2013-0422: INDUCTION OF ANTITUMOR RESPONSE IN MELANOMA PATIENTS USING THE ANTIMICROBIAL PEPTIDE LL37

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Abstract

Tumors are potentially immunogenic, however they fail to spontaneously induce immune responses capable of rejecting tumors. A major reason for this is that the tumor microenvironment lacks adequate innate immune activation required to initiate strong adaptive antitumor immunity. Plasmacytoid dendritic cells (pDCs) are highly specialized components of the innate immune system that are capable of sensing microbial nucleic acids via intracellular Toll-like receptors. During viral infection, pDCs accumulate in infected tissues and are activated by viral nucleic acids to produce large amounts of type I interferons (IFNs) and generate protective immunity against the virus by activating myeloid dendritic cells, T cells, and natural killer cells. Tumors also contain pDCs but do not provide molecular signals to activate pDCs, although tumors contain self-DNA released in the extracellular environment at high concentrations as a result of increased tumor cell turnover. pDCs, though activated by viral nucleic acids, are normally not able to sense tumor-derived DNA and thus are unable to initiate strong innate anti-tumor immune responses. We recently found that pDCs can, in fact, sense and respond to self-DNA when combined with an endogenous peptide called LL37. LL37 can bind to self-DNA fragments released by dying cells to form aggregates and condensed structures that are delivered to and retained within early endosomes of pDCs. In these intracellular compartments, LL37/self-DNA can interact with Toll-like receptor 9 to trigger robust type I IFN production similarly to viral DNA. Because tumors release large amounts of self-DNA and contain pDCs but do not express LL37, our hypothesis for the proposed phase I/II outcome-adaptive Bayesian dose finding clinical trial described herein is that exogenous LL37 can be used to target tumor-derived self-DNA and convert it into a “danger signal” that triggers pDC activation and type I IFN production at the tumor site in patients with melanoma. This then induces T-cell-mediated immunity against melanoma by using the same mechanism by which anti-viral-immune responses are induced.

1. Objectives

1.1 Primary Objective

To determine the optimal biologic dosing of LL37 based on toxicity and efficacy.

1. 2 Secondary Objectives

To evaluate antitumor immune responses and clinical efficacy of intra-tumoral injection of LL37 in patients with melanoma.

2. Background and Rationale

2.1 Natural anti-tumor 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 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, all 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 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-α and IFN-β), 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.

2.4 Cancer vaccines have significant potential to generate anti-tumor responses, 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% [23]. 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 enhanced antitumor responses *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

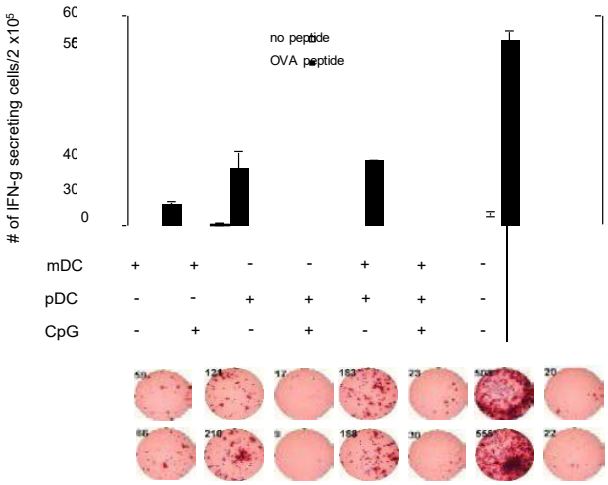

Figure 1. Immunization with a combination of TLR9-activated pDC plus mDC significantly enhances antigen-specific CTL responses. Mice were immunized s.c. with purified pDC, mDC, or a combination of pDC and mDC (1:1) which were first co-cultured for 4 hours with CpG and $OVA_{257-264}$ peptide (the total 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.

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).**

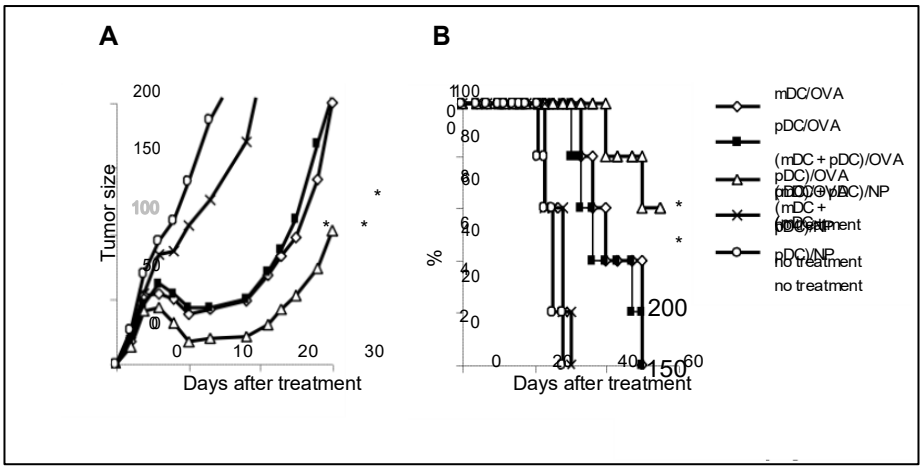

Figure 2. Immunization with a combination of TLR9-activated pDC plus mDC improves therapeutic efficacy against tumor. Mice were inoculated s.c. with E.G7 (OVA+) tumor cells. Four days later, the tumor-bearing mice were immunized s.c. with TLR9-activated, OVA peptide-pulsed pDCs or mDCs alone, or a combination of pDCs plus mDCs, as described in Figure 5. Tumor growth (A) and mouse survival (B) was monitored. (* =Ps<0.05).

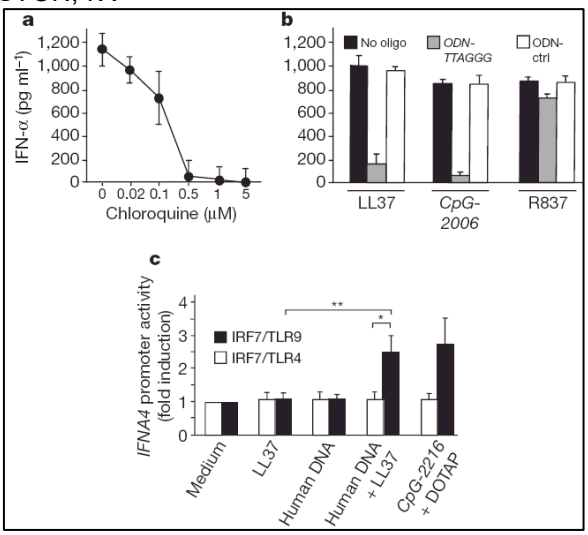

Figure 3. Self-DNA coupled with LL37 triggers pDCs via TLR9. IFN-α produced by pDCs after stimulation with (A) LL37 plus chloroquine at increasing concentrations or (B) LL37, CpG-B-2006, or R837 after pretreatment with ODN-TTAGGG or a control (ctrl-ODN). (C) IFN-α4 promoter activity of IRF7/TLR9 (solid bars)- or IRF7/TLR4 (empty bars)-transfected HEK293 cells measured using a luciferase assay after stimulation under the indicated conditions. CpG-2216 complexed with cationic lipids (DOTAP) was used as a positive control. The data in A and B are representative of four independent experiments; the error bars represent the SD of triplicate wells. The data in C were statistically analyzed using an unpaired two-tailed Student *t*-test and presented as the mean ± the standard error of the mean for five independent experiments. *p=0.01; **p=0.003.

2.6 Self-DNA Coupled with LL37 Triggers TLR9

Because pDCs sense DNA through TLR9 [24], we examined TLR9 to determine whether it is involved in the recognition of the LL37/self-DNA complex by pDCs. Chloroquine, which blocks endosomal TLR signaling, potently inhibited the IFN-α expression induced by LL37/DNA **(Figure 3A).** To specifically inhibit TLR9 in pDC, we used short oligonucleotides (ODN-TTAGGG) that block type I IFN induction by CpGs (TLR9 agonists) but not by imiquimod (R837; TLR7 agonist) **(Figure 3B)**. Pretreatment of pDCs with ODN-TTAGGG specifically inhibited IFN-α induction by LL37/DNA. We confirmed TLR9-mediated recognition of LL37/DNA by showing that the LL37/DNA complex activated the IFN-α4 promoter in IRF7/TLR9-transduced HEK293 cells but not in HEK293 cells expressing an irrelevant TLR **(Figure 3C).** As in pDC cultures, the LL37/DNA complex but not DNA alone activated TLR9-transduced cells. However, in contrast with pDC cultures, LL37 alone was unable to induce activation of TLR9-transduced cells, which is consistent with the absence of cell death in these cultures and hence lack of DNA release. Taken together, these data demonstrate that in complexes with LL37, self-DNA can activate pDCs through TLR9 [25].

2.7 Human Melanomas Contain pDCs in the Vicinity of Dying Tumor Cells but Do Not Express LL37

Human blood pDCs can be identified according to their unique surface expression profiles lacking common lineage markers for T, B, NK cells and monocytes with

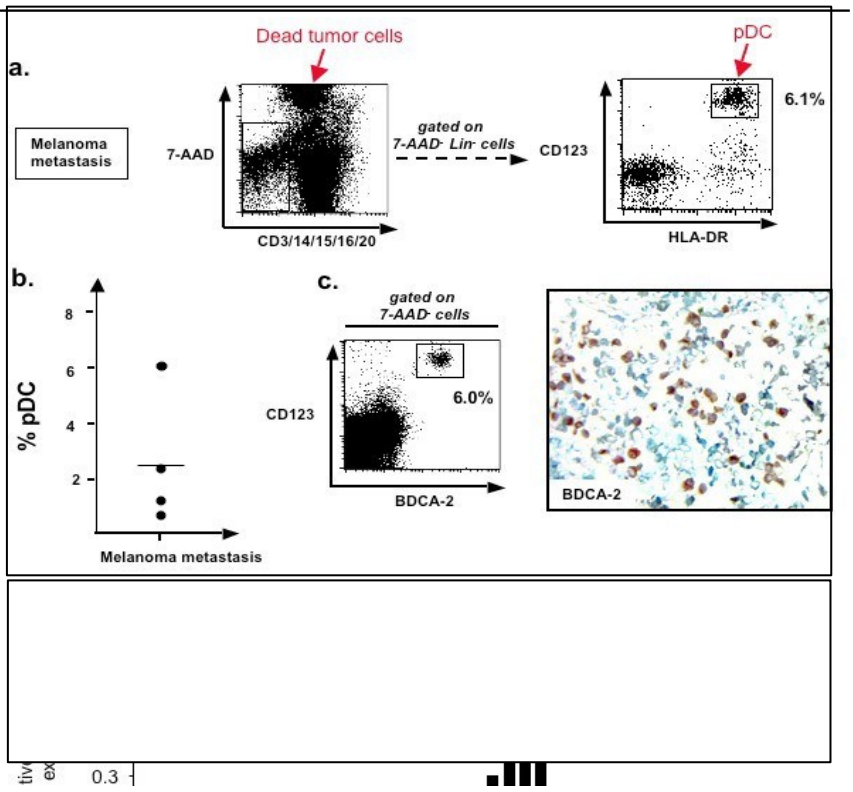

Figure 4. **Melanoma metastases contain pDCs and dying tumor cells but do not express LL37.** (A) pDCs of $^{-}$HLADR^{+} CD123^{+} lineage in mononuclear cell suspensions of a subcutaneous melanoma metastasis. Tumor pDCs co-exist with dying 7-AAD^{+} tumor cells. (B) Percentage of pDCs among mononuclear cells in melanoma metastases in four independent specimens measured as described in A. (C) pDC identification using flow cytometry (left panel) and immunohistochemistry for BDCA-2.

exions obtained from DCs of Hs 4A and **4B**pDCs siells ofHLADR+ CD123+ lineage **(Figure 4C).** Immunohistochemical analysis of BDCA-2 confirmed that substantial numbers of pDCs can infiltrate the tumor microenvironment of human melanoma metastases. Tumor-infiltrating pDCs had nonactivated phenotypes as described previously [35] and were able to produce type I IFNs in response to CpGs as demonstrated by stimulation of tumor-derived mononuclear cell suspensions (data not shown). As aggressively growing tumors, melanomas are typically characterized by high numbers of dying tumor cells. This phenomenon is well known to pathologists in particular because the high degree of cell death makes immunohistological analysis of tumor specimens difficult to interpret[26]. Using flow cytometry, we indeed found considerable numbers of dead tumor cells identified according to their typical large size with high forward/side scatter and staining for 7-AAD. The presence of dying tumor cells suggests the presence of self-DNA released into the extracellular tissue compartment.

Interestingly, we typically found pDCs in areas of tumors where structural integrity is lost because of tumor cell death (**Figure 4C**).

2.8 LL37 Can Bind Self-DNA Released by Dying Tumor Cells

LL37 has the ability to bind to DNA and protect it from nuclease degradation [25, 27]. To determine the ability of LL37 to bind to and protect self-DNA released by dying tumor cells from nuclease degradation, we generated apoptotic melanoma cells in the presence or absence of LL37 and measured the DNA content in culture supernatants using electrophoresis. We confirmed the presence of apoptosis induction by using Annexin V plus propidium iodide staining and using electrophoresis, we detected DNA exclusively in supernatants of irradiated melanoma cells cultured with LL37 (data not shown). These results indicated that irradiated melanoma cells release self-DNA that is bound to LL37 and protected by LL37.

2.9 Intratumoral Injection of LL37 Elicits Local Antitumor Activity

We inoculated 3 x 10^5 B16 melanoma cells into shaved flanks of C57BL/6 mice. We allowed the resulting tumors to grow for 7 days. On day 7, we injected tumors with a single dose of LL37 (20 µmol) or injected them with saline as a control. We monitored tumor size using a caliper and estimated tumor volume using the formula π/6 x length x width^2. We stopped the experiment 12 days after injection because all of the mice in the control group had died or their tumors had reached at least 20 mm in maximal diameter. We found that a single intratumoral injection of LL37 significantly delayed the growth of established B16 tumors **(Figure 5)**. Thus, intratumoral LL37 injection induces potent antitumor activity in melanomas**.**

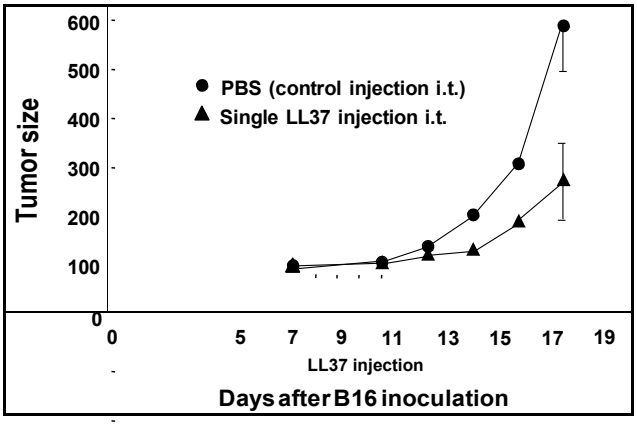

Figure 5. Single intratumoral injection of LL37 delays growth of pre-established B16 tumors. Mice bearing 7- day subcutaneous B16/F10 melanomas were injected with 20 µmol of LL37. PBS injections were performed as controls. Tumor size was monitored using caliper every second day. The data represent the mean for four mice per group. i.t., intratumoral.

2.10 Vaccination with LL37 plus Dying Tumor Cells Elicits Systemic Antitumor Activity

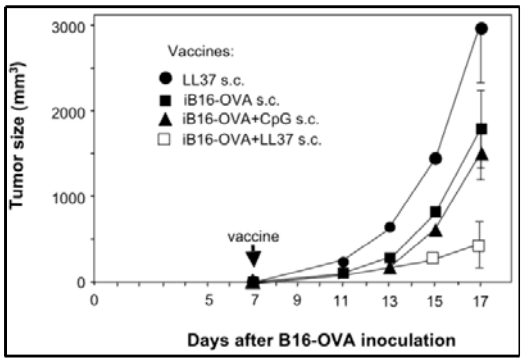

Figure 6. Single vaccination with LL37 plus irradiated B16 cells expressing OVA delays the growth of pre-established B16-OVA tumors. Mice bearing 7-day subcutaneous B16 melanomas transfected with a gene encoding OVA (B16-OVA) were vaccinated subcutaneously with LL37 alone, irradiated B16-OVA tumor cells (iB16-OVA), irradiated B16-OVA tumor cells mixed with 20 μmol of CpG-2216 (iB16-OVA+CpG), or irradiated B16-OVA tumor cells with 20 μmol of LL37 (iB16-OVA+LL37). Tumor size was monitored using a caliper every other day. The data represent the mean for four mice per group.

We performed vaccine studies using the B16 model of melanoma. B16 cells can be transfected with ovalbumin (OVA) to produce an immunogen that allows easy tracking of antitumor immune responses. We subcutaneously implanted 3 x 10^5 B16-OVA cells in the flanks of C57BL/6 mice and allowed them to grow. Seven days later, we gave the mice single subcutaneous injections of LL37 mixed with irradiated B16-OVA cells. Control injections included LL37 alone, irradiated B16-OVA cells alone, and irradiated B16-OVA cells mixed with the synthetic TLR9 agonist CpG. A detailed method for the generation of these vaccines is described below in Research Design and Methods. We monitored the sizes of the resulting tumors using a caliper and estimated the tumor volumes using the formula π/6 x length x width^2 as described previously. We stopped the experiment 10 days after the injections because all of the mice in the control group had died or had tumors that were at least 20 mm in maximal diameter. Vaccination with LL37 plus irradiated tumor cells significantly delayed the growth of 7-day established B16 tumors more than the control groups and more than the mice that received irradiated B16-OVA cells mixed with CpG (**Figure 6**). These findings indicate that vaccination with LL37+dying tumor cells elicit potent systemic antitumor activity. LL37 appears to be more potent than CpG, which is among the most potent adjuvantscurrently tested in clinical vaccination trials [35]. We performed these experiments using CpG-2216, which is the most potent CpG sequence for inducing type I IFNs in both murine and human pDCs [28].

Having demonstrated that LL37, which is capable of stimulating innate immunity by activating pDCs and inducing antitumor immune response, we propose to evaluate this peptide in patients by injecting LL37 into accessible tumor sites followed by evaluations of tumor size, blood samples, and biopsies of injected and non-injected tumors.

2.11 Rationale for Current Protocol

In this study, we will inject melanoma tumor deposits with LL37 in order to activate the innate immune response at the tumor site. We hypothesize that this will lead to a strong systemic T-cell response resulting in immune activation and tumor regression at LL37 injected as well as non-injected sites. The primary objective will be to ascertain the optimal biologic dose of LL37 which will be defined based upon toxicity and efficacy

We anticipate that these studies may lead to principles in cancer immunotherapy that may be widely applicable to the treatment of melanoma as well as other cancer types.

3. Clinical Pharmacology

3.1 LL37 Peptide Preparation and Administration

Synthetic LL37 will be manufactured in GMP conditions and supplied as a lyophilized bulk powder by Bachem Americas. The lyophilized powder will be reconstituted to a concentration of 8 mg/mL in sterile saline and stored frozen in aliquots of at -20°C. The peptide solution will be thawed and diluted as required in sterile saline to achieve peptide concentrations of 8 mg/mL (2000 µg/250 µL) or 4 mg/mL (1000 µg/250 µL) or 2 mg/mL (500 µg/250 µL) or 1 mg/mL (250 µg/250 µL). Peptide solution will be prepared in P14 CTL/GMP laboratory by trained personnel. The peptide solution will be prepared from the bulk LL37 received from Bachem Americas according to an approved SOP. The solution will be vialed and the lot will be tested per the SOP. Analytical testing is performed by the contracted testing laboratory SGS MScan per GMP peptide specific procedures and a QA reviewed/approved report is provided to the MD Anderson GMP. The MD Anderson GMP will prepare a CoA and if all test specifications are met, the lot will be released and sent to investigational pharmacy.

Since it is assumed that the average size of a melanoma nodule is approximately $1cm^3$ which is equivalent to 1mL, use of a vialed peptide solution at 2000µg/250µL would produce a final concentration of 2000µg/250 µL injected into the tumor. Similar mathematics is utilized for all dose levels and injection of 250µL of LL37 administered into tumor sites will achieve concentrations of 250, 500, 1000, or 2000 µg/tumor. Up to four lesions will be treated per patient.

3.2 Dose Justification

In our murine studies we injected 100 µg into 0.1-0.2 cm^3 tumors. Assuming that tumors are a self-contained mass in which the injected peptide will diffuse, we hypothesize concentrations of **500-1,000 µg/ml** are in the range that is needed for pDC activation and antitumor function. This range is also close to the levels seen in psoriasis patients (mean 1,520 µg/ml), in which robust pDC activation is seen[29]. Therefore, in the clinical trial we propose to inject 250, 500, 1,000, or 2,000 µg of LL37 peptide into tumors. Assuming that the average size of injected human melanoma nodules is $1cm^3$ (= 1ml) the concentrations in the tumors will be **250, 500, 1,000 or 2,000 µg/ml.** We estimate that these levels should be well tolerated by patients based on the murine data and levels found in psoriasis patients.

4. Eligibility Assessment and Enrollment

4.1 Inclusion Criteria

4.1.1 Patients with histologically documented metastatic melanoma with at least 3 cutaneous lesions measuring over 5mm diameter. At least two lesions must be at least 10mm in diameter to serve as the injected disease. At least one other lesion measuring at least 5mm in diameter may serve as the non-injected lesion that will be measurable disease. Patients will have stage IIIB or IIIC (in-transit lesions with or without nodal metastases) or stage IV M1A disease with cutaneous or nodal lesions assessable for administration of LL37. Patients are only eligible if their melanoma deposits are not amenable to complete surgical excision. Skin lesions that are 5mm or greater are deemed measurable however lesions that are at least 10mm in diameter will be preferentially utilized for LL37 injection.

4.1.2 Age greater than or equal to 18 years.

4.1.3 Clinical performance status of ECOG 0 – 2 within 30 days of signing informed consent.

4.1.4 Total bilirubin less than or equal to 2.0 mg/dl, except in patients with Gilbert's Syndrome who must have a total bilirubin less than 3.0 mg/dl.

4.1.5 Platelet count greater than or equal to 100,000/mm^3

4.1.6 WBC $\geq$3000/mm^3

4.1.7 Serum ALT and AST less than three times the upper limit of normal

4.1.8 Serum creatinine $\leq$ 2.0 mg/dl

4.1.9 Seronegative for HIV antibody

4.1.10 Patients with a negative pregnancy test (urine or serum) must be documented within 28 days of of starting treatment for women of childbearing potential (WOCBP). A WOCBP has not undergone a hysterectomy or who has not been naturally postmenopausal for at least 12 consecutive months (i.e. who has not had menses at any time in the preceding 12 consecutive months).

4.1.11 Unless surgically sterile by bilateral tubal ligation or vasectomy of partner(s), the patient agrees to continue to use a barrier method of contraception throughout the study such as: condom, diaphragm,

hormonal, IUD, or sponge plus spermicide. Abstinence is an acceptable form of birth control.

4.1.12 Patients must consent for protocol PA13-0291 for potential immunologic evaluations on biopsy specimens.

4.2 Exclusion Criteria

4.2.1 Active autoimmune disease requiring disease modifying therapy.

4.2.2 Concurrent systemic steroid therapy.

4.2.3 Any form of active primary or secondary immunodeficiency.

4.2.4 Prior malignancy except 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 LL37.

4.2.6 Active systemic infections requiring intravenous antibiotics.

4.2.7 Prior systemic therapy, radiation therapy, or surgery within 28 days of starting study treatment

4.2.8 Patients who are pregnant or nursing.

4.3 Pretreatment Evaluation

At the screening visit, patients will be assessed for study eligibility. All patients must sign an informed consent form and a negative pregnancy test (urine or serum) must be documented for women of childbearing potential before enrollment and being registered in CORE/PDMS. Consent will be obtained within 28 days of therapy initiation.

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

4.3.1 Complete history, demographics, concurrent medication usage and physical examination including vital signs, height, weight, noting in detail the exact size and location of any lesions that exist will be performed.

4.3.2 Chemistries to include serum electrolytes, BUN, creatinine, glucose, albumin, alkaline phosphatase, ALT, AST, LDH, calcium, and total bilirubin.

4.3.3 CBC, differential, PT/PTT, and platelet count will be performed.

4.3.4 β-HCG pregnancy test (urine or serum) on all women of childbearing potential will be performed.

4.3.5 EKG.

4.3.6 HIV serology.

4.3.7 Baseline radiological studies to evaluate the status of disease (CT scans of chest, abdomen, pelvis: MRI/CT of brain) to evaluate the status of disease. Ultrasound or CT of area of in transit lesions is required.

The following pre-treatment studies must be completed **within 7 days** of treatment initiation:

4.3.8 Medical photography and measurements of in-transit lesions and/or cutaneous lesions.

4.3.9 Baseline adverse event recording, physical exam and concurrent medication usage will be documented

4.3.10 Baseline biopsy of tumor site to be injected with LL37

4.3.11 If patient agrees to the optional procedure, leukapheresis consisting of 7.5-10 liter exchange lasting approximately 3 hours to obtained baseline immunologic sampling

5. Treatment Plan

5.1 Phase I (dose escalation) Overview

The dose escalation schema is listed in table 5.1.

Table 5.1: dose escalation schema for phase I

Dose Level	LL37
1 (starting dose)	250 µg/tumor per week
2	500 µg/tumor per week
3	1000 µg/tumor per week

A total of 36 patients will be enrolled into the study. Two patients will be entered at each dose level beginning with cohort 1 (starting dose 250 µg/tumor). The number of subjects at each dose level will be assigned adaptively as described in the Statistics Section (section 10) but to summarize, the efficacy and toxicity of

each dose will be monitored simultaneously and will be used to determine the desirability of each dose. Once two patients have been treated on the same dosing level, pre-treatment and 24 hour post treatment biopsies will be analyzed for amount of interferon alpha upregulation by RT-PCR analysis. DLT assessment will be done within the first two weeks of each patient's therapy. Once the efficacy and toxicity data are available, the EffTox program will be utilized and the next cohort of patients can be assigned.

There will be no intra-patient dose escalation above the assigned dosing.

Dose Limiting Toxicity in a given patient is defined as:

a. Any grade 3 or 4 non-hematologic toxicity by NCI CTCAE Version 4.03 regardless of duration, including:
   Grade 3 skin reactions at injection sites including experiencing severe tissue damage or require operative intervention and Grade 3 fever
b. Grade 3 or 4 hematologic toxicity as per the NCI CTCAE Version 4.03

DLT monitoring will be done within the first two weeks of therapy. Patients will discontinue administration of LL37 if they experience a DLT.

LL37 will be administered intratumorally in cutaneous or subcutaneous tumors at least 1 cm in diameter. Patients will receive weekly intratumoral injections of LL37 for up to 8 weeks. The injections will be given every 7 days (+/- 48 hours).

Dose administration will be delayed based on toxicities including development of intolerable Grade 2 toxicities with a specific focus on fevers and injection site reactions. Doses can be held for a maximum of 2 weeks. Protocol therapy will be discontinued if toxicities do not reduce to at least Grade 1 within this specified time.

Biopsy samples of injected tumors will be obtained before the initial injection, 24 hours (+/- 8 hours), and 4 weeks (+/- 48 hours) after the initial injection. Minimum biopsy sample size is a 5mm by 5mm punch biopsy performed in clinic by mid-level providers or attending physicians. The samples will be obtained by either a punch biopsy or an excisional biopsy. Biopsy samples of distant non-treated tumors will be obtained at week 4 (+/- 48 hours).

Peripheral blood, approximately 60 ml, will be collected before the initial injection and then every 2 weeks (+/- 48 hours) to assess systemic immune responses to tumors. If patient agrees to the optional procedure, leukapheresis will be performed prior to initial treatment and at week 8 for more extensive immune monitoring.

5.2 Study Calendar

	Pre-study (within 28 days of start)	Pre-study (within 7 days of start)	Start (Day 1, week 1)	Day 2 week 1[8]	Week 2[9]	Week 3[9]	Week 4[9]	Week 5[9]	Week 6[9]	Week 7[9]	Week 8[9]	Off study[10]
Informed consent	x											
EKG	x											
HIV serology	x											
Medical photography		x					x				x	x
Demographics	x											
Medical history	x											
Concurrent meds	x	x			x	x	x	x	x	x	x	x
Physical exam + vitals + PS[1]	x	x	x		x	x	x	x	x	x	x	x
Adverse event evaluation		x	x		x	x	x	x	x	x	x	x
CBC with diff	x		x		x	x	x	x	x	x	x	x
Serum chemistries[2]	X		x		x	x	x	x	x	x	x	x
PT, PTT	x											
B-hcg[3]	x											
Biopsy injected site[4]		x		x			x					x
Biopsy noninjected site[5]							x					
Leukapheresis[6]		x									x	
Research blood[7]			**x**			**x**		**x**		**x**		x
Radiologic evaluations [11]	x										x	x
Clinical tumor measurements		x					x				x	x

1. Full physical exam including vital signs and Eastern Cooperative Group Performance Status
2. Serum chemistries include: albumin, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, glucose, LDH, phosphorous, potassium, total protein, SGOT (AST), SPGT (ALT), sodium
3. Serum or urine β-hcg test in women with child bearing potential
4. If feasible, biopsy of LL37 injection site week 4 will be performed. Biopsy will only be done if there is evidence of clinical tumor persistence.
5. If feasible, biopsy of non-LL37 injected lesion will be performed at week4. Biopsy will only be performed at week 4 if clinically evident tumor remains
6. If patient agrees to the optional procedure, leukapheresis will be performed prior to treatment and 8 weeks (+/- 48 hours) after initial LL37 injection. Each leukapheresis will consist of a 7.5-10 liter exchange lasting approximately 3 hours
7. Approximately 60mL of blood will be taken for research purposes including 1 10mL red top tube for serum, 1 green top tube for plasma and 2 20mL green top tubes for peripheral blood mononuclear cells
8. 24 hours +/-8 hours after initial LL37 injection. It is preferable that this biopsy site be different from the site of the initial biopsy if possible
9. All subsequent time measurements are weekly +/- 48 hours from time of initial LL37 injection
10. Off study is at time of documented progression as per the immune mediated response criteria, withdrawal of consent or significant noncompliance on the protocol or grade IV adverse event
11. Radiologic evaluations in the form of CT scans of affected disease sites will be performed every 8 weeks (+/- 14 days) while on study.

6. Evaluation during Study

6.1 Clinical evaluation:

Patients will be evaluated every week at the time of the injections for up to 8 weeks. Physical examination, update of concurrent medications and adverse event recordings will be done during weekly visits prior to LL37 injections. CBC with differential and serum chemistries during weekly visits for up to 8 weeks and when off study will be performed. Medical photography at baseline, week 4, 8 and off study will be performed. After completion of LL37 injections, patients will be followed routinely in clinic for a period of one year to assess ongoing response to therapy.

6.2 Immunological Evaluations

6.2.1 For blood and tissue studies, specimens will be collected as part of the protocol as specified in the Study Calendar (section 5.2), processed and/or stored for later analysis.

6.2.2 If patient agrees to the optional procedure, leukapheresis will be utilized for blood collection instead of phlebotomy to obtain peripheral lymphocytes at two time points when feasible. Leukapheresis will be performed prior to treatment and 8 weeks (+/- 48 hours) after initial LL37 injection for assessment of specific T-cell reactivity in peripheral blood.

Each leukapheresis will consist of a 7.5 to 10 liter exchange lasting approximately 3 hours.

6.3 Biopsies

Two areas will be biopsied during the course of the study including LL37 injected and non-injected sites. The first biopsy will be taken as a base line at any time after enrollment onto the trial, but prior to the first vaccination. Tumor biopsy of an injected site will be taken approximately 24 hours (+/- 8 hours) after initial LL37 injection to allow for analysis of interferon alpha levels which will help inform us of the optimal biologic dose. At week four, biopsy of both LL37 injected and non-injected sites will be taken if tumor tissue is still present. Tumor biopsies may be taken using any biopsy method to optimize tumor yield using standard sterile techniques. We anticipate most biopsies will be done via a 5mm x 5mm skin punch biopsy performed in clinic. At least one injected or one non-injected deposit must not be biopsied to allow for preservation of measureable disease. Fresh tumor biopsies will be sent to the Immunotherapy Platform or Melcore lab.

If patient agrees to the optional procedure, blood and tissue specimens collected in the course of this research project may be banked and provided in the future to investigators with IRB approved research protocols.

6.4 Off study is at time of documented progression as per the immune mediated response criteria, withdrawal of consent or significant noncompliance on the protocol or grade IV adverse event. Off study procedures will include full physical exam, documentation of adverse events and concurrent medications, scans for documentation of progression, tumor biopsy, research blood, standard of care laboratories, medical photography and clinical tumor measurements. Off study procedures will be completed within 14 days of patient going off-treatment. Patients will be contacted by phone or clinic visit at 30 days (+7 days) from last drug administration for all drug-related toxicities which were present at the end of study.

6.5 Duration of Treatment

Patients who tolerate the drug without documented evidence of progression (as defined by Immune Mediated Response Criteria) will be treated for eight weeks. After completion of all 8 sets of LL37 injections, patients will be monitored off of therapy with routine clinic visits and restaging scans every 8-12 weeks per the discretion of the treating physician.

6.6 Duration of Follow Up

After patients are removed from the study, they will be followed every 3 months for 1 year to assess status of disease if possible. If the patient does not plan to continue to receive medical care at MDACC, they will be contacted by phone/email/letter to assess disease status.

6.6 Priority of assays and quantitative parameters for analysis

Tissue Type	Parameter	Modality	Assay	Quantitative Measurement	Priority
Blood	T-cell phenotype	Flow cytometry	Specific antibodies for effector-memory panel and homing panel	Percentage of cells expressing markers	1
	T-cell cytokine production	Luminex and ELISPOT	T-cell production of IFN-Γ and other cytokines	Specific cytokine release or number of antigen-specific spots per 10_6 CD8+ (for gp100) or CD4+ (for MAGE-3) T cells	2
	T cell specificity for melanoma antigens	Flow cytometry	Tetramer staining for MART-1, gp100, and tyrosinase	Percentage of antigen-specific CD8+ (MART-1, gp100, tyrosinase) T cells	3
	T-cell specificity for melanoma antigens (if no tetramer+ CD8+ cells are found)	Overlapping peptide libraries	Cytokine production against *de novo* antigens	Specific cytokine release per 10_6 T cells	4
Tumor (LL37 treated and untreated)	Immune cell function	qRT-PCR	qRT-PCR for cytokine gene expression	Relative expression of cytokine genes compared to housekeeping genes	1
	Immune cell infiltration	IHC	Antibodies to pDCs, mDCs, NK cells, and T cells	Number of positive cells per high-power field	2
	T-cell specificity and phenotype	Flow cytometry	Tetramer and T-cell phenotype antibodies	Percentage of antigen-specific CD8+ (MART-1, gp100, tyrosinase) T cells positive for tetramer and other markers	3
	Immune cell phenotype	Flow cytometry	Antibodies specific for pDCs, mDCs, and Treg (Foxp3); effector-memory and homing panel	Percentage of cells expressing markers	4

7. Evaluation of Toxicity

7.1 This study will utilize the National Cancer Institute Common Terminology Criteria (CTC) for Adverse Events version 4.03 for toxicity and Adverse Event reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.03.

7.2 Dose Limiting Toxicity in a given patient is defined as:

b. Any grade 3 or 4 non-hematologic toxicity by NCI CTCAE Version 4.03 regardless of duration including: grade 3 skin reactions at injection sites including experiencing severe tissue damage or require operative intervention and Grade 3 fever
Grade 3 or 4 hematologic toxicity as per the NCI CTCAE Version 4.03 regardless of duration.

7.3 Adverse event recording will occur weekly prior to LL37 injections

7.4 The principal investigator will monitor the data and toxicities to identify trends. The principal investigator will be responsible for revising the protocol as needed to maintain safety. The MD Anderson IRB will review serious adverse events as they are submitted. Serious adverse events will be submitted to the FDA by the IND Safety Project Manager in the IND Office. The principal investigator will also review serious adverse events and evaluate trends. Whenever a trend is identified, the principal investigator will determine an appropriate follow up plan. The investigator or physician designee is responsible for verifying and providing source documentation for all adverse events and assigning the attribution for each event for all subjects enrolled on the trial.
Prior to advancing the dose level cohort, a cohort summary will be submitted to the clinical research monitor.

7.5 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:

7.5.1 Death.

7.5.2 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.

7.5.3 Inpatient hospitalization or prolongation of existing hospitalization.

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

7.5.5 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.

7.5.7 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).

7.5.8 **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.

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

7.5.10 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.

7.5.11 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.

7.5.12 Reporting to FDA

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

7.5.13 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.

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

7.5.14 IND safety reports as required under 21CFR312.32©(1).

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

7.5.16 Careful evaluation to ascertain the toxicity, immunologic effects and anti-tumor efficacy of therapy will be performed.

8. Criteria for Response

8.1 Tumor measurements will be performed at week 0, then every 4 weeks while the patient remains on the study. For visible cutaneous or palpable subcutaneous tumors, target sites will be assessed by measurements of tumor size on clinical exam and photographically documented and placed in the patient's electronic chart. Tumor measurements and photography will be performed every 4 weeks while on study.

8.2 For visceral or other subcutaneous or soft tissue metastases, radiologic evaluations in the form of CT scans will be performed every 8 weeks while the patient remains on the study. Percentage decrease in the size of individual tumor lesions will be monitored and compared for different time points during the study.

8.3 Tumor response to therapy for this study will be assessed using immune-related response criteria (irRC) which is a modified version of the WHO criteria [30].

8.4 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 > 5 mm. The area will be defined as the product of the largest diameter with its perpendicular. Injectable lesions will be at least 10mm in diameter.

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

8.5 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."

8.6 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.

8.7 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

8.8 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 8.6. 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. Criteria for Removal from the Study

Patients will be taken off the study if: (a) the patient voluntarily withdraws, (b) there is significant noncompliance (failure to appear to more than 2 protocol specified procedures), or (c) there is progression of disease in those patients after treatment.

Any patient who develops a DLT or Grade IV toxicity due to treatment will be taken off protocol.

10. Statistical Considerations and Data Analysis

The primary objective of this trial is to determine the optimal biological dose (OBD) of LL37 based upon toxicity and efficacy. The trial will be conducted based on a phase I/II outcome-adaptive Bayesian dose-finding procedure (EffTox) described in Thall and Cook [31] and Thall, Cook and Estey [32]. Under the EffTox procedure, the efficacy and toxicity of each dose will be monitored simultaneously and will be used to determine the desirability of each dose (I.e. trade-off between efficacy and toxicity) based on the accumulating dose-outcome data. Each time a dose must be chosen, the desirability of each dose is determined based on the current interim data and the next cohort is given the most desirable dose. Efficacy will be defined by IFN-alpha expression at the treated tumor site 24 hours after the first injection of LL37 (positive is defined as at least 2-fold higher IFN than baseline levels). Toxicity will be defined as a DLT (defined in Section 7.2) experienced within the first two weeks.

Each successive cohort of 2 patients (N=36) will be assigned adaptively to one of four dose levels of LL37, with the first cohort starting at dose level 1: 250 µg (per 1 cm^3 tumor). Dose levels 2-4 will be 500, 1000, and 2000 µg, respectively. Simulation results establishing the design's properties under each of five potential dose-outcome scenarios are summarized in Table 10.1 (EffTox dose-finding v4.0). The lowest acceptable probability of efficacy (pE) used in the simulation was 0.20 [Pr (pE > 0.20 | data) > 0.05]. The highest acceptable probability of toxicity (pT) used in the simulation was 0.30 [Pr (pT < 0.30 | data) > 0.05]. Each scenario was simulated 1000 times. For each scenario, Table 10.1 includes the desirability (see technical details), the proportion of trials that each dose is selected, and the average number of patients treated at each dose. Of note, if there is no evidence of efficacy at any dose (Scenario 3), the percent of selecting no OBD is 80% and only 14 patients will be treated before the study will be stopped.

Table 10.1:Operating Characteristics of the Design

Scenario		Dose Level				
		1 (250 µg/tumor)	2 (500 µg/tumor)	3 (1000 µg/tumor)	4 (2000 µg/tumor)	None
1						
	True pT, pE	.02, .05	.03, .15	.05, .30	.08, .75	-
	Desirability	-0.20	-0.08	0.09	0.55	-
	% selected	1	1	26	72	0
	# Patients Treated	2.9	2.4	9.4	21.2	-
2						
	True pT, pE	.40, .20	.50, .40	.60, .60	.70, .80	-
	Desirability	-0.82	-0.95	-1.14	-1.38	-
	% selected	26	7	4	2	62
	# Patients Treated	10.4	3.5	2.3	0.5	-
3						
	True pT, pE	.02, .01	.03, .01	.05, .01	.08, .01	-
	Desirability	-0.25	-0.25	-0.27	-0.30	-
	% selected	2	0	0	18	80
	# Patients Treated	2.5	2.0	2.5	7.0	-
4						
	True pT, pE	.10, .20	.20, .40	.30, .60	.40, .80	-
	Desirability	-0.11	-0.10	-0.20	-0.39	-
	% selected	34	29	28	4	5
	# Patients Treated	11.8	10.5	9.9	2.0	-
5						
	True pT, pE	.05, .50	.10, .65	.15, .80	.20, .95	-
	Desirability	0.33	0.40	0.40	0.32	-
	% selected	49	22	25	4	0
	# Patients Treated	19.6	7.3	7.6	1.5	-

Data Analyses

At the end of the trial, the OBD will be determined using the algorithm above. Safety data will be summarized by dose level.

Analyses of response will be performed on patients treated at the OBD. The rates of CR, PR, SD, and PD will be estimated with 95% confidence intervals. Additionally, response will be defined as experiencing either a complete or partial response (CR or PR), and the association between response and disease characteristics and T-cell responses will be assessed using logistic regression. Because we are using an adaptive design, the number of patients that will be treated at the OBD is unknown up front, but we expect this number to be between 19 and 27 (from the table above). If we have 19 patients treated at the OBD, we will have 80% power to detect an odds ratio of 3.6 associated with a particular covariate, assuming a logistic regression model with a normally distributed covariate and a Type I error rate of 5%. If we have 27 patients treated at the OBD, we will have 80% power to detect an odds ratio of 2.9 under the same assumptions. If the number of patients is between 19 and 27, the detectable OR will fall between 2.9 and 3.6.

Time to progression and overall survival will be estimated by the Kaplan-Meier method. Cox proportional hazards regression models will be used to assess the association between survival parameters and disease and demographic factors of interest.

11. 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 CORe utilizing a two-turnstile registration before any study specific tests are performed. The EffTox procedure will be implemented using the Biostatistics Department Clinical Trial Conduct Website. Concomitant medications will be captured in the medical record.

The principal investigator agrees to keep all information and results concerning the study confidential. The confidentiality obligation applies to all personnel involved with this clinical trial. The Investigator must ensure that each participant's anonymity will be maintained in accordance with applicable laws. The principal investigator should keep a separate log of ID numbers, names and addresses. Documents that contain the names associated with these ID numbers (e.g., written consent/assent forms), should be maintained by the Investigator in strict confidence except to the extent necessary to allow auditing by regulatory authorities, auditing or monitoring by the IRB.

The Principal Investigator shall obtain all such permissions and authorizations as may be necessary or desirable to allow the collection and use of information protected under federal privacy laws and state privacy laws, including permission/authorization for monitoring and analysis (including re-analysis in combination with results of other studies), for regulatory submission purposes and for applicable reporting (if any).

12. Administrative Procedures

12.1 Changes to the Protocol:

Any change or addition to this protocol requires a written protocol amendment that must be approved by the IND Office and the IRB. A copy of the written approval of the IRB must be received by the IND Office and the principal

investigator before implementation of any changes. The IRB must review and approve all amendments to the protocol. This study will be monitored for compliance by the IND office.

12.2 Ethics and Good Clinical Practice:

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

12.2.1 ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996.

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

12.2.3 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).

12.2.4 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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