

2.1 Natural antitumor immune responses are often weak compared with antiviral immune responses

The notion of whether the immune system is capable of recognizing, responding to, and eradicating established tumors was at one time a rather contentious and controversial issue [1,2]. However, much evidence has accumulated over the past two decades, both in humans and in mouse models, and at the cellular and molecular level, to establish that immune cells can play an important role in inducing successful cancer regression [3,4]. Multiple studies using different tumor models have shown that administration of immune cytokines, specific vaccines, and adoptive transfer of immune cells can all lead to effective tumor eradication in the appropriate setting [5-7]. Nonetheless, it is true that all of these treatments rely on specific immune interventions for their success. The generation of effective natural immunity against established tumors is likely to be a very infrequent event, as evidenced by the clinical manifestation of tumors in non-immunocompromised hosts and by the fact that spontaneous regressions are very rarely observed.

By contrast, the natural generation of effective immunity against viral infections remains the rule rather than the exception. Multiple studies have demonstrated that viral infections frequently lead to the spontaneous generation of strong immune responses that are often not only capable of inducing viral clearance, but also in generating long-lived memory responses capable of protecting the host against re-infection [8-10]. In the past ten years, a number of seminal research studies have shed much light on why viruses and other pathogens can elicit such potent and effective natural immune responses. We believe that harnessing and adapting the mechanisms used by pathogens to induce effective specific immunity represents a very promising approach to improving specific antitumor immune responses.

2.2 Activation of innate immunity is critical for the generation of effective adaptive immune responses

Effective antiviral immune responses are initiated through activation of innate immune cells, including NK cells, conventional myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs), by specific TLR ligands [11-13]. Activation of innate immunity induces the production of proinflammatory cytokines which can directly activate cells important for the initiation of adaptive immune responses. Type I interferons (IFNs) and tumor necrosis factor (TNF-β), for example, are potent inducers of mDC maturation, inducing upregulation of major histocompatibility complex (MHC) and costimulatory molecules as well as production of IL-12, both of which are important for the priming of naïve T cells [14,15]. In addition, the activation of NK cells by pDC, cytokines and TLR ligands may lead to increased lysis of virally-infected cells or tumors which, in turn, can provide antigen to mDCs for presentation to T cells. Activation of innate

immunity is important not only for the generation of antigen-specific T-cells, but also to induce inflammation at the pathogen site which leads to enhanced migration of antigen-specific T-cells to the infected tissue.

2.3 Plasmacytoid DCs represent a critical link between innate and adaptive immunity

As the major producer of type I IFNs (represented by IFN-□ and IFN-□), pDCs represent one of the most important links between innate and adaptive immunity [16-20]. Upon triggering of TLR7 or TLR9 by virus, pDCs rapidly produce large amounts of Type I IFNs, activate a variety of immune cells such as B cells, natural killer (NK) cells and macrophages, and differentiate into antigen presenting cells (APCs) to induce antigen-specific T cell responses [21]. Both mDCs and NK cell activation can also be partially mediated by Type I interferons. IFN□Rc -/- mDCs are defective in the ability to adequately respond to viral infections [22], suggesting that interferon-producing pDCs may be critical for the activation of mDCs and subsequent development of adaptive immunity. A number of lines of evidence suggest that pDCs may interact with mDCs to induce an enhanced adaptive immune response in the development of antiviral immunity [23-25]. Activation of mDCs by double stranded RNA or viral infection has been shown to be dependent on exposure to IFN-□ [22,25-27]. In addition, HIV was found to be able to activate pDC, which could subsequently activate mDC upon co-culture [24]. In addition, it has been recently demonstrated that pDCs may interact with lymph node mDCs in the generation of anti-HSV CTL [28].

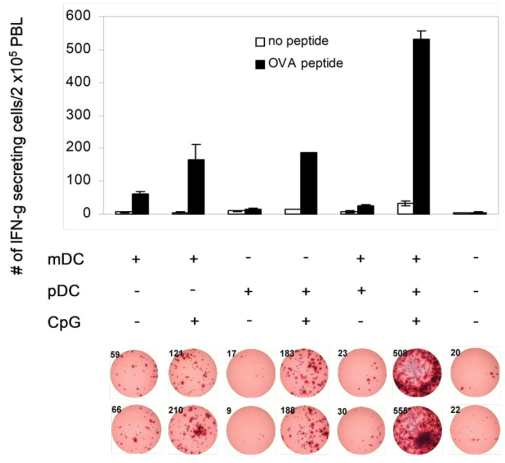


Figure 1. Immunization with a combination of CpG-activated pDC plus mDC significantly enhances antigen-specific CTL responses. Mice were immunized s.c. with purified pDC, mDC, or a combination of pDC and mDC (1:1) which were first co-cultured for 4 hours with CpG and $OVA_{257-264}$ peptide (the total number of DCs injected were kept at the same level for all groups). Seven days after immunization, mice were bled and PBL were isolated. Ag-specific IFN-γ production by immune cells was assessed by ELISPOT assay upon *in vitro* restimulation with or without OVA peptide. ELISPOT picture represents duplicate wells in each immunized group in the presence of OVA peptide stimulation.

2.4 Cancer vaccines have significant potential, but require considerable optimization

The identification of tumor antigens recognized by T-cells has allowed the development of rational cancer vaccine strategies. Current evidence suggests that cancer vaccines have the ability to increase the levels of circulating T-cells capable of recognizing tumor antigens. However, this has not led to significant tumor regressions in patients. For example, an analysis of the response rate in over 500 patients with metastatic melanoma treated with vaccines was under 3%.[29] Our hypothesis is that this is due to inadequate activation of innate immunity at the site of immunization as well as at the tumor site itself. Lack of adequate inflammation at the vaccine site may lead to suboptimal T-cell priming, while equally

important; the lack of inflammation at the tumor site may lead to inefficient migration of T-cells back to the tumor.

2.5 Plasmacytoid dendritic cells and myeloid dendritic cells synergize in their ability to generate antigen specific immune reactions, resulting in an enhanced antitumor response in vivo

We hypothesized that by their capacity to activate innate immunity, pDCs would potentiate the function of mDCs when used in combination. Indeed, when purified murine pDCs and mDCs were co-cultured in the presence of antigen and a TLR activation stimulus, such as CpG, followed by in vivo administration, antigen-specific T-cell levels were higher than with the administration of either DC subset alone (figure 1). Since the total number of DCs was kept constant for all groups, the interaction between pDC and mDC in their ability to stimulate T-cells was synergistic and not simply additive.

Importantly, tumor reduction was also enhanced when mice were treated with the combination of activated, antigen-pulsed pDC and mDC compared to either DC subset alone (Figure 2). Treatment of tumors with a combination of pDC and mDC resulted in mice with both smaller tumors (Figure 2A) and enhanced survival (Figure 2B).

Since pDC and mDC synergize in the generation of antigen-specific T-cells, we propose to use a TLR agonist, R848, which is capable of activating both pDC and mDC through TLR 7 and 8, respectively, in combination with vaccine in order to induce antigen-specific T-cells in melanoma patients.

2.6 TLR agonists can activate pDC and mDC and can induce inflammation

a. R848 (Resiquimod)

Resiquimod (also known as R-848) is a novel heterocyclic amine that acts as a dual TLR7 and TLR8 agonist and therefore is capable of activating both plasmacytoid dendritic cells (pDC), which express TLR7, and myeloid dendritic cells (mDC), which express TLR8. Resiquimod is a member of the imidazoquinoline family of IRMs, of which imiquimod has been most extensively characterized (and is in active clinical use for the treatment of actinic keratosis and basal cell cancer). Resiquimod is consistently more potent than imiquimod (50-100 fold) based on median minimum concentration required to elicit a 3-fold increase in IFN-α, TNF-α, and IL-12 production by human PBMC. Resiquimod can promote functional maturation of DC subsets as defined by enhanced expression of co-stimulatory molecules important for antigen presentation and T-cell activation.

Resiquimod is available in a gel formulation for topical use and has been used previously in clinical trials of herpetic lesions, actinic keratosis and anogenital warts. These studies revealed that topical resiquimod is well tolerated and capable of inducing inflammatory reactions consistent with activation of plasmacytoid and myeloid dendritic cells. As we have shown synergy between pDC and mDC in the induction of antigen-specific T-cell priming, we propose to utilize topical resiquimod over the vaccine site.

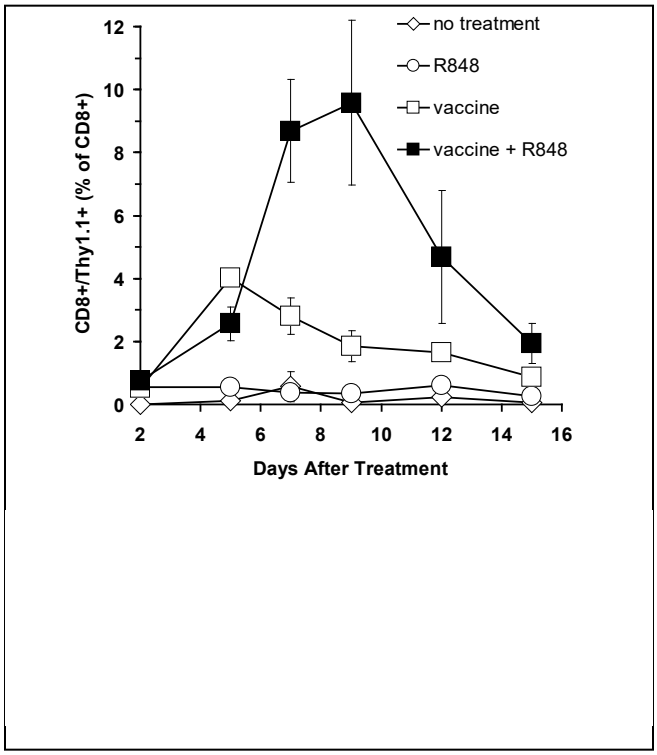
In a study of multiple doses of topical resiquimod applied to intact skin of the upper arm (1246-R848), with a maximum exposure of 2.5mg (1gm of 0.25% gel) 2x/week for 3 weeks, dose-related , but tolerable, systemic adverse effects consistent with systemic cytokine induction were observed. These included fever, chills, and transient neutropenia. Application site reactions including erythema, stinging, and pruritis were also observed. Dose-related increases in local cytokine production and T-cell infiltration were also reported.

Because the 0.25% dose provided the highest level of immune cell infiltration, we will plan to utilize a dose of 0.2% R848 2 times per week in our study (currently, this is the highest clinical grade formulation available), with the option of dose reducing to 1 time per week should patients not tolerate the treatment due to adverse reactions.

In murine models, we have found that TLR agonists, given at the site of vaccine, can enhance the generation of antigen-specific T-cells (T-cell priming).

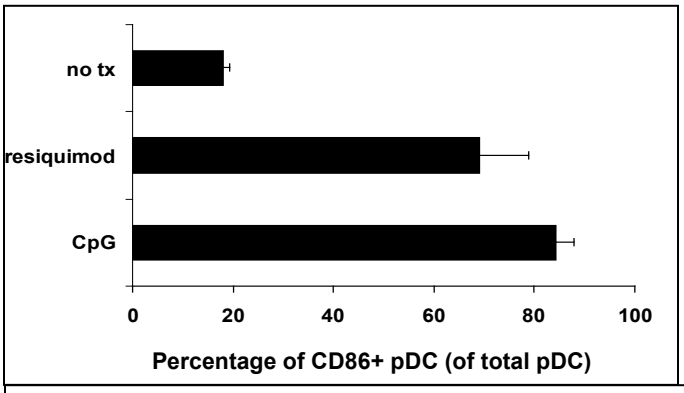
2.7 TLR 7/8 agonist R848 enhances T-cell priming against gp100(g209-2M)

While activated pDC at the site of immunization resulted in enhanced T-cell priming, use of purified activated pDC would be challenging in the clinic due to the extensive and personalized nature of the preparation. Although interferon-□ is a major mediator of the effects of pDC, it is clear from our studies that cell-cell contact between pDC and mDC also play an important role and providing interferon as an adjuvant will be insufficient to obtain the full benefits of pDC activation. Therefore, we have focused on the use of toll-like receptor agonists capable of activating pDC in vivo. Because our preliminary data indicates that activated pDC and mDC synergize in the priming of specific T-cells, we have selected a novel molecule, Resiquimod (R848), which stimulates both TLR 7 and 8, thereby activating both pDC and mDC, respectively. In order to evaluate the ability of R848 to enhance antigen specific vaccination, we first administered naïve pmel T-cells, which are transgenic to express a T-cell receptor specific for the gp100(g209-2M) antigen. We found that administration of R848 gel over a vaccine site using the melanoma antigen gp100(g209-2M) enhances the proliferation of gp100(g209-2M). Specific pmel T-cells compared to the vaccine alone (Figure 3).



Besides enhancing T-cell priming, we have found that the administration of TLR agonists at the tumor site induces pDC activation, and subsequent T-cell infiltration, tumor regression, and generation of T-cells against other (“de novo”) antigens.

2.8 TLR agonist administration results in activation of pDC at the tumor site.



To determine the ability of TLR agonists to activate pDC at the tumor site, Resiquimod (R848) or CpG, capable of activating pDC through TLR 7 or TLR 9, respectively, was injected intratumorally and 24 hours later tumor was harvested and pDC were evaluated by flow cytometry. Intratumoral injection of both Resiquimod and CpG resulted in pDC activation, as determined by CD86 expression (Figure 4). Similar results

Figure 4. B16 tumor bearing mice (3 mice per group) received either CpG (37 ug/mouse) or resiquimod (40 ug/mouse) intratumorally. One day later mice were sacrificed and a single cell suspension was prepared from tumors. The percentage of CD86 positive pDC was analyzed using flow cytometry.

were seen using CD40 as a marker of pDC activation (not shown).

Having demonstrated that administration of TLR agonists resulted in pDC activation at the tumor site, we next studied whether this led to enhanced infiltration of T-cells into the tumor. To study this, mice bearing subcutaneous B16-ova melanoma tumors were injected with naïve pmel T-cells, which express a T-cell **receptor specific for the gp100(g209-2M) melanoma antigen, expressed by B16 tumor. Use of the pmel** system allows the tracking of vaccine-induced

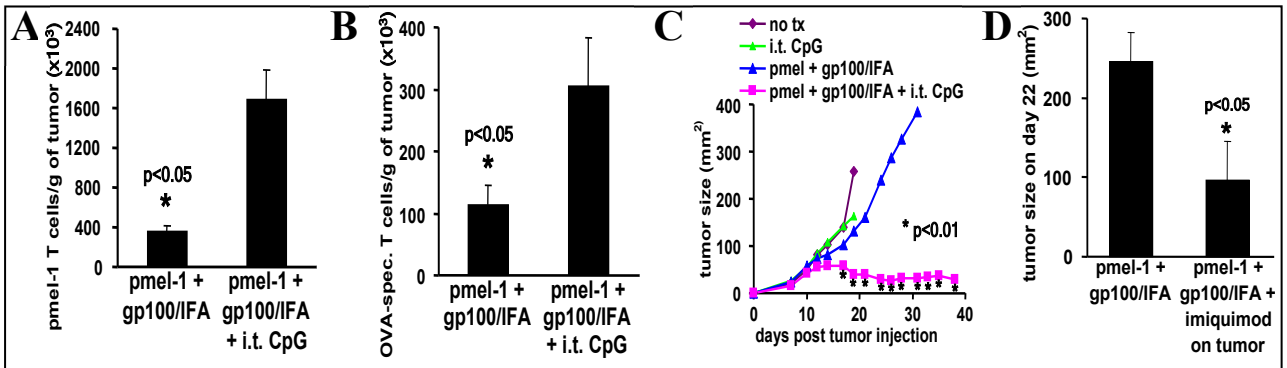


Fig. 5. *Intratumoral TLR triggering enhances accumulation and anti-tumor effect of vaccine-induced and de novo induced tumor-specific T cells.* C57BL/6 mice were injected with $3x10^5$ B16.OVA cells on day 0, 10 □g Flt3L plasmid i.v. on day 2 (to increase circulating pDC), $1x10^7$ Thy1.1$^+$ pmel-1 cells and hgp100(g209-2M) /IFA peptide vaccination on day 6 and i.t. injection of 100 □g CpG on days 13, 15 and 17. On day 18 tumors were excised (n=3) and infiltrating pmel-1 (**A**) and OVA-specific (**B**) T cells were counted by FACS. (**C**) Five mice per group continued to receive i.t. CpG every other day and tumor growth was followed. (**D**) Mice were treated as in (C) but instead of i.t. CpG injections, topical imiquimod (Aldara cream) was applied on the s.c. tumors every three days. Tumor size on day 22 after tumor injection is shown.

lymphocytes in vivo, as well as the simulation of the circulating levels of T-cells we find in patients following immunization. Mice were then immunized with gp100(g209-2M) peptide in IFA. TLR activation at the tumor site was performed by giving intratumoral CpG every other day. Five days later, tumors were harvested and the percentage of Pmel and ova-specific T-cells were enumerated by flow cytometry. Pmel T-cells could be distinguished from endogenous T-cells by expression of the congenic marker Thy 1.1. This experiment allowed us to determine whether TLR activation at the tumor site increased vaccine induced gp100(g209-2M) specific T-cells as well as non-vaccine induced T-cells against a de novo antigen (ova in

this model) through cross-priming. High levels of vaccine-induced, antigen-specific Pmel T-cells were found to infiltrate tumors only in mice receiving TLR activation at the tumor site (Fig 5a). Moreover, using tetramer analysis, endogenously derived ova-specific T-cells were found in high numbers in tumors receiving TLR activation compared to those that did not (Fig 5b). This suggests that TLR activation at the tumor site results in increased accumulation of vaccine-induced T-cells, as well as cross-priming of endogenous T-cells against other antigens present in the tumor. Finally, **mice receiving a combination of vaccine and intratumoral TLR activation exhibited tumor regression compared to control groups** (Fig 5c and 5d). This was evaluated using both intratumoral CpG administration (TLR 9 agonist, Fig 5c), as well as topical imiquimod (TLR7 agonist, Fig 5d).

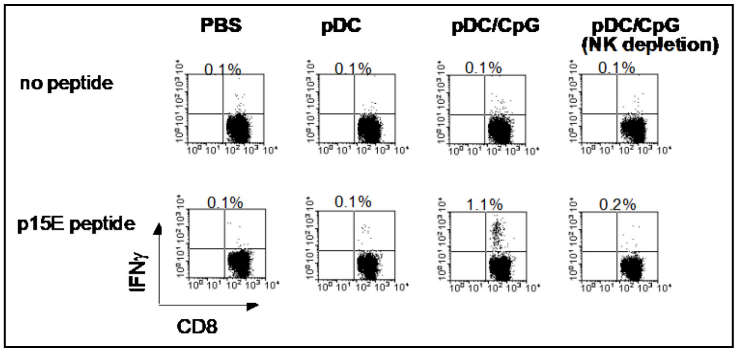


Figure 6: *Activated pDC at the tumor site can lead to cross-priming of tumor antigens*. C57Bl/6 mice were injected subcutaneously with $3x10^5$ B16 melanoma cells. 10 days later, tumors were injected with pDC that were either unactivated or activated for 6 hours with the TLR 9 agonist CpG. NK cells were depleted in the indicated groups by i.p. injection of 200 ug anti-asialo-GM1 Ab on days -2, 0 and 2 (in repeat experiments, anti-NK1.1 was used with similar results). Five days after pDC injection, splenocytes were harvested, and pulsed with an H2-K^b restricted peptide derived from p15E, an endogenous retroviral antigen expressed by B16. IFN-γ production was measured by intracellular staining and flow cytometry.

These studies suggest that the induction of high numbers of circulating vaccine-induced, antigen specific T-cells followed by TLR activation at the tumor site can result in migration of the vaccine-induced T-cells to the tumor. In addition, this can result in increased cross priming against other antigens expressed by the tumor, and, importantly, tumor regression.

2.10 Activated pDC can enhance cross-priming of tumor antigens

To specifically evaluate the role of activated pDCs at the tumor site, we injected activated purified pDC into tumors then evaluated splenic T-cells one week later for their ability to specifically recognize tumor antigens. Following the intratumoral injection of TLR-activated but not non-activated pDC, T-cells were found to be reactive against the **native tumor antigen** p15E, an endogenous retroviral antigen which has been found to be upregulated in murine tumors (Figure 6).

Cross-primed T-cells were found to infiltrate **and induce regression of both pDC injected and non-injected tumors**. Interestingly, we found this cross-priming to be NK cell dependent, since generation of pDC-induced p15E specific T-cells was not seen following depletion of NK cells (Fig 6). In further studies, we found that activated pDC chemoattract NK cell migration into the

tumor site (Fig 7a). We hypothesize that this leads to NK cell activation and IFN□ production, tumor lysis (Fig 7b), uptake and cross-priming of tumor antigen by mDC, and activation of specific T-cells in the draining lymph nodes. Indeed, antigen-specific T-cells were decreased when activated pDC were intratumorally injected in mice deficient for either perforin or IFN□. Furthermore, mDCs isolated from lymph nodes draining pDC injected, GFP-expressing tumor were found to be GFP positive, and capable of activating antigen-specific T-cells. Therefore, we further hypothesize that TLR-mediated activation of pDC at the tumor site will result in NK cell infiltration and cross-priming of antigens to generate T-cells with de novo specificities.

Testing our hypothesis in patients, that vaccines in combination with TLR agonists will enhance both T-cell priming and migration/effector function, requires vaccines which can be used to elicit antigen specific T-cells that can be reliably measured in immune monitoring assays.

2.11 Gp100(g209-2M) peptide vaccine

Gp100(g209-2M) is a melanocyte differentiation antigen with high levels of expression in melanomas. A synthetic peptide of gp100(g209-2M) with the replacement of threonine by methionine at the second position of the immunodominant peptide, referred as g209-2M appeared to be more immunogenic in vitro than the native peptide, due to enhanced binding to the HLA-A*0201 molecule. This peptide has been studied in vivo in patients with metastatic melanoma. Tumor-specific immune responses to gp100(g209-2M) peptide vaccines have been demonstrated.

Previously our group has reported that the immunization of patients with g209-2M emulsified in Incomplete Freund' adjuvant (IFA) could generate T cell responses against the native peptide and melanoma cells[5].As shown in a characteristic experiment in Table 1, PBMCs obtained from patients prior to immunization exhibited no in vitro sensitization against peptide after 11 days of incubation with g209 or g209-2M peptide, but developed

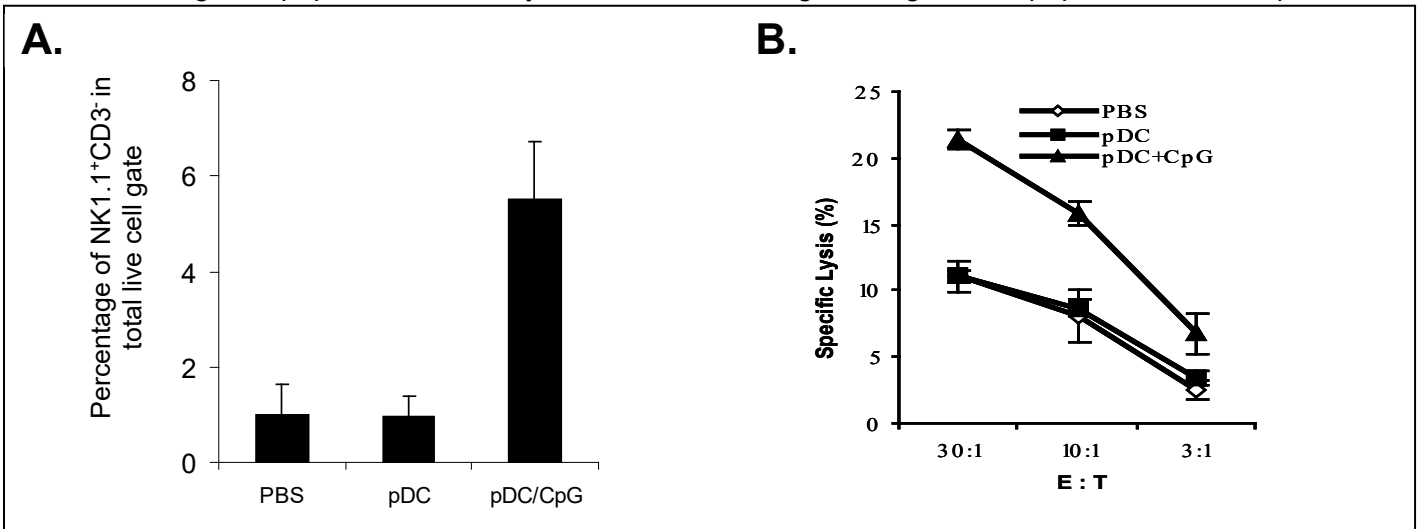


Figure 7: ***Activated pDC induce NK cell migration and activation.*** ***A.*** *Infiltration of NK cells in tumor induced by intratumoral injection of pDC.* Mice (3 mice per group) were first inoculated with 3 x 10^5 B16 tumor cells. Ten days later, mice received intratumoral injection of non-activated or CpG-activated pDC. Thirty six hrs after pDC intratumoral injection, mice were sacrificed and the percentage of NK cells in tumor were evaluated by staining cells with NK1.1 and CD3. The percentage shown is the percent of $NK1.1^+$ $CD3^-$ cells in total live cell gate after excluding dead cells by PI staining.
B. *Activation of NK cells in vivo by pDC.* Mice (3 mice per group) received i.v. injection of non-activated or CpG-activated pDC. Mice were sacrificed 36 hrs later and NK cells were enriched from spleen using DX5 microbeads and then FACS-sorted for NK1.1+/CD3-. Cytotoxicity of NK cells was tested in 4hrs Chromium release assay by using ^{51}Cr labeled B16 tumor cells as target cells and with different effector:

specific anti-influenza reactivity ater incubation with the control flu peptide. However, PBMCs obtained following two immunizations with g209-2M in IFA at a 3-week interval could be specifically sensitized in vitro to recognize native peptide as well as two HLA-A*0201$^+$ melanoma cell lines. Furthermore, administration of g209-2M with concurrent IL-2 therapy resulted in tumor regression in 13 of 31 patients (42%) with metastatic melanoma. This response rate is higher than previous studies of IL-2 alone (17%) [31].

Table 1. Specificity of reactivity against g209-2M peptide and HLA-A*0201$^+$ melanomas.

		Simulator (IFN-γ□ pg /ml)					
No. of immunizations	**In vitro sensitization with peptide**	**T2**	**T2 (flu)**	**T2 (209)**	**501 mel (A2+)**	**SK23.mel (A2+)**	**888 mel (A2-)**
None	209-2M	135	230	146	195	180	115
	209	118	238	84	148	231	261
	Flu	178	35,570	124	290	284	282
2	209-2M	86	61	24,150	72,780	43,250	33
	209	86	165	9,890	25,710	19,480	98
	Flu	121	36,460	132	313	448	362

In a study designed to identify the optimal schedule for immunization, patients received g209-2M peptide vaccines in three different schedules: once weekly for three weeks, every day for 4 days every three weeks, or once every 3 weeks (figure 8).

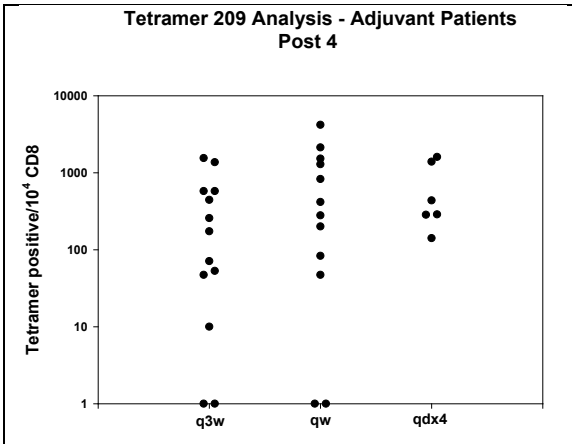


Figure 8. Antigen-specific CD8$^+$ T cells were detected at similar levels following three different vaccination schedules.

1mg of gp209-2M in IFA was injected subcutaneously every 3 weeks, once weekly, or every day for 4 days every 3 weeks. PBMCs from each group of patients were collected and analyzed at 3-month (one course) following immunization. Antigen-specific CD8$^+$ T cells were evaluated by tetramer assay.

For this trial, we will adopt the weekly peptide vaccination schedule based on its efficacy (Figure 8) and feasibility.

In a group of patients who received vaccination every 3 weeks for 12 months, antigen-specific CD8$^+$ T cells were analyzed at 3, 6, 9, and 12 months by tetramer assay. As shown in Figure 9, the number of antigen-reactive CD8+ T cells gradually increased over this period.

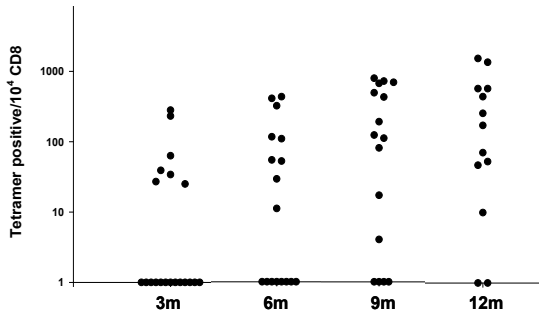


Figure 9. Levels of antigen-reactive CD8+ T cells gradually increased over 12-month vaccination period.

PBMCs from patients receiving vaccine q3w were collected at 4 different time points and analyzed by tetramer assay.

To test whether the frequency of antigen-specific T cells analyzed by tetramer assay correlates with that of ELISPOT, PBMCs were also analyzed by IFN-□γ ELISPOT. Similar to the tetramer assay, increased levels of IFN-□γ producing $CD8^+$ T cells specific for gp209-2M and melanoma cells was detected at 3-months after initiation of vaccine, with higher levels at 12 months (data not shown). Antigen-specific T cell reactivity analyzed by tetramer assay correlated well with IFN-□□γ ELISPOT assay as shown in Figure 10.

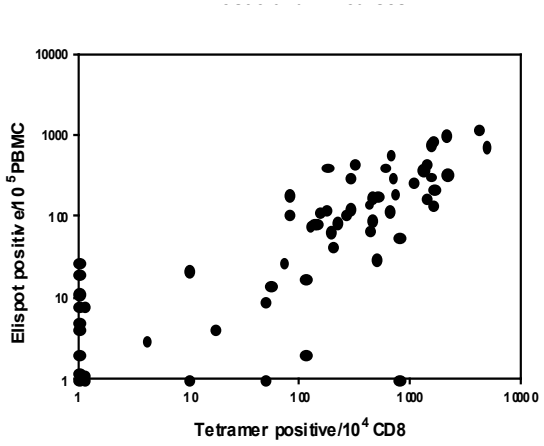


Figure 10. Comparison of tetramer and ELISPOT in patients immunized with gp209-2M peptide.

PBMCs were collected at different time points, both tetramer and ELISPOT assays were performed to test the levels of antigen-specific $CD8^+$ T cells.

2.12 MAGE-3 peptide vaccines

MAGE family antigens are also attractive targets for the development of cancer vaccines, because they are tumor specific, and are shared by different types of cancers. Among these, MAGE-3 is the most frequently expressed in melanomas, with 55% expression in melanoma primary lesions, and 74% in metastatic lesions [46]. Several MAGE-3 peptides were identified that can be recognized by $CD4^+$ T cells that are restricted by MHC class II molecules. Specifically, MAGE-$3_{243\text{-}258}$ peptide (KKLLTQHFVQENYLEY) was identified to be presented by HLA-DP4 molecules[47], a HLA class II allele expressed by approximately 76% of Caucasians. Exposure of MAGE-$3_{243\text{-}258}$ peptide presented by DP4 molecules to specific $CD4^+$ T cell clones resulted in a robust production of IFN-γ. Furthermore, this specific $CD4^+$ T cell clone exhibited lytic activity against melanoma cells mediated by the recognition of MAGE-$3_{243\text{-}258}$ antigen. MAGE-3 antigens have been used in several small-scale therapeutic trials. The vaccines consisted of an antigenic peptide, a protein, a pox family recombinant

virus carrying a MAGE-3 sequence, or dendritic cells (DCs)-pulsed with an antigenic peptide[48-51]. No significant toxicity was observed. Clinical responses were observed in a small number of patients[52].

3.0 Clinical Pharmacology

3.1 gp100(g209-2M): 209-217 amino acid sequence: IMDQVPFSV:

This class I HLA-A2 restricted peptide will be manufactured and purchased from a commercial source (Merck Sciences, Clinalfa). This peptide will be provided as sterile, pyrogen-free lyophilisate, aliquoted in 3 ml serum vials. Each vial will contain 1mg gp100(g209-2M) peptide with ≥ 95% purity. The vial will be individually labeled and store at –20°C.

3. 2 MAGE-$3_{243-258}$ peptide amino acid sequence: KKLLTQHFVQENYLEY:

This class II HLA-DP4-restricted peptide will also be manufactured and purchased from a commercial source (Merck Sciences, Clinalfa). Similarly, this peptide will be provided as sterile, pyrogen-free lyophilisate, aliquoted in 3 ml serum vials. Each vial will contain 2 mg MAGE-$3_{243-258}$ peptide with ≥ 95% purity. The vial will be individually labeled and stored at –20°C.

3.3 Peptide preparation and administration:

On the day of injection, peptides, I mg of the class I peptide (gp100(g209-2M) and 500 mcg of the class II peptide (MAGE-3), will be emulsified individually with 1mL saline and 1.5 ml Incomplete Freund's Adjuvant (IFA) formulated as Montanide ISA-51. Each injection will consist of intradermal and subcutaneous administration at the same site. The class I and class II peptides will be injected in separate extremities and the same extremity will be used for each peptide throughout the 8 weeks of immunizations. Extremities that have undergone regional lymph node dissection will not be used for vaccine administration since we believe that T-cell priming occurs in the regional lymph nodes. In addition, in order to obtain a prime-boost effect, the same extremity will be used for serial administration of each individual class I and class II peptides.

3.4 R848 Resiquimod (gel)

Resiquimod is solubilized in a single phase, translucent gel formulation composed of propylene glycol, colloidal silicon dioxide, and triacetin. The 0.2% Resiquimod gel is packaged in 3.5g multidose tubes.Topical resiquimod gel preparations are to be stored between 15C (59F) and 30C (86F) with excursions permitted to 4C (39F) and 40C (104F). R848, 0.2%, will be applied over the gp100(g209-2M) vaccine injection site each week.

4.0 Eligibility Assessment and Enrollment

4.1 Inclusion Criteria

4.1.1 HLA-A*0201 positive (to enable immunization with the HLA class I restricted gp100(g209-2M) peptide). Stage IIB or IIC patients will be

enrolled after review and approval by the PI. (a tool to determine the projected survival at 5 years, like, but not limited to, the nomogram at www.melanomaprognosis.org. If the projected survival is less than 50% at 5 years, then the patient is considered for enrollment. This is with the recognition that the adjuvant, if effective offers a significant impact in that group of stage II patients.)

4.1.2 Patients $\geq$ 18 years old with histologically documented melanoma with:

a.(Metastatic disease cohort) Measurable disease, stage IIIB, IIIC (in transit lesions with or without nodal metastases) or stage IV M1A with disease that includes lesions accessible for biopsies or IV M1B

b. (Adjuvant cohort) subjects who are NED and stage III or IV. This includes patients with stage IV disease resected to NED. Stage IIB or IIC patients will be enrolled after review and approval by the PI.

4.1.3 ECOG performance status of 0-1

4.1.4 At least 2 biopsiable easily accessible cutaneous and subcutaneous lesions in patients in the metastatic disease cohort

4.1.5 WBC $\geq$ 3000/mm^3 (part 1 & 2)

4.1.6 Platelet count $\geq$ 90,000/mm^3 (part 1 & 2)

4.1.7 Serum ALT and AST $\leq$ 3 X upper limit of normal (ULN) (part 1 & 2)

4.1.8 Total bilirubin $\leq$ 2 X ULN, except for patients with Gilbert's syndrome who must have a total bilirubin less than 3.0 mg/dl (part 1 & 2)

4.1.9 Seronegative for HIV antibody. (The experimental treatment being evaluated in this protocol depends on an intact immune system. Patients who are HIV seropositive can have decreased immune competence and thus may be less responsive to the experimental treatment and more susceptible to its toxicities.)

4.1.10 Negative pregnancy test for women of childbearing potential (WOCBP). A WOCBP is a woman who has not undergone a hysterectomy or who has not been naturally postmenopausal for at least 12 consecutive months (i.e., who has had mense at any time in the preceding 12 consecutive months)

4.1.11 Patients of both genders must practice a barrier method of birth control while participating in this trial.

4.2 Exclusion Criteria

4.2.1 Active autoimmune disease requiring active therapy with any form of steroid or immunosuppressive therapy or a documented history of any of the following: inflammatory bowel disease; regional enteritis; systemic lupus erythematosis; Sjogren's syndrome; inflammatory neurologic disorder such as multiple sclerosis; or any immune mediated disease that can cause life-threatening symptoms or severe organ/tissue damage in the opinion of the principle investigator.

4.2.2 Concurrent systemic or inhaled steroid therapy or herbal preparations

4.2.3 Any form of active primary or secondary immunodeficiency

4.2.4 Prior malignancy except for the following: adequately treated basal cell or squamous cell skin cancer, in-situ cervical cancer, thyroid cancer (except anaplastic) or any cancer from which the patient has been disease-free for 2 years

4.2.5 History of immunization with gp100**(g209-2M)**
4.2.6 Active systemic infections requiring intravenous antibiotics
4.2.7 Women who are breastfeeding
4.2.8 Prior systemic therapy, radiation therapy or intracavitary surgery (intra-thoracic, intra-abdominal or intracranial) within 28 days of starting study treatment
4.2.9 Patients on chronic anticoagulation such as Aspirin, Plavix, or Coumadin who cannot have anticoagulation held for procedures are not eligible due to the need for leukapheresis

4.3 Inclusion of Women and Minorities

Both men and women and members of all ethnic groups are eligible for this trial. Women who are breastfeeding are ineligible.

5.0 Pretreatment Evaluation

At the screening visit, patients will be assessed for study eligibility. All patients must sign an informed consent before any protocol specific testing for eligibility and be registered in CORE/PDMS.

The following baseline studies must be completed within 14 days of treatment initiation:

5.1 Complete history and physical examination
5.2 CBC, differential, PT/PTT, platelet count
5.3 Chemistries to include serum electrolytes, BUN, creatinine, glucose, albumin, alkaline phosphatase, ALT, AST, LDH, calcium, and total bilirubin
5.4 β-HCG pregnancy test on all women of child-bearing potential or who have not undergone sterilization procedure and have had menstruation in the past 12 months.
5.5 EKG
5.6 HIV serology

The following baseline studies must be completed within 28 days of treatment initiation:

5.7 Radiological studies (CT scans of chest, abdomen, pelvis; or PET/CT scan, MRI/CT of brain) to evaluate the status of disease.

The following test will be performed with no time restriction

5.8 HLA-A* and HLA-DP4 typing

6.0 Treatment Plan

All patients will receive the class I, HLA-A2 restricted gp100(g209-2M) peptide vaccine. Patients who are HLA-DP4 positive will also receive the class II restricted MAGE-3 peptide, in separate extremities. This will allow us to generate melanoma-specific CD8 + and CD4+ T-cells. The gp100(g209-2M) immune response will be our primary endpoint for assessment of T-cell priming, since we have extensive previous experience with this vaccine and believe that CD8+ responses are critical to the development of a strong antitumor immune response. The MAGE-3 peptide will be given in order to stimulate antigen-specific CD4+ T-cells, which we believe are also important in the generation of strong antitumor immunity. Patients positive for both HLA types will

receive both vaccines, gp100(g209-2M) and MAGE-3, for eight weeks Patients who are HLA-A2 positive and HLA-DP4 negative will recieve only the gp100 peptide for 8 weeks. There will be two arms in Part 1: Arm A is the peptide vaccines plus the TLR7/8 agonist, R848 and Arm B is the peptide vaccines only. All patients will be randomized to either Arm A or Arm B by CORe during the registration process. The randomization will be stratified by cohort (metastatic disease or adjuvant). In Arm A, R848 gel will be applied to the gp100(g209-2M) vaccine injection site immediately following the injection. The R848 gel will be allowed to air dry for 30 minutes and then covered with a gauze dressing. All vaccines will be administered subcutaneously and intradermally. The gp100(g209-2M) peptide will be administered in the same extremity weekly (every 7 days +/- 2 days) for 8 weeks. Similarly, in a separate extremity of eligible patients, the MAGE-3 peptide will be administered weekly (every 7 days +/- 2 days) for 8 weeks. Each syringe (a total of 4, 2 for gp100 and 2 for MAGE-3) will contain 1ml of peptide vaccine; 0.5ml of the vaccine in each syringe will be administered intradermally and the other 0.5ml will be administered subcutaneously at each injection site for a total of 4 sites.

Blood samples will be obtained serially in order to determine the immune response against the gp100(g209-2M) and MAGE-3 vaccines (weeks 0, 2, 4, 8, 12, 16, 20, and 24: weeks 8 and 16 will not be collected when leukopheresis is performed; blood samples will be collected by leukapheresis at weeks 0 and 8 (+/- 1 week), and 16 when feasible). To evaluate the inflammatory infiltrate at the site of immunization, biopsies will be obtained of vaccination sites as outlined in section 7.3

<u>Subjects in the adjuvant cohort.</u>
Vaccination will continue for the designated 8 weeks. Blood draw and pheresis times will be the same as subjects in the metastatic disease cohorts. Subjects will be monitored for disease recurrence, but if recurrence occurs prior to the completion of blood draws or pharesis these tests can still be obtained if deemed valid by the PI.

<u>Subjects in the metastatic disease cohort:</u>
At the end of 8 weeks, we will allow tumor progression since patients will have only received vaccine at this point. We estimate that of a total of approximately 24 patients in this cohort, 17% will drop off at this point, leaving a total of 20 patients to be treated in Part 2. To have enough patients to evaluate the secondary endpoints, 20 additional patients may be enrolled in the metastatic disease cohort so that at least 30 patients (10/arm) with gel and 10 are available for Part 2.

At the end of 8 weeks of vaccine, cutaneous lesions (at least 2 and up to 10 lesions) will be assigned by the physician according to region to receive or not to receive 0.2% R848 gel. The CORe randomization system will not be used in Part 2. The physician will designate the regions and which tumors will be treated. The area of cutaneous lesions will be divided into two regions, Region A and Region B, striving to equalize tumor burden in each designated region as best as possible. When feasible, Region A will be a proximal group of lesions, and B distal. When this proximal – distal differentiation is not feasible because of the characteristics of the metastatic pattern, then A and B will be assigned to regions in no particular order. If the two regions coalesce during the vaccination phase, the coalesced tumor may be divided into two regions. Each target tumor whether it is treated or untreated will be marked, measured and photographed for the medical record. Because this dose provides the highest level of immune cell

infiltration, we plan to utilize this dose of R848 two times per week every 3-4 days (+/- 2 days), with the option of dose reducing from twice to once a week should patients experience any grade 3 or greater toxicity.

The physician will designate which of the two regions to receive or not to receive R848. (For example, if the cutaneous disease is limited to the thigh, the area of lesions will be divided in two with one region proximal and one region distal. Then the proximal region is assigned region A and the distal assigned region B. Resiquimod to be applied to region A or B. A 5mm margin will be included in the treatment area around each of the lesions at a spread rate of 10mcg/cm^2. The sum of the area of all treated lesions will not exceed 100cm^2. The treatment area will be allowed to air dry for 30 minutes and then covered with a gauze dressing which can be removed the following day. The R848 gel will be applied in the clinic by the research nurse for the first two weeks of administration, and then the subject will apply the gel at home thereafter. The patient will be trained for gel self-administration by the research staff. Sachets of gel will be supplied for home use and the subject will be instructed on maintaining a home record of administration. This home record or a progress note by one of the research team reviewing home administration may each act as a source document for all matters regarding home administration during this period. The gel may be applied in the clinic for longer than the first two weeks at the discretion of the investigators.

Tumor biopsies of treated and untreated lesions will be obtained as outlined in section 7.3 when feasible in order to assess T-cell infiltration and function at the site.

In order to give patients the opportunity to receive R848 gel on all their lesions, we will allow cross-over of patients who do not respond in untreated lesions by week 16 (8weeks after initiating gel on the tumors) in the untreated lesions to receive R848 gel on all of the lesions, but these patients will not be included in the final analysis of clinical endpoints that occur following week 24. Patients will not cross-over until after all blood, biopsies, and data are recovered for assessment of the primary endpoints in any subject. Treatment with R848 gel will continue for up to 4 months beyond week 24 as long as there is no disease progression.

Subjects in either cohort will be followed for 12 months after the completion of therapy (vaccination in the adjuvant cohort, Resiquimod in the metastatic cohort) unless they meet criteria for withdrawal as per section 10.0.

7.0 Evaluation During Treatment

7.1 Clinical Evaluation

Patients will be evaluated every 2 weeks (+/- 2 days) through week 4, then every 4 weeks (+/- 1 week) during the vaccination/treatment. For subjects in the adjuvant cohort, this schedule will continue through week 8, for patients in the metastatic cohort, this will continue until Resiquimod treatment is discontinued. With each patient visit, the evaluation will consist of complete interim history and physical examination, including measurement of vital signs, performance status, and weight. Patients will be evaluated for toxicity and concommitant medications at each visit. Toxicities will be recorded on the toxicity sheet and the medications will be recorded in the patient's electronic medical record. Patients will have a complete blood count (CBC), with differential, PT/PTT, serum electrolytes, BUN, creatinine, glucose, albumin, alkaline phosphatase, ALT, AST, LDH, calcium, and total bilirubin tested at baseline and then tested approximately every 2-4 weeks (using pediatric blood collection tubes if feasible).

For patients in the adjuvant arm,

CT scans of the chest, abdomen, and pelvis will be done at baseline, week 8, (+/- 1 week) and then repeated at 8 week (+/- 1 week) intervals during resiquimod treatment. MRI/CT of the brain will be done at baseline and repeated at the time of the CT scans if clinically indicated.

At discontinuance of vaccination in the adjuvant cohort or resiquimod treatment in the metastatic cohort, clinical follow-up, labs, and staging studies (CT, MRI, PET scans) will be obtained at the discretion of the treating physician following standard clinical pathways.

Subjects in either cohort will be followed on study for 12 months after the completion of therapy (vaccination in the adjuvant cohort, Resiquimod in the metastatic cohort) unless they meet criteria for withdrawal as per section 10.0. Clinical events will be tracked from the physician determined routine clinical follow-up during this period. Clinical events that occur after this period on study may be retroactively recorded from the patient's medical record including tumor progression and death.

7.2 Immunological Evaluations

Sixty mL (4 tablespoons) of peripheral blood will be obtained at weeks 0, 2, 4, 8, 12, 16, 20, and 24 (+/- 1 week). Weeks 0, 8 and 16 will collect **only** ten mL (2/3 tablespoon) of peripheral blood by phlebotomy in addition to the leukopheresis. If the leukopheresis is not performed for those time points, then we will collect Sixty mL (4 tablespoons) of peripheral blood. When feasible, biopsies of the vaccine site will be obtained and tumor will be obtained as outlined in section 7.3.

7.2.1 Flow cytometric analysis
Flow cytometry will be utilized to determine the percentage of antigen specific $CD8^+$ (gp100(g209-2M) or $CD4^+$ (MAGE -3) T-cells and the percentage of cells expressing phenotypic markers to assess activation status of T-cells in the circulation and at the tumor site. In addition, this technique will be used to determine the nature of the inflammatory infiltrate at the tumor site in the presence or absence of TLR agonist.

7.2.2 Immunohistochemistry (IHC)
IHC will be performed on vaccine site and tumor biopsies and will be used to assess the nature of the inflammatory infiltrate at these sites in the presence or absence of TLR agonists. Specifically, antibodies specific for pDCs, mDCs, NK cells, and T-cells will be used.

7.2.3 ELISPOT/ Luminex
These assays will be utilized to determine specific cytokine release or number of antigen specific spots per 10^4 $CD8^+$ (for gp100(g209-2M) or $CD4^+$ (for MAGE-3) T-cells. In addition, Luminex assays will be utilized to determine the cytokine profile of antigen-specific T-cells.

7.2.4 Quantitative RT-PCR
This technique will be used to determine the gene expression of specific cytokines at the vaccine and tumor sites.

7.2.5 Leukapheresis will be utilized for blood collection together with a ten mL phlebotomy to obtain peripheral lymphocytes and plasma at three time points when feasible. Leukapheresis will be performed prior to treatment, after treatment at week 8 (+/- 1 week), and at week 16 (+/- 1 week). This is for both cohorts. Each leukapheresis will consist of a 7.5 to 10 liter exchange lasting about three hours.

7.2.6 Plasma collected from phlebotomy will be used to measure reactivity of patient's antibodies against tumor antigens.

7.3 Biopsies: Two areas will be biopsied during the course of this study, the vaccine site and the tumor.

Vaccine biopsies: Vaccine sites will be biopsied from all patients in the study when feasible. The vaccine site biopsied will be a GP100 vaccine site. The vaccine site will be identified and a punch biopsy taken of full thickness skin with standard sterile technique and closure. Vaccine sites will be biopsied at 24 hrs (+/- 6 hours) after the first gp100(g209-2M) vaccine and at week two of vaccination. These biopsies will occur the day following the vaccination for that week. Failure to obtain vaccine biopsies due to refusal, discretion of the investigators or infeasibility will not constitute a violation of the trial.

Tumor biopsies will be taken from patients in the cohort with metastatic disease. There will be no tumor biopsies in patients enrolled in the adjuvant cohort. The first

biopsy will be taken as a base line at any time after enrollment onto the trial, but prior to the first vaccination. A second tumor biopsy will be taken at week 8 prior to the first dose of R848 on the tumors, and at week 12 (4 weeks after initiating R848 therapy on the tumors, but at any time during that week) Tumors will be randomly selected when biopsied, but when feasible, a biopsy will be taken of both a treated lesion and untreated lesion at week 12. Tumor biopsies may be taken using any biopsy method to optimize tumor yield using standard sterile techniques. Tumor volume may not allow for all biopsies to be obtained in all individuals, in this case pre-vaccine biopsies and week 12 will take precedence. Tumor biopsies are at the option of the enrolled subject and the investigators, and failure to obtain tumor biopsies due to refusal, inadequate remaining tumor volume, discretion of the investigators or infeasibility will not constitute a violation of the trial.

7.4 Samples for Research

Leftover blood, tissue, and T-cells (and possibly genetic material extracted from these samples) will be stored for research. These samples may be analyzed by MD Anderson and other qualified investigators. These samples will be de-identified. The research studies involving these samples will be performed following IRB approved protocols. The samples used in these research studies will be to gain further information about the disease and will not be used for future treatment decisions.

8.0 Serious Adverse Event Reporting (SAE)

Vaccines are usually well-tolerated. They may cause discomfort, swelling, and/or redness around the injection site. While there has not been significant autoimmune symptoms caused by gp100(g209-2M) or MAGE-3 peptide vaccines alone, patients who experience any Grade III or greater autoimmune reactivity, except for local skin reactions, will be taken off the study. The occurrence of 3 patients with Grade III or greater toxicity will stop the entry of the study until an assessment of the risks, coordination with MD Anderson IRB and appropriate modifications of the informed consent can be completed.

The most frequent systemic toxicities associated with Resiquimod are fever, fatigue, headache, pain, dizziness, myalgia, chills, and a small increase in absolute lymphocyte count. Localized toxicities reported are erythema, edema, ulceration, weeping, scabbing, flaking, pruritis, and numbness/tingling. Other infrequent toxicities reported that may be related are abnormal LFT, abdominal cramping, nausea, vomiting, diarrhea, backache, neck pain, rhinitis, and uriticaria.

All adverse events will be recorded according to this table:

Attribution	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Unrelated			X	X	X
Unlikely			X	X	X
Possible	X	X	X	X	X
Probable	X	X	X	X	X
Definitive	X	X	X	X	X

Exceptions to this rule will include events and laboratory abnormalities that represent common symptoms and abnormalities of melanoma and chemotherapy and/or have no clinical significance:

- Abnormalities in hematologic parameters due to myelosuppressive therapeutic effect:

  i. Anemia, neutropenia, lymphopenia, thrombocytopenia
  ii. Epistaxis or bleeding except for catastrophic CNS or pulmonary hemorrhage

- Common symptoms of cancer (unless grade >/= 3) including:

  i. Fatigue
  ii. Weakness
  iii. Bone, joint or muscle pain
  iv. Alopecia
  v. Loss of appetite, Nausea, Vomiting
  vi. Chemistry abnormalities (phosphorus, calcium, glucose)
  vii. Coagulation abnormalities (shortened PT, PTT, increased fibrinogen)
- Laboratory abnormalities:
-

  i. LDH (increased or decreased)
  ii. Alkaline phosphatase (increased or decreased)
  iii. Low levels of the following: AST, ALT, creatinine, BUN, uric acid, bilirubin, albumin, total protein
  iv. Electrolyte abnormalities (sodium, potassium, bicarbonate, CO2, magnesium

Abnormal laboratory test results will be captured if intervention is required.

Based on the CTCAE v.4, any grade 3 or greater toxicity will result in a dose reduction of the application of R848 from twice a week to once a week, however we will still report this AE. In addition, if this is required in 3 patients, we will temporarily halt accrual so that we can reassess the study as indicated. Abnormal lab values that are considered clinically significant by the principal investigator will be tracked. The criteria used is based on our experience with this assay in patients, was that biologically significant response is considered to be $\geq$ 0.1% gp100(g209-2M) tetramer + cells in the CD8 + T-cell population, and an increase of $\geq$ 2 fold of gp100(g209-2M) tetramer + cells at any time point over the pre-vaccine levels. This is based on the sensitivity of detection using our current tetramers and immunologic reagents.

8.1 Serious Adverse Event Reporting (SAE)

An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or the sponsor, it results in any of the following outcomes:

· Death

· A life-threatening adverse drug experience – any adverse experience that places the patient, in the view of the initial reporter, at immediate risk of death from the adverse experience as it occurred. It does not include an adverse experience that, had it occurred in a more severe form, might have caused death.

- Inpatient hospitalization or prolongation of existing hospitalization.

· A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.

· 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 patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse (21 CFR 312.32).

· Important medical events as defined above, may also be considered serious adverse events. Any important medical event can and should be reported as an SAE if deemed appropriate by the Principal Investigator or the IND Sponsor, IND Office.

· All events occurring during the conduct of a protocol and meeting the definition of a SAE must be reported to the IRB in accordance with the timeframes and procedures outlined in “The University of Texas M. D. Anderson Cancer Center Institutional Review Board Policy for Investigators on Reporting Unanticipated Adverse Events for Drugs and Devices”. Unless stated otherwise in the protocol, all SAEs, expected or unexpected, must be reported to the IND Office, regardless of attribution (within 5 working days of knowledge of the event).

Investigator may include protocol specific reporting criteria here if desired.

· All life-threatening or fatal events, that are unexpected, and related to the study drug, must have a written report submitted within 24 hours (next working day) of knowledge of the event to the Safety Project Manager in the IND Office.

· Unless otherwise noted, the electronic SAE application (eSAE) will be utilized for safety reporting to the IND Office and MDACC IRB.

· Serious adverse events will be captured from the time of the first protocol-specific intervention, until 30 days after the last dose of drug, unless the participant withdraws consent. Serious adverse events must be followed until clinical recovery is complete and laboratory tests have returned to baseline, progression of the event has stabilized, or there has been acceptable resolution of the event.

· Additionally, any serious adverse events that occur after the 30 day time period that are related to the study treatment must be reported to the IND Office. This may include the development of a secondary malignancy.

Reporting to FDA:

· Serious adverse events will be forwarded to FDA by the IND Sponsor (Safety Project Manager IND Office) according to 21 CFR 312.32.

It is the responsibility of the PI and the research team to ensure serious adverse events are reported according to the Code of Federal Regulations, Good Clinical Practices, the protocol guidelines, the sponsor's guidelines, and Institutional Review Board policy

Investigator Communication with Supporting Companies:

Serious adverse event reporting requirements to supporting companies can be added here if needed.

8.2 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 patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or development of drug dependency or drug abuse (21 CFR 312.32).

a. Important medical events as defined above, may also be considered serious adverse events. Any important medical event can and should be reported as an SAE if deemed appropriate by the Principal Investigator or the IND Sponsor/the IND office.
b. All events occurring curing the conduct of a protocol and meeting the definition of a SAE must be reported to the IRB in accordance with the timeframes and procedures outlined in "University of Texas M.D. Anderson Cancer Center Institutional Review Board Policy on Reporting Serious Adverse Events". Unless stated otherwise in the protocol, all SAEs, expected or unexpected, must be reported to the IND office regardless of attribution.
c. All grade 3-5 reactions to the vaccination and all unexpected SAEs will be reported in an expedited fashion.
d. All life-threatening or fatal events, expected or unexpected, and regardless of attribution to the study drug, must have a written report submitted within 24 hours(next working day) of knowledge of the event to the Safety Project Manager in the IND office.
e. The MDACC "Internal SAE Report Form for Prompt Reporting" will be used for reporting to the IND office
f. Serious adverse events will be captured from the time the patient signs consent until 30 days after the last application of R848. Serious adverse events must be followed until clinical recovery is complete and laboratory tests have returned to baseline, progression of the event has stabilized, or there has been acceptable resolution of the event.
g. Additionally, any serious adverse events that occur after the 30 day time period that are related to the study treatment must be report to the IND office. This may include the development of a secondary malignancy.
h. The adverse events will be recorded in PDMS according to the following guidelines.

8.3.1 Reporting to FDA - Serious adverse events will be forwarded to FDA by the IND Sponsor (Safety Project Manager in the IND office) according to 21 CFR 312.32.

8.4 It is the responsibility of the PI and the research team to ensure serious adverse events are reported according to the Code of Federal Regulations, Good Clinical Practices, the protocol guidelines, and Institutional Review Board policy.

Recommended Adverse Event Recording Guidelines					
Attribution	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Unrelated	Phase I	Phase I	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III
Unlikely	Phase I	Phase I	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III
Possible	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III
Probable	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III
Definitive	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III	Phase I Phase II Phase III

Tumor response to therapy this study will be done using immune-related response criteria (irRC) which is a modified version of WHO criteria.

9.1 Definition of Measureable and Non-Measurable Lesions Measurable Lesions are lesions that can be accurately measured in two perpendicular diameters, with at least one diameter > 10 mm. The area will be defined as the product of the largest diameter with its perpendicular. Skin lesions can be considered measurable. Cutaneous lesions that are > 5 mm in diameter can be considered measurable.

Non-Measurable (evaluable) Lesions are all other lesions, including unidimensionally measurable disease and small lesions.

9.2 Definition of Index/Non-Index Lesions

In many patients, all measurable disease will be cutaneous, so we will allow all 10 index lesions to be in a single organ. All measurable lesions, up to a maximum of ten lesions in total, should be identified as *index* lesions to be measured and recorded on the medical record at baseline. The *index* lesions should be representative of all involved organs. In addition, *index* lesions should be selected based on their size (lesions with the longest diameters), their suitability for accurate repeat assessment by imaging techniques, and how representative they are of the patient's tumor burden. A sum of the products of diameters (SPD) for all *index* lesions will be calculated and considered the baseline sum of the products of diameters. Response criteria to be followed are listed below. The baseline sum will be used as the reference point to determine the objective tumor response of the *index* lesions at tumor assessment (TA). Measurable lesions, other than *index* and all sites of non-measurable disease will be identified as *non-index* lesions. *Non-index* lesions will be recorded on the medical record and should be evaluated at the same assessment time points as the *index* lesions. In subsequent assessments, *non-index* lesions will be recorded as "stable or decreased disease," "absent", or "progression."

9.3 Definition of Tumor Response Using irRC

The sum of the products of diameters at tumor assessment using the immune-related response criteria (irRC) for progressive disease incorporates the contribution of new measurable lesions. Each net Percentage Change in Tumor Burden per assessment using irRC criteria accounts for the size and growth kinetics of both old and new lesions as they appear.

Definition of Index Lesions Response Using irRC

irComplete Response (irCR): Complete disappearance of all *index* lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.

irPartial Response (irPR): Decrease, relative to baseline, of 50% or greater in the sum of the products of the two largest perpendicular diameters of all *index* and all new measurable lesions (i.e., Percentage Change in Tumor Burden). Note: the appearance of new measurable lesions is factored into the overall

tumor burden, but does not automatically qualify as progressive disease until the SPD increases by ≥25% when compared to SPD at nadir.

irStable Disease (irSD): Does not meet criteria for irCR or irPR, in the absence of progressive disease.

irProgressive Disease (irPD): At least 25% increase Percentage Change in Tumor Burden (i.e., taking sum of the products of all *index* lesions and any new lesions) when compared to SPD at nadir.

Definition of Non-Index Lesions Response Using irRC

irComplete Response (irCR): Complete disappearance of all *non-index* lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria

irPartial Response (irPR) or irStable Disease (irSD): *non-index* lesions are not considered in the definition of PR, these terms do not apply.

irProgressive Disease (irPD): Increases in number or size of *non-index* lesions does not constitute progressive disease unless/until the Percentage Change in Tumor Burden increases by 25% (i.e., the SPD at nadir of the index lesions increases by the required amount).

Impact of New Lesions on irRC
New lesions in and by themselves do not qualify as progressive disease. However, their contribution to total tumor burden in included in the SPD which in turn feeds into the irRC criteria for tumor response. Therefore, new non-measurable lesions will not discontinue any subject from the study.

9.4 Definition of Overall Response Using irRC

Overall response using irRC will be based on these criteria:

Immune-Related Complete Response (irCR): Complete disappearance of all tumor lesions (index and non-index together with no new measurable/unmeasurable lesions) for at least 4 weeks from the date of documentation of complete response.

Immune-Related Partial Response (irPR): The sum of the products of the two largest perpendicular diameters of all index lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the sum of the products of the two largest perpendicular diameters of all index lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irSPD). A decrease, relative to baseline of the irSPD compared to the previous SPD baseline, of 50% or greater is considered an immune Partial Response (irPR).

Immune-Related Stable Disease (irSD): irSD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease.

Immune-Related Progressive Disease (irPD): It is recommended in difficult cases to confirm PD by serial imaging. Any of the following will constitute progressive disease:

- At least 25% increase in the sum of the products of all index lesions over baseline SPD calculated for the index lesions.
- At least a 25% increase in the sum of the products of all index lesions and new measurable lesions (irSPD) over the baseline SPD calculated for the index lesions.

Immune-Related Response Criteria Definitions

Index Lesion Definition	Non-Index Lesion Definition	New Measurable Lesions	New Unmeasurable Lesions	Percent Change in Tumor Burden	Overall irC Response
Complete Response	Complete Response	No	No	-100%	irCR
Partial Response	Any	Any	Any	≥-50% <50%to<+25% >+25%	irPR irSD irPD
Stable Disease	Any	Any	Any	<50%tp<+25% >+25%	irSD irPD
Progressive Disease	Any	Any	Any	≥+25%	irPD

9.5 Immune-Related Best Overall Response Using irRC (irBOR)

irBOR is the best confirmed irRC overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local palliative radiotherapy for painful bone lesions) for the individual subjects in the study. For assessment of irBOR, all available assessments per subject are considered.

If a lesion is surgically resected or treated with definitive radiosurgery, the size of the lesion prior to the definitive local therapy will be included in the calculated irBOR.

Primary evidence of antineoplastic activity will be evaluated as a function of objective tumor response following the vaccination period. An overall objective assessment of all measurable and non-measurable disease will be performed as outlined in 9.1. Tumor assessments should be performed by physical exam, ultrasound, MRI or CT scan, throughout the study. The treating physicians will perform tumor measurement. Radiological studies must account for all lesions that were present at baseline and must use the same techniques as used at baseline. All

complete and partial responses must be confirmed by a second assessment at least four weeks later.

9.6 Baseline measurement

In this trial a period of immunization opens the treatment, and immunization alone mediates tumor response rarely.(Cancer immunotherapy: moving beyond current vaccines. Rosenberg SA, Yang JC, Restifo NP. Nat Med. 2004 Sep;10(9):909-15.) So with this sequence, as has been seen with other immunologic mediators, we should expect that a period of progression may precede any response from the treatment.

Therefore, because subjects are likely to progress during the vaccination portion of the study, the baseline will be that measurement during the first 10 weeks of the study at which the tumors are at their maximum size (based on sum of LD). Progression will be allowed during these first 10 weeks without withdrawal of the subject from treatment or from the study. This includes increase in the size of the lesions as well as the appearance of new lesions.

10.0 Criteria for Removal From The Study

10.1 Disease progression:

All patients who develop recurrent disease will be taken off the study. Patients in the either cohort may complete their laboratory evaluation (blood draws, biopsies and pharesis) if they recurr/progress before week 16. Patients will be allowed to continue on the study if their disease progresses during the first 10 weeks of study, since they will have only received vaccine +/- local TLR agonist at this point. For subsequent evaluations, patients with progressive disease will be removed from the treatment.

10.2 Withdrawal of consent:

The patient's desire to withdraw from the study may occur at any time. If a patient withdraws consent, the investigator will assess whether the reason for withdrawal is actually an adverse event, in which case the adverse event should be noted as the reason for withdrawal.

10.3 Adverse event:

Including unacceptable toxicity or exacerbation of underlying disease associated with treatment and necessitating discontinuation of treatment, usually associated with events that pose a threat to the patient's life or functioning. For example, any grade III or greater toxicity, attributable to the peptide vaccines or R848 (Resiquimod)

10.4 Withdrawal by the physician:

For clinical reasons not related to treatment.

10.5 Violation of the study protocol:

Including patient failure to return for required visits. Every effort should be made to contact such patients to determine their reason for withdrawal, and to assess any adverse events that may have contributed to the patient's failure to return. Patients who miss dose(s) because of holidays and/or clinic closures will not be withdrawn from the study.

10.6 End of study

Subjects in either cohort who do not meet any of the above criteria will be followed for 12 months after the completion of therapy (vaccination in the adjuvant cohort, Resiquimod in the metastatic cohort) and will be removed from the study thereafter.

11.0 Statistical Considerations

Overview

This is a randomized phase II trial enrolling melanoma patients who are HLA-A2+ with or without HLA-DP4+. Patients of two separate types will be enrolled in separate cohorts. One cohort includes patients who are NED but at high risk for recurrence. The second cohort will include patients with non-visceral metastatic disease and must have at least two evaluable and biopsiable lesions to be eligible for the trial.

The trial will have two parts. In Part 1 (both cohorts), we will fairly randomize patients to receive either the gp100(g209-2M) +/-MAGE3 peptide vaccine plus a topical TLR 7 /8 agonist, R848, (Arm A) or the gp100(g209-2M) +/- MAGE3 vaccine alone Arm B). The randomization will be stratified by cohort. A total of 48 patients will be enrolled, 24 per arm. All patients will receive the gp100(g209-2M) vaccine (those who are HLA-DP4 + will also receive the MAGE3 vaccine) weekly (for 8 weeks) and the patients randomized to receive R848 gel will have the gel applied to the gp100(g209-2M) vaccine site weekly.

After 8 weeks of treatment on Part 1, Part 2 of the trial begins. In Part 2 (only patients in the metastatic disease cohort), the treating physician will divide the area of cutaneous lesions into regions, Region A and Region B and designate the two regions to receive or not to receive R848. At least one lesion will be randomly assigned to receive R848 from weeks 8 through 24, and one will not. One lesion from each region will be chosen for biopsy as outlined in section 7.3. If a patient has more than two evaluable lesions, we will stratify by lesion size to assure that the lesions will be comparable.

As a safety measure, if a patient's untreated lesions grow while treated lesions are stable or shrink, all lesions will be treated with R848. This will occur after the assessment of the primary endpoints of the study, so it will not affect the analysis of these endpoints.

In April 2015, the trial is being further modified to add an additional 20 patients with metastatic disease, all of whom will receive the the gp100(g209-2M) +/-MAGE3 peptide vaccine plus R848 (Arm C). These patients will not be part of the primary comparative study analysis of T-cell responses in the blood. The purpose of these patients is to provide additional information on characterizing tumor response, defined as at least a 50% shrinkage in tumor size by irRC criteria.

Part 1

The primary objective of Part 1 of the trial is to compare T-cell responses in the blood between the vaccine+R848 and vaccine alone treatment arms. This will be assessed in the two cohorts separately and combined. The primary outcome of this part of the trial will be T-cell response to gp100(g209-2M) +/- MAGE3 measured at 8 weeks using a tetramer/multimer assay measured by flow cytometry. Based on the number of gp100(g209-2M) +/- MAGE3 T-cells measured, when the study began, patients were to be categorized as either immune responders or immune non-responders. From previous vaccine studies, a ≥ 0.1% response was thought to be a reasonable target. Thus, T-cell response to the gp100(g209-2M) + /- MAGE3 peptide in each patient was

defined as ≥0.1% gp100(g209-2M) tetramer+ cells in the CD8+ T-cell population or ≥0.1% MAGE3 multimer cells in the CD4+ T-cell population. In September 2013, due to problems in obtaining the multimers necessary to perform the CD4+ T-cell assays, these assays have not yet been run, although more than 24 patients have reached week 24 on the trial. Based upon the aggregate CD8+ responses, no patients have met the definition of response according to the above definition. Therefore, the study team is changing the definition of the primary endpoint to be the actual change from baseline for gp100(g209-2M) tetramer+ cells in the CD8+ T-cell population. The study will be stratified by cohort (NED/metastatic), but we expect the same immune response in each cohort.

For part 1 the 8-week primary immune outcome values CD8+ T-cell response will be compared between the two arms by using a Wilcoxon rank-sum test. Patients who discontinue from the study before week 8 will be assigned the lowest rank in this analysis. With one interim analysis, using an O'Brien-Fleming boundary to stop for efficacy, a one-sided significance level of 0.05, with 24 patients per arm, we will have 80% power to be able to detect an effect size of 0.73*SD(CD8+ T-cell response).

Part 2

The primary outcomes for part 2 of the trial are immune cell infiltration for pDCs, mDCs, and NK cells in the tumor biopsy samples. This can be assessed in the metastatic disease cohort only, since those subjects in the adjuvant therapy cohort are free of disease. Tumor samples will be taken as outlined in section 7.3 Three different types of analyses will be performed on these tumor biopsies: (1) Immunohistochemistry (IHC) for the degree of pDC, mDC, and NK cell infiltration, (2) Flow cytometry (FACS) analysis for infiltrating melanoma specific CD8+ T cells, and (3) Quantitative RT-PCR (qRT-PCR) assays for IFN-γ, IFN-α (and other type I interferons), IL-12, and TGF-β. For each of these outcomes, we will determine the differences in each of these parameters between the pretreatment and post-treatment samples for each of the biopsied lesions for each patient. Mixed-effect models will be fit for each parameter to assess the effects of treatment, time, and the initial randomization to gp100(g209-2M) +/- MAGE-3 vs. gp100(g209-2M) +/- MAGE-3 +R848. If there is no evidence of an effect due to the initial randomization in Part 1, the changes from baseline for the R848 treated lesion and the untreated lesion will be calculated, and these differences will be compared between the R848 and non-R848 lesions by using a paired t-test.

A sample size argument for Part 2, based upon a 2-sample t-test (which represents a lower bound on the mixed-effects model given above), is as follows: we assume that a total of 24 patients will be enrolled in the metastatic disease cohort, and of these, 17% of the patients initially enrolled will discontinue the trial at 8 weeks due to progressive disease and that dropout will be equally distributed across the 2 arms, leaving a total of 20 patients (10/arm) for Part 2. The 2-sample t-test will assess differences in the change between the two treatment groups with respect to the 3 outcomes described previously. With 20 patients, we will have 80% power to detect an increase of 1.2 standard deviations in the R848 group for each outcome at 8 weeks, assuming a one-sided 2-sample t-test with a 5% Type I error rate. One of the benefits of our mixed-effects modeling approach is that it allows for the analysis of the data using complete likelihood methods (Beunckens, Molenberghs, and Kenward, 2005; Clinical Trials). In addition, we will perform 2 types of sensitivity analysis: 1) We will use last-observation-carried-forward; 2) We will explore how deviations from the Missing at Random (MAR)

assumption of the direct likelihood approach may impact inference using methods developed in Verbeke and Molenberghs (2000).

Additional Cohort of Non-Randomized Patients

As noted above in the Overview, after the initial 48 patients have been enrolled in the randomized portion of the trial, 20 additional patients with metastatic disease will be enrolled into Arm C in order to further characterize tumor response of R848. These patients will not be included in the primary analysis. Tumor response as measured by a 50% shrinkage in tumor size by irRC criteria will be the primary endpoint for these patients. The response rate associated with the standard of care in these patients is no more than 5%. We expect a total of 10 metastatic disease patients in Arm A from the initial randomized portion of the trial. Combined with the additional 20 patients (Arm A and C, with a total of 30 patients, we will have 82% power to detect an increase to 22%, assuming a one-sided exact binomial test with a 5% Type I error rate.

Overview of Analysis of Secondary Endpoints

In addition to the Part 1 and Part 2 primary endpoints described above, several additional secondary endpoints will be investigated. Blood will be drawn at baseline, 2, 4, 8, 12, 16, 20, and 24 weeks, and every 4 weeks after the 24-week draw. A secondary analysis for data generated from Part 1 will measure *ex vivo* IFN-□ via ELISPOT assay. This outcome is measured as a continouous variable. Our first step will be to plot each laboratory parameter over the 24 weeks of follow-up to visually inspect the data for any interesting trends between the two clinical trial arms. Separate graphical displays will be created for each treatment arm (gp100(g209-2M)/MAGE vaccine+R848 and gp100(g209-2M)/MAGE vaccine alone). Subsequent to the initial exploratory data analysis discussed above, a repeated measures model will be fit for each measured parameter in the blood to the data using Proc Mixed in SAS to model the repeated laboratory measurements over the 24 weeks of follow-up. Tumor biopsies will be taken as outlined in section 7.3 In addition, a punch biopsy will be taken at the vaccine site for all patients as outlined in section 7.3. To evaluate the ability of **locally administered** TLR agonist (R848 gel) to activate innate immune cells at the **vaccine site** we will evaluate the inflammatory microenvironment at the site of the immunization. Immune cell infiltration for pDCs, mDCs, NK cells, and T-cells will be measured along with immune cell function using IHC and quantitative RT-PCR (qRT-PCR) by Core B. For each patient, changes from baseline will be calculated for both the lesions treated with R848 and for the non-treated lesions. The 4 immune cell infiltration measures and qRT-PCR relative expression measures are all continuous variables. We will use Wilcoxon signed rank test to compare the measures between the lesions within patient. Significance will be assessed at the 5% level.

To evaluate the ability of R848 gel, administered at the tumor site to induce inflammation and up-regulation of adhesion molecules on tumor vasculature we will focus on the tumor biopsies taken in R848 treated and untreated tumors at pre and post-treatment timepoints outlined in section 7.3. These biopsies will be used to investigate the effect of R848 treatment on tumors compared to untreated tumors. We will measure inflammation and activation of tumor vasculature using Immuno-histochemistry (IHC) and flow cytometry (FACS), We will use mixed models to assess differences between treatment groups. Our mixed model will include fixed effects for treatments, and random effects for

time, treatment-by-time interaction and an additional random effect to model the within patient variability. Significance will be assessed at the 5% level.

To evaluate the ability of R848 gel, administered at the tumor site to enhance immune-cell infiltration into tumor we will utilize antibodies to PDC, mDC, NK, and T cells. Since these are continuous measures (assessed at baseline, 2, 4, 8, 12, 16, 20, and 24 weeks, and every 4 weeks after the 24-week draw) we will use mixed models to assess differences between treatment groups. Our mixed model will include fixed effects for treatments, and random effects for time, treatment-by-time interaction and an additional random effect to model the within patient variability. Significance will be assessed at the 5% level.

To evaluate the ability of R848 gel, administered at the tumor site to generate T-cells against additional tumor antigens, not present in the vaccine (i.e., antigen spreading) we will assess cytokine production against de novo antigens. Since these are continuous measures (assessed at baseline, 2, 4, 8, 12, 16, 20, and 24 weeks, and every 4 weeks after the 24-week draw) we will use mixed models to assess differences between treatment groups. Our mixed model will include fixed effects for treatments, and random effects for time, treatment-by-time interaction and an additional random effect to model the within patient variability. Significance will be assessed at the 5% level.

To evaluate the association between clinical response and laboratory parameters ofT-cell priming, T-cell migration to tumor, and inflammation at the vaccine and tumor sites we will explore the relationship between immune cell activation measures at the vaccine site (vaccine site measures) and at the tumor site (tumor site measures). Additionally we will assess the effect of the TLR agonist R848 upon clinical response. Clinical response will be assessed using the immune-related response criteria (irRC) and will be assessed at week 8, 16, and every 8 weeks thereafter. Since both vaccine site measures and tumor site measures are continuous variables we will assess association between these two types of variables using Pearson's or Spearman's correlation coefficient. In addition, we will use logistic regression to model the relationship between either vaccine site or tumor measures and best clinical response assessed over a 16 weeks period after initial vaccination.

To assess the association between R848 treatment on lesions and response, the irRC will be used to assess response separately by lesion type (lesions receiving or not receiving R848 cream). That is, using the irRC, we will, for each patient, assess if the lesions not treated with R848 cream responded and also determine if the lesions treated with R848 cream responded. Thus, for each patient we will record 4 possible joint outcomes ++,+-,-+, and --, where the ++ indicates that both lesions receiving and not receiving R848 cream responded; -- indicates neither types of lesions responded. Similarly +- and -+ indicate one type of lesion responded while the other did not. We will assess differences between treatments (based on within patient differences) using McNemar's test. A statistically significant difference with respect to the McNemar's test will indicate if cream administered to the lesion results in improved outcome (i.e., immune-related rsponse criteria) relative to lesions for which cream was not administered.

Study Stopping Criteria

We will conduct an interim analysis after half of the patients have been enrolled and have reached week 8 on the study. At the interim analysis, we will test whether there is strong evidence that the immune responses differ between the two treatment arms. We will use an O'Brien-Fleming stopping boundary to stop for efficacy, with a nominal significance level of 0.0056. With the alpha spent at the interim look, the nominal significance level at the final analysis will be 0.0482.

12.0 Data Entry and Protocol Management

For the purposes of this study at M. D. Anderson Cancer Center, the Protocol Data Management System (PDMS) will be employed. All patients will be registered in PDMS/CORE. This study will be monitored by the MDACC Compliance Office. The randomization will be done by the CORE system and the data will be reviewed annually by the institution's Data Safety Monitoring Board (DSMB). The statisticians will prepare the report for review by the DSMB.

13.0 Administrative Procedures

13.1 Changes to the protocol:

Any change or addition to this protocol requires a written protocol amendment that must be approved by the IRB. A copy of the written approval of the IRB must be received before implementation of any changes. The IRB must review and approve all amendments to the protocol.

13.2 Ethics and good clinical practice:

This study must be carried out in compliance with the protocol and Good Clinical Practice, as described in:

13.2.1.1.1 ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996.

13.2.1.1.2 US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations).

13.2.1.1.3 Declaration of Helsinki, concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research
Involving Human Subjects, Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996). The investigator agrees, when signing the protocol, to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice.

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