Multi-Compartmental Delivery Systems for Peptide and DNA Vaccines in Melanoma Immunotherapy

Thesis presented

by

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ABSTRACT

The vaccine delivery systems containing adjuvants modulate magnitude, breadth, quality, and longevity of immune responses to antigen vaccinations aimed to induce cell-mediated immunity. For vaccines targeting tumor antigens, adjuvant-based delivery systems are necessary to overcome various tolerance mechanisms and facilitate induction of cytotoxic T lymphocytes (CTLs) that can traffic to and lyse malignant cells. The objective of this doctoral thesis project was to develop a multi-compartmental vaccine delivery system for safe and efficient delivery of a variety of melanoma antigens for immunotherapy.

Water-in-oil-in-water (W/O/W) multiple emulsions-based multi-compartmental vaccine delivery systems containing the gp100 peptide or hgp100 plasmid DNA was prepared by a two-step emulsification method. Morphological evaluation of the W/O/W multiple emulsions demonstrated that the oil-droplets were homogenously dispersed with an antigen encapsulated in an inner aqueous-phase. In addition, in vitro transfection and transgene expression ability of the hgp100 plasmid DNA vaccine was investigated in murine immune cells. In vivo immunization effectiveness of the novel gp100 antigen vaccines was evaluated in the murine B16 melanoma model. Immunization of C57BL/6 mice using the W/O/W multiple emulsions encapsulated gp100 antigen vaccines provided increased protection against tumor challenge. In addition, serum Th1 cytokine levels and immuno-histochemistry of excised tumor tissues indicated the vaccine evoked of antigen-specific cell-mediated anti-tumor immunity. Moreover, the newly developed vaccine
formulation was well tolerated \textit{in vivo}. Further enhancement of immune responses by preventing adenosine-mediated immune-suppression was investigated by concurrent administration of adenosine A$_{2A}$ receptor antagonist such as caffeine. A considerable increase in protection against tumor challenge due to co-administration of caffeine with gp100 peptide or hgp100 plasmid DNA immunization was not observed. Therefore, adjunct therapy using potent adenosine A$_{2A}$ receptor antagonists such as ZM-241385 or istradefylline (KW-6002) is recommended to validate the hypothesis.

The W/O/W multiple emulsions-based vaccine formulation efficiently delivers the gp100 peptide and hgp100 plasmid DNA antigens to evoke cell-mediated anti-tumor immunity and protects C57BL/6 mice against subsequent tumor challenge. Thus, the novel multi-compartmental vaccine delivery system offers an alternate, safe antigen delivery for melanoma immunotherapy.
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OBJECTIVE AND SPECIFIC AIMS

A significant challenge amid therapeutic strategies against cancer is to precisely target tumor cells while minimizing collateral damage to normal tissues. Immunotherapy in cancer treatment has potential to accomplish this clear and critical need due to inherent biologic specificity of the immune system. Identification and characterization of melanoma-associated antigens have shifted a treatment paradigm from conventional therapy to immunotherapy. A current approach to melanoma immunotherapy involves administration of the tumor specific antigens emulsified in the Incomplete Freund’s Adjuvant (IFA) to enhance an immune response. Despite its effectiveness, use of IFA in vaccine formulations imposes additional challenges such as severe adverse reactions and poor injectability. Therefore, it is desirable to develop a safe and effective delivery system for melanoma vaccines to induce enhanced anti-tumor immunity. Water-in-oil-in-water (W/O/W) multiple emulsions have potential to enhance an immune response due to reservoir effect of an antigenic peptide incorporated in the inner aqueous-phase. In addition, being a particulate delivery system it is internalized by immune cells thereby transfecting them with encapsulated plasmid DNA to produce encoded protein antigen endogenously. Moreover, multiple emulsions offer notable advantages of improved tolerability, low viscosity, and ease of injection. Therefore W/O/W multiple emulsion-based multi-compartmental vaccine delivery system was selected for this research thesis.

The first objective of this doctoral thesis project is to develop a novel water-in-oil-in-water (W/O/W) multiple emulsions-based multi-compartmental delivery system for gp100 peptide and plasmid DNA vaccines in melanoma immunotherapy. The second
objective is to evaluate effectiveness of the W/O/W multiple emulsion-based vaccines to induce anti-tumor immunity \textit{in vivo} in murine B16 melanoma model using prophylactic and active treatment approaches.

The six specific aims of this thesis project were:

- **Aim 1**: Preparation and optimization of squalane oil-containing W/O/W multiple emulsions-based vaccine delivery system for gp100 peptide and plasmid DNA administration.
- **Aim 2**: \textit{In vitro} characterization of W/O/W multiple emulsions based gp100 peptide and plasmid DNA vaccine.
- **Aim 3**: \textit{In vitro} evaluation of murine immune cell-specific transfection capability of the W/O/W multiple emulsions encapsulated plasmid DNA vaccine.
- **Aim 4**: \textit{In vivo} evaluation of anti-tumor effectiveness of gp100 peptide and plasmid DNA vaccines in murine B16 melanoma model in prophylactic treatment approach.
- **Aim 5**: \textit{In vivo} evaluation of anti-tumor effectiveness of gp100 peptide vaccine in murine B16 melanoma model in active treatment approach.
- **Aim 6**: \textit{In vivo} evaluation of vaccine tolerability in C57BL/6 mice by measuring body weight change, liver enzymes, and histopathology of liver and spleen tissues.
CHAPTER 1
MELANOMA DISEASE AND CANCER IMMUNOTHERAPY

1.1 Melanoma Disease

Melanoma is the most serious form of a neoplasm that originates from epidermal melanocytes of the skin. Melanocytes produce melanin, a pigment responsible for skin, hair, and eye color and that protects from the harmful ultraviolet (UV) rays in sunlight. Melanoma is a leading cause of death from the skin disease. In addition, melanoma is one of several cancers that exhibits growing incidence in recent years globally\(^1\). This is mainly due to change in lifestyle habits and due to gradual decrease in the stratospheric ozone that provides shielding from direct ultraviolet radiation. During the year of 2011, The National Cancer Institute (NCI) estimated about 70,230 new cases and 8,790 deaths from melanoma in the United States\(^2\). Estimates suggest the melanoma incidence rates will double every 10-20 years\(^3\). Melanoma is curable during early stage if diagnosed and treated. However, metastasis and relapse occur frequently and the prognosis for patients with metastatic melanoma is poor\(^4,5\). Conventional therapy such as surgery, radiation, and chemotherapy induces a complete therapeutic response in a minority of patients. Moreover, melanoma is a devastating illness and treatment options are limited. Therefore, there is a critical need for an effective therapy for melanoma treatment\(^6\).

1.2 Immunotherapy for the Treatment of Cancer

Cancer immunotherapy, including the development of prophylactic and therapeutic vaccines, is considered a novel approach for prevention and treatment of cancer. Immunotherapy involves a wide range of therapeutic interventions that can ultimately stimulate the body’s immune system to target and eradicate neoplastic cells\(^7\).
The exquisite specificity of the immune system can be utilized to precisely target the cancer cells while minimizing collateral damage to surrounding normal cells. The ideal goal for cancer vaccine is to elicit potent anti-tumor immunity involving both innate and adaptive immune systems that can recognize and subsequently destroy the tumors mass. During past several years, researchers have developed a variety of immunization modalities; including whole tumor cells, tumor lysates, specific tumor antigens, tumor peptides, heat shock proteins, DNA vaccines, exosomes, and dendritic cells (DCs)-based vaccines. Despite initial disappointments, the rapid increase in knowledge of the immune system and its regulation led to a resurgence of interest in cancer immunotherapy that resulted in several approved products. In June 2006, the United States Food and Drug Administration (FDA) approved viral oncoprotein-based immunotherapy, known as Gardasil® (or Silgard by Merck & Co., Inc., Whitehouse Station, NJ). Gardasil® is indicated for the prevention of certain types of human papilloma virus (HPV) infection that is responsible for most HPV-induced anal, vulvar, vaginal, and penile cancers. Gardasil® does not treat existing infection and, therefore, it is used primarily as prophylactic vaccination before adolescence and potential sexual activity. In April 2010, the FDA-approved the DCs-based adaptive immunotherapy, commercially known as Provenge® (or Sipuleucel-T by Dendreon Corporation, Seattle, WA) for the treatment of advanced prostate cancer in men. Provenge® is the first FDA-approved cancer immunotherapy that is targeted towards tumor antigens that exist in the specific patient; therefore it is used as therapeutic cancer vaccine. A schematic showing induction of anti-tumor immunity using DCs-based immunotherapy such as Provenge is illustrated in the Figure 1.
Figure 1: Generation of Antitumor DC Vaccines from Peripheral Blood Monocytes. Elutriated monocytes from a leukapheresis are cultured with GM-CSF and IL-4 to produce dendritic cells (DCs), which are then matured with CD40 ligand (CD40L) or other agents, pulsed with peptide or tumor lysate, or transduced with an expression vector and then injected into the patient as an autologous DC vaccine to induce a T cell immune response against the tumor.

1.2.1 Anti-Tumor Immunity

It is well established that the immune system has capacity to attack malignant cells. During malignant transformation cells acquire numerous molecular and biochemical changes converting them vulnerable to immune cells. Yet, it is self-evident that a growing tumor has managed to evade the host defense mechanisms. The exact ways in which the immune system interacts with tumor cells and how cancers are able to escape immunological eradication have only recently started to be fully explained. It is crucial to understand relationship between the tumor and the anti-tumor immune response and how that can be altered to develop successful immunotherapy for cancer patients. Although anti-tumor immunity involves both the innate and adaptive immune systems, it is generally recognized that CD8+ cytotoxic T lymphocytes (CTL) are the most potent anti-tumor effector cells. The T-cell immune response can be broken down into the following steps. All of the steps need to be satisfied for effective anti-tumor immunity: (1) tumor antigen(s) must be present, and (2) they must be seen as dangerous by the immune system; (3) antigens must be acquired and presented by antigen presenting cells (APC) in the draining lymph node; (4) specific T-cells must then recognize and respond to tumor antigen by proliferation, enter into systemic circulation and reach to the tumor site as CTL; (5) where they need to overcome local immunosuppressive environment before killing tumor cells. In addition, the memory cells may need to be generated to produce a long-lasting response. Failure of the anti-tumor immune response can occur at one or more of these steps. Targeting rate-limiting steps with therapies designed to boost the immune response can improve anti-tumor immunity.
1.2.2 Tumor Antigens

Tumors typically express two types of antigen: neo-antigens and self-antigens. Neo-antigens (tumor-specific antigens- TSA) are derived from mutated self-proteins that are not expressed in normal tissues. Malignant cells express numerous neo-antigens as a result of genomic instability\textsuperscript{10}. Most of these mutations do not have functional significance for the tumor cell, but may still provide potential antigenic targets for immune cells. In addition, tumors can also express normal self proteins, but in abnormal quantities or locations (tumor-associated antigens- TAA). During T-cell development, T-cell precursors with a strongly self-reactive T-cell receptor are deleted in the thymus, resulting in a T-cell repertoire with a high affinity for foreign antigens and a weak affinity for self antigens. Thus, tumor neo-antigens being foreign induce strong immune responses whereas tumor associated antigens are considered self and therefore induce weak immune responses. CTL responses can be generated against weak antigens, but require higher antigen concentrations and prolonged duration of exposure\textsuperscript{11}. It is imperative to identify the ideal tumor-specific antigen targets for an effective anti-tumor immunotherapy\textsuperscript{12}.

1.2.3 Role of Antigen Presenting Cells

Although T-cell receptors bind with variable affinity to self- and non-self antigens, they are not competent to discriminate dangerous from harmless antigen by themselves. The antigen presenting cells (APCs), especially DCs, satisfy this crucial role by acquiring antigens and responding to associated danger signals. Subsequently, they present the antigens to naïve T-cells in the context of major histocompatibility molecules (MHC) with the appropriate information about the level of danger present as shown in
The ‘professional’ APCs present the processed antigen bound to MHC class I molecules to naïve CD8+ T-cells and provide the additional co-stimulation needed to activate CTLs. In addition, helper (CD4+) T-cells recognizes the antigen presented on MHC class II molecules and allows DCs to release co-stimulatory signal to promote CTL activation. Thus, helper T-cells offer a ‘second opinion’ to the DCs so that antigens recognized as dangerous are promoted as immunogenic. The fate of the T-cells whether it becomes primed or inactivated as a result of encounter with DCs critically dependent on state of the DCs maturation. Immature DCs are inefficient at cross-presenting antigen and do not express the co-stimulatory molecules required for T-cells activation. DCs maturation is initiated by ‘danger signals’ from antigen and by inflammatory cytokines such as IFN-γ. DCs maturation results in increased antigen uptake, up regulation of MHC expression, and expression of co-stimulators CD80 (B7-1) and CD86 (B7-2). During maturation, DCs migrate from tissues to draining lymph nodes where they activate naïve T-cells.

1.2.4 Role of Anti-Tumor Cytotoxic T-Lymphocytes

Cellular immune responses such as activated CD4+ and CD8+ (also known as T-Cells, killer T-cells, or cytotoxic T-lymphocyte) CTLs, a sub-group of T-cells play an important role in anti-tumor immunity and in maintaining immune system homeostasis. The final effector cells in the most anti-tumor models are the CD8+ CTLs. Thus, the majority of cancer immunotherapy efforts are devoted to stimulate cellular immune responses against tumors. The immunologic destruction of established tumors depends on three criteria: (i) sufficient numbers of immune cells with highly avid recognition of tumor antigens must be generated in vivo (ii) these cells must traffic to and
infiltrate the tumor stroma, and (iii) the immune cells must be activated at the tumor site to manifest appropriate effector mechanisms such as direct lysis or cytokine secretion capable of causing tumor cell destruction. In addition, T-cells must be in the correct state of activation and differentiation in order to mediate the antitumor effects\textsuperscript{16}. Activation of anti-tumor CD8\textsuperscript{+} CTLs by mature DCs in the draining lymph node requires several signals: T-cell receptor binding to antigen coupled to MHC class 1, ligation of CD28 on the T-cells with CD80 or CD86 on the DCs and release of inflammatory cytokines such as IL-12 and type 1 INF-\(\gamma\). In addition, an effective anti-tumor immunity also requires that CTLs proliferate, survive in the circulation, and enter into the tumor site to execute their effector function\textsuperscript{17} as shown in Figure 3. Activated CD8\textsuperscript{+} CTLs infiltrate the tumor site, encounter tumor antigens, and release perforin and granulysin: cytotoxins that form pores in the target tumor cell's plasma membrane. The pore formation allows ions and water to flow into the cells and cause it to burst or lyse\textsuperscript{18}. Additionally, CD8\textsuperscript{+} CTLs release granzyme, a serine protease that enters cells via pores to induce apoptosis (cell death) through Fas-Fas ligand (FasL) pathway\textsuperscript{19-21}.

Persistent CD4\textsuperscript{+} help and IL-2 secretion are required to maintain CD8\textsuperscript{+} T-cells function and numbers. Direct cell–cell contact from CD4\textsuperscript{+} T-cells also protects the effector CD8\textsuperscript{+} T-cells from activation induced cell death. Importantly, CD4\textsuperscript{+} T-cells secrete cytokines promoting clonal expansion of CD8\textsuperscript{+} CTLs thus cause immune rejection of tumor cells\textsuperscript{22-24}. Combining tumor antigen-specific CD4\textsuperscript{+} T-cells with CD8\textsuperscript{+} T-cells in adoptive transfer treatment in mice demonstrated increased accumulation of tumor-specific CTLs in tumor and lymphoid tissues compared to CD8\textsuperscript{+} T-cells transfer alone. Thus, CD4\textsuperscript{+} T-cells play a critical role in effector function of CD8\textsuperscript{+} T-cells\textsuperscript{25}. 
Figure 2: Induction Phase of Anti-Tumor CD8+ Cytotoxic T-Lymphocytes.
Immature dendritic cells (DCs) acquire tumor antigen migrate to the draining lymph node. Antigen is processed by the DCs and presented to CD4+ T-cells on MHC class II molecules and cross-presented to CD8+ T-cells on MHC class I molecules. DCs activation is promoted by danger signals, IFN-γ and ligation of CD40 by helper T cells. On activation DCs express co-stimulatory molecules and cytokines, leading to activation of tumor antigen-specific T-cells.
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Figure 3: Effector Phase of Anti-Tumor CD8+ Cytotoxic T-Lymphocytes.
Cytotoxic CD8+ T-cells (CTLs) exit the circulation and enter into tumor site. CD4+ T-cells facilitate tumor infiltration and may promote secondary expansion of CTLs. Following recognition of the cognate antigen presented on the tumor cells surface, CTLs execute the effector cell function that results in tumor cell killing. Local immunosuppressive mechanisms may inhibit the anti-tumor response, including suppression by regulatory T-cells and inhibitory cytokines.
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1.3 Immunotherapy for the Treatment of Melanoma

Melanoma is known to exhibit an inherent immunogenicity\(^\text{27}\). Therefore melanoma immunotherapy is viable for prevention of tumor growth, metastasis, and relapse. As shown in the Table 1\(^\text{28}\) melanoma antigens can be divided into two broad categories: tumor-associated antigens (TAA) and melanoma-associated antigens (MAA). The TAA, MAGE-3 is expressed in melanoma and other tumors such as colon and lung cancer. They can be cell-surface products seen in embryonic tissues, proto-onco gene products, or antigens associated with viral transformations\(^\text{29}\). MAA are found predominantly in melanomas, but also are often expressed in normal melanocytes. MAA are usually proteins or glycoproteins such as tyrosinase-related protein (TRP) and melanoma-antigen recognized by T cells-1 (MART-1, e.g. gp100) respectively and are often associated with melanogenesis\(^\text{30-33}\). The CTLs derived from melanoma patients are able to recognize melanoma antigens and sensitize the tumor cells \textit{in vitro} suggesting antigens can be targeted to induce anti-tumor immunity\(^\text{34}\).

Table 1: Immunogenic Antigens Identified in Human Melanoma Cells

<table>
<thead>
<tr>
<th>Tumor-associated antigens (TAA)</th>
<th>Melanoma-associated antigens (MAA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary TAA (glycoprotein 90)</td>
<td>Lipoprotein 180</td>
</tr>
<tr>
<td>Fetal antigen (glycoprotein 70)</td>
<td>Tyrosinase</td>
</tr>
<tr>
<td>810 peptide (43 kDa)</td>
<td>MART-1/Melan A</td>
</tr>
<tr>
<td>MAGE-1</td>
<td>Glycoprotein 75 (gp75.TRP)</td>
</tr>
<tr>
<td>MAGE-3</td>
<td>Glycoprotein 100 (gp100/pmel 17)</td>
</tr>
<tr>
<td>GM(_2)</td>
<td>High molecular weight melanoma antigen</td>
</tr>
<tr>
<td>GD(_2)</td>
<td></td>
</tr>
<tr>
<td>O-acetyl GD(^3)</td>
<td></td>
</tr>
<tr>
<td>GM(_3)</td>
<td></td>
</tr>
</tbody>
</table>

Reprinted from John Wiley & Sons, Inc. © 1996\(^\text{28}\).
Native gp100 is a member of melanocyte differentiation antigens family that is strongly expressed in most melanomas. It is a hydrophobic glycoprotein of 661 amino acids with a molecular mass of 70 kDa (GenBank Acc. No.NM_006928). gp100 protein includes a variety of immunogenic epitopes that are recognized by CTLs recovered from peripheral blood of melanoma patients and from tumor infiltrating lymphocytes (TILs). While gp100-derived peptides have been used in many melanoma vaccination studies, the full-length gp100 protein has rarely been used because of difficult purification method and its hydrophobic nature. Compared with the peptide-based approach, there are several notable advantages with use of entire protein as vaccines: (i) they are non-HLA-restricted, (ii) may contain multiple antigenic epitopes, and (iii) may stimulate both CD4+ and CD8+ T-cells. Therefore, gp100 peptide was selected as one of the melanoma antigens in this doctoral thesis project.

### 1.4 Challenges in Cancer Immunotherapy

Tumors are already engaging with the immune system in the patients with cancer. The goal of immunotherapy is to boost the immune response such that the balance shifts from tolerance to rejection. An immunotherapy may fail due to limiting factors at any point in the induction or effector phase shown in Figure 2 and Figure 3, respectively. Such factors include inadequate quantity of tumor antigens or inadequate danger signals to generate strong immune stimulation. Vaccination targeting self-antigens may produce only weak T-cell responses that may be insufficient to cause tumor regression. The recent clinical trial involving human papilloma virus vaccine showed that anti-tumor immunity mediated through a robust effector T-cells response is essential for successful immunotherapy. In this study the measured T-cells responses strongly correlated with
regression of tumor lesions\textsuperscript{39}. However, in other clinical trials evaluating tumor vaccines against larger, invasive malignancies the effective generation of tumor antigen-specific CTLs in peripheral blood has not predicted clinical efficacy\textsuperscript{40}. This difference may reflect the weaker activity of T-cells generated by cancer vaccines targeting shared self-tumor antigens compared to those directed against viral neo-antigens. It may also reflect the presence of a number of barriers to effective immunotherapy in established invasive tumors compared to premalignant lesions. The anti-tumor T-cells response may fail downstream of the induction phase because of a variety of possible conditions: (1) CTLs may remain in the periphery or in the draining lymph node without actually infiltrating the tumor site, (2) CTLs may disseminate to the tumor but are unable to mediate anti-tumor activity, (3) Activated T-cells may fail to continue expansion and maintain effector function, (4) Activated T-cells may be switched off by immune suppressors secreted by tumors, (5) Immune suppressive action of regulatory T-cells on activated T-cells, (6) Tumors may alter their microenvironment to escape immune surveillance.

\subsection*{1.5 Role of Adjuvants in Vaccine Formulation}

Newer generation of vaccines, particularly those based on recombinant proteins and DNA, have a favorable safety profile, but they are less immunogenic than attenuated organisms. Therefore, vaccine adjuvants have been used to increase the magnitude of an adaptive immune response to an antigen. The immune-adjuvant is an agent that can stimulate the immune system and increase response to a vaccine, without having any specific antigenic effect of its own. Whereas, immuno-stimulatory adjuvants are predominantly derived from pathogens and often represent pathogen-associated molecular patterns, such as lipopolysaccharides and CpG nucleic acid motifs. The
adjuvants activate and engage components of the innate immune system to enhance T- and B-cell responses\textsuperscript{41,42}. However, recently adjuvants have been employed to guide the immune system to produce the most effective forms of immunity for each specific pathogen; for example T helper 1 (Th1) cell versus T helper 2 (Th2) cell, CD8+ versus CD4+ T-cells, and specific antibody isotypes. Adjuvants are used in cancer immunotherapy to: (1) increase the immune response to a weak tumor-antigen; (2) facilitate the use of smaller doses of antigen; and (3) permit immunization with fewer doses of vaccine. Very few vaccine adjuvants have been licensed for use in human. Alum (aluminum salts) has been widely used for more than 70 years and until recently represented the only approved adjuvant in the United States. MF59 and AS03 (squalene oil-in-water emulsions) are licensed for adjuvant-containing influenza vaccines in Europe. AS04, a combination adjuvant composed of monophosphoryl lipid A (MPL) adsorbed to alum is approved for hepatitis B virus (HBV) and HPV vaccines in Europe and has been recently licensed in the USA.

Vaccine delivery systems are generally nano- and micro-particulate systems, such as liposomes, oil-in-water emulsions, and polymeric micro-particles that can encapsulate the antigen payloads and deliver them to the target site and possibly APC’s – either systemically or upon mucosal administration. Increasingly, more complex formulations are being developed in which delivery systems are exploited both for the delivery of antigens and also for the delivery of co-administered immune-stimulatory adjuvants. The rationale for this approach is to ensure that both antigen and adjuvant are delivered into the same population of APCs. Moreover, delivery systems can focus effect of the adjuvants onto the key cells of an immune system and limit systemic distribution to
minimize potential adverse effects. The potent immune-adjuvants encapsulated in particulate vaccine delivery system may allow the development of therapeutic vaccines against cancers.

### 1.5.1 Squalane Oil as Vaccine Adjuvant

Squalene is a linear hydrocarbon precursor of cholesterol found in many tissues, notably liver of sharks (Squalus) and other fishes. Squalene is a fully saturated analog that is prepared by hydrogenation of squalene. Thus, it offers increased stability against auto-oxidation. Squalene is a natural product present in sebaceous secretions. Squalene is free-flowing oil and has been used in pharmaceuticals as a skin lubricant, as an ingredient in suppositories, and as a vehicle for lipophilic drugs. Both squalene and squalane can be metabolized and have a good record in toxicology studies.

Several reports in the literature suggest squalene oil-based emulsions demonstrated potent and safe vaccine adjuvant effects in preclinical and clinical evaluation including a number of trials carried out in infants. In the 1980s, a vaccine adjuvant containing squalene oil emulsion (MF59) was developed by Syntex (U.S.A.) Inc. (Palo Alto, CA) to replace more commonly used Freund’s adjuvant. MF59 enhanced the mucosal and systemic immunogenicity of subunit influenza vaccine when administered intranasally in mice. A squalene oil-containing O/W emulsion with encapsulated influenza vaccine was approved in Italy in 1997, and in several additional countries in the year 2000. This formulation showed enhanced immunogenicity of influenza vaccine in small animal models. Moreover, use of squalene oil emulsion resulted in a potent adjuvant effect compared to alum for hepatitis B vaccine in baboon and in humans. Studies involving labeled squalene oil-based formulations showed
that it is internalized by macrophages and dendritic cells at the site of injection and in local lymph nodes via mucosal administration\(^59\).

Both squalene and squalane were found to be equally effective as vaccine adjuvant, and squalane is preferred because of its greater stability\(^45\). Our group has previously demonstrated enhanced mucosal and systemic immune response stimulation using squalane oil multiple emulsions as delivery system in mice\(^60\). To leverage adjuvant effect of squalane oil, it was selected as dispersed oil-droplets phase in the W/O/W multiple emulsions.

### 1.5.2 Pluronic® Surfactant as a Vaccine Adjuvant

Pluronic\(^\circledast\) (poloxamer) is a non-ionic, hydrophilic polyoxyethylene–polyoxypropylene (POE–POP) ABA-type block copolymer used for its surfactant and protein stabilizing properties. Pluronic\(^\circledast\)-based systems exhibit a variety of useful biological attributes, such as excellent biocompatibility. For example, W/O/W emulsions formulated using Pluronic\(^\circledast\) block copolymers have demonstrated the immune-adjuvant properties\(^61,62\). Pluronic\(^\circledast\) block copolymers can significantly enhance both cell-mediated and humoral immune response against broad spectrum of antigens. The Pluronic\(^\circledast\) F127-based vaccine delivery system in combination with CpG motifs or chitosan was evaluated for multiple protein antigens\(^63\). IgG antibody responses were significantly enhanced by the F127/CpG and F127/chitosan combinations compared to antigens mixed with CpGs or chitosan alone. In another study ProJuvant\(^TM\) (Pluronic\(^\circledast\) F127/chitosan) enhanced the immune response to intranasally administered tetanus toxoid\(^64\). Because of its immune-adjuvant effect Pluronic\(^\circledast\) F127 was selected as surfactant to stabilize the dispersed oil-droplets during multiple emulsions preparation.
CHAPTER 2
PREPARATION AND CHARACTERIZATION OF MULTI-COMPARTMENTAL WATER-in-OIL-in-WATER EMULSION FOR gp100 PEPTIDE ANTIGEN DELIVERY

2.1 Introduction

The multi-compartmental, water-in-oil-in-water (W/O/W) multiple emulsions are three-phase systems in which an internal aqueous-phase (nanometer size) encapsulating oil-droplets are stabilized and dispersed in an external aqueous-phase\textsuperscript{60,65}. A schematic representation of the dispersed oil-droplet in the W/O/W multiple emulsions is shown in Figure 4. The entrapped internal aqueous-phase acts as a reservoir and can be used to prolong release of the payload\textsuperscript{66}. In addition, the dispersed oil-droplets act as particulate adjuvants and have comparable dimensions to the pathogens; therefore, they are naturally targeted for uptake by APCs to facilitate potent immune response stimulation. Moreover, the W/O/W multiple emulsions possess low viscosity due to the outer aqueous-phase and therefore offer improved tolerability and notable ease of injection\textsuperscript{45,46}. The multiple emulsions also offer an advantage of encapsulating several active agents in a single formulation and sequestering the different agents in selective compartments for enhanced stability\textsuperscript{67-69}. Therefore, the W/O/W multiple emulsions have been used in drug delivery applications including vaccination\textsuperscript{65,70-72}. 
Figure 4: Schematic Representation of the W/O/W Multiple Emulsions.
Structure of the W/O/W multiple emulsion droplet showing the role of the various components.
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2.2 Materials and Methods

Extra pure grade squalane oil and Pluronic® F-127 were provided as a gift by Jedwards International (Quincy, MA) and BASF Corporation (Florham Park, NJ), respectively. IFA and Span 80™ were purchased from Sigma Chemicals, Inc. (St. Louis, MO). All other chemicals were procured from Fisher Scientific (Fair Lawn, NJ) and were used as received.

2.2.1 Preparation of the W/O/W Multiple Emulsions Vaccine

Squalane oil-containing the W/O/W multiple emulsions were prepared by two-step emulsification method described by Okochi60,65 for water soluble payload. The gp100 peptide solution (3 mg/mL) was emulsified with squalane oil-Span 80™ mixture (9:1) using a homogenizer (Silverson’s Model: L4RT-A; Silverson Machines, East Longmeadow, MA) at 10,000 rpm for 5 minutes. The resulting primary water-in-oil (W/O) emulsion was further emulsified with Pluronic® F127 solution (0.5% w/v) using the homogenizer at 10,000 rpm for 10 minutes to create the W/O/W multiple emulsions.

2.2.2 Optimization of the W/O/W Multiple Emulsions Vaccine

The W/O/W multiple emulsions preparation method was optimized using ovalbumin as a surrogate payload. The W/O/W multiple emulsions comprising different grades of Pluronic® such as L64, P123, F68, and F127 were prepared using identical homogenization conditions (10,000 rpm for 10 min) to identify the suitable grade. Additionally, the effect of second emulsification step homogenization speed (5,000 - 10,000 rpm) and time (5 - 10 minutes) on dispersed oil-droplets size and polydispersity index was studied to identify the optimum homogenization conditions. The composition
and homogenization condition that created the smallest size oil-droplets with narrow size distribution was considered suitable composition and optimum condition.

2.2.3 Preparation of Incomplete Freund’s Adjuvant W/O Emulsion Vaccine

The IFA-containing W/O emulsion vaccine formulation was prepared by homogenization method. The gp100 peptide solution (1.5 mg/mL) was emulsified with IFA using the homogenizer (Silverson’s Model: L4RT-A) at 10,000 rpm for 10 min to produce W/O emulsion.

2.2.4 Determination of the Oil-Droplets Size, Surface Charge, and Morphology

The dispersed oil-droplets size was measured by the photon correlation spectroscopy using a Zetasizer ZS (Malvern Instruments Ltd, UK). The W/O/W multiple emulsions samples were diluted 100-fold with Pluronic® F127 solution (0.5% w/v) and the hydrodynamic diameter of oil-droplets (50-150 kcps) was measured at 90° fixed angle at 20 °C. The surface charge (zeta potential) on the W/O/W multiple emulsions dispersed oil-droplets were measured using the Zeta-sizer ZS instrument. About 100-fold diluted (Pluronic® F127 solution (0.5% w/v)) samples were connected to an electrode at 4 V and 2 Hz field frequency at 25°C. The zeta potential values were derived from the electrophoretic mobility of the dispersed oil-droplets using the Smoluchowski–Helmholtz equation.

The dispersed oil-droplets morphology was investigated by optical microscopy. Additionally, the internal aqueous-phase and dispersed oil-phase were stained using Evans blue (water-soluble dye) and Sudan red (oil-soluble dye) to ascertain the W/O/W phase configuration. About 100-fold diluted sample in Pluronic® F127 solution (0.5%
w/v) was placed on a glass slide covered slip and the optical images were captured using an Olympus microscope (Model: IX51, Olympus America Inc., Chelmsford, MA) at 40x magnification. The acquired digital images were processed using the Image J software (National Institutes of Health, Bethesda, MD).

2.2.5 Qualitative Determination of gp100 Peptide Encapsulation

The payload encapsulation in the inner-aqueous phase of the W/O/W multiple emulsions was investigated using fluorescently-tagged gp100 peptide. Fluorescein isothiocyanate (FITC)-labeled gp100 (FITC-gp100) was custom synthesized at Tufts University Peptide Core Facility (Boston, MA). The FITC-gp100 was dissolved in DMSO and incorporated into the inner-aqueous phase of the W/O/W multiple emulsions. About 100-fold diluted sample in Pluronic® F127 solution (0.5% w/v) was placed on a glass slide covered slip and fluorescence light excited images were captured using a Nikon fluorescence microscope at 40x magnification. The acquired digital images were processed using the Image J software (National Institutes of Health, Bethesda, MD). Qualitative assessment of the FITC-gp100 peptide encapsulation was performed by evaluating relative fluorescence density in the dispersed oil-droplets compared to outer-continuous phase.

2.2.6 Determination of the W/O/W Multiple Emulsions Viscosity and Syringe Glide Force

Viscosity of the W/O/W multiple emulsions formulation was measured using the Kinexus-pro® rotational rheometer (Malvern Instruments Ltd, UK) installed with 40 mm diameter parallel plate geometry. About 0.75 mL sample was placed between 0.5 mm plate gap and viscosity measurement was performed at 25°C using shear rate of 100 S⁻¹.
Mean viscosity value from twelve data points measured in 1 min was determined. In addition, the glide force required for a syringe plunger to drive 25 mm distance at 100 mm/min speed while injecting the vaccine formulations through a 1 mL syringe with a 27G needle was measured using an Instron Model 5565 (Instron, Inc., Norwood, MA) fitted with a T101320-1002 syringe testing attachment.

2.2.7 Stability of the W/O/W Multiple Emulsions Vaccine

The W/O/W multiple emulsions were stored in a refrigerator at 4°C in the dark for up to 8 weeks. The W/O/W multiple emulsions stability was evaluated by monitoring formulation attributes such as visual appearance, oil-droplets size, surface charge, and morphology were monitored to evaluate formulation stability. At each stability time point the samples were vortex mixed for 60 sec followed by characterization using characterization methods described in Section 2.2.4. In addition, visual inspections were performed to record qualitative attributes such as creaming and phase separation.

2.3 Results and Discussion

2.3.1 Preparation and Optimization of the W/O/W Multiple Emulsions Vaccine

The two-step emulsification method\textsuperscript{65} shown in Figure 5 created the W/O/W multiple emulsions comprised of distinct and stable three-phase system. The first emulsification step involved homogenization of gp100 peptide-containing internal aqueous-phase with Span\textsuperscript{TM} 80 containing squalane oil-phase to form W/O primary emulsion. The second emulsification step involved homogenization of the resulting W/O
primary emulsion with an outer aqueous-phase containing Pluronic® F127 to form the W/O/W multiple emulsions. Morphological evaluation of the W/O/W multiple emulsions, shown in Figure 5, demonstrate that the oil-droplets were discrete, round, and homogenously dispersed in outer aqueous-phase. In addition, staining of the W/O/W multiple emulsions using water-soluble (Evans blue) and oil-soluble (Sudan red) dyes ascertained that it contained blue inner aqueous-phase [1] encapsulating, pink dispersed oil-phase [2] stabilized in clear outer aqueous-phase [3]. Thus, the three-phase configuration of the W/O/W multiple emulsions was established.

Ovalbumin was used as a surrogate to optimize the W/O/W multiple emulsions composition and the preparation method. Based on the preliminary experiments the optimum ratio of internal aqueous-phase to oil-phase to outer aqueous-phase was identified as 1:2:3. Subsequently, the same aqueous- to oil-phase ratios were used in all the W/O/W multiple emulsions preparation. Non-ionic surfactant such as Pluronic® was selected due to its immuno-adjuvant effects. The multiple emulsions prepared using the Pluronic® P123 was not stable and therefore formulation attributes characterization was not performed. In addition, during solution preparation Pluronic® P123 imposed enormous challenges due to extremely high viscosity. The oil-droplets size and polydispersity index values for the W/O/W multiple emulsions prepared using different grades of Pluronic® are outlined in Error! Reference source not found.. The Pluronic® 64, F68, and F127 created stable the W/O/W multiple emulsions having comparable size of the dispersed oil-droplets. Results demonstrated that the oil-droplets mean hydrodynamic diameter was about 1 µm and polydispersity index values ranged between 0.2 and 0.6. Importantly, the composition containing Pluronic® F127 demonstrated
Figure 5: Two-Step Emulsification Method and the Bright-Field Images of the W/O/W Multiple Emulsions System.

(a) The W/O/W multiple emulsions-based vaccine formulation was prepared by two-step emulsification method. The first emulsification step involved homogenization of gp100 peptide containing internal aqueous-phase with squalane oil-phase containing Span™ 80 to form W/O primary emulsion. The second emulsification step involved homogenization of W/O primary emulsion with outer aqueous-phase containing Pluronic® F127 to form the W/O/W multiple emulsions. (b) Staining with Evans blue and Sudan red 7B ascertained the phase configuration of the W/O/W multiple emulsions. An internal aqueous-phase (blue) encapsulating, dispersed oil-phase (pink) that was stabilized in an outer aqueous phase (grey). (c) The bright-field image of the W/O/W multiple emulsions system with encapsulated gp100 peptide antigen.
lowest polydispersity of oil-droplets. Therefore, Pluronic® F127 was selected as water-soluble surfactant to stabilize the oil-droplets that are dispersed in outer-aqueous phase.

The effect of homogenization parameters on the oil-droplets size and size distribution was examined to select the optimum conditions for the W/O/W multiple emulsions preparation method. The oil-droplets size and polydispersity index values for the multiple emulsions homogenized at 5,000 and 10,000 rpm speed for 5 and 10 minutes are outlined in Table 2. The oil-droplets mean hydrodynamic diameter ranged from 1.1 µm to 1.7 µm and polydispersity index values ranged between 0.2 and 1.0. The results demonstrated that slowing homogenization speed generally created larger oil-droplets, whereas shorter homogenization duration resulted in increased polydispersity of the oil-droplets size. Therefore, multiple emulsions homogenization at 10,000 rpm speed for 10 min was considered optimal and that was used to prepare gp100 peptide containing vaccine for in vivo effectiveness evaluation. Moreover, surface charges on the oil-droplets in the W/O/W multiple emulsions were measured between -30 mV to -40 mV.

The gp100 peptide-containing the W/O/W multiple emulsions were prepared using optimized composition and preparation method for in vivo vaccine effectiveness evaluation. The formulation attributes for six independent the W/O/W multiple emulsions-based gp100 peptide vaccines are outlined in Table 3. The oil-droplets mean hydrodynamic diameters were between 1.4-1.9 µm and surface charges were about -40 mV. The coefficient of variance for polydispersity index values was 0.4 (n = 5). Notably, the two-step emulsification method was reproducible as indicated by coefficient of variance for polydispersity index value and created stable gp100 peptide-containing the W/O/W multiple emulsions.
### Table 2: The Effect of the Composition and the Preparation Method Parameters on the W/O/W Multiple Emulsions Attributes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Pluronic®</th>
<th>Homogenization</th>
<th>Emulsion Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Speed (rpm)</td>
<td>Time (min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prototype – 1</td>
<td>L64</td>
<td>10,000</td>
<td>10</td>
</tr>
<tr>
<td>Prototype – 2</td>
<td>P123</td>
<td>10,000</td>
<td>10</td>
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<tr>
<td>Prototype – 3</td>
<td>F68</td>
<td>10,000</td>
<td>10</td>
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<tr>
<td>Prototype - 4</td>
<td>F127</td>
<td>10,000</td>
<td>10</td>
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<tr>
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<tr>
<td>Prototype – 7</td>
<td>F127</td>
<td>5,000</td>
<td>10</td>
</tr>
</tbody>
</table>

The results are shown as mean ± S D, n = 3.

### Table 3: gp100 Peptide-Containing the W/O/W Multiple Emulsions Formulation Attributes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Emulsion Attributes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oil-Droplets Size</td>
</tr>
<tr>
<td></td>
<td>Mean Diameter (µm)</td>
</tr>
<tr>
<td>Lot - 1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Lot - 2</td>
<td>--</td>
</tr>
<tr>
<td>Lot - 3</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Lot - 4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Lot - 5</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Lot - 6</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

The results are shown as Mean ± SD, n = 3.
2.3.2 gp100 Peptide Encapsulation

The gp100 peptide encapsulation into the inner-aqueous phase of the W/O/W multiple emulsions was qualitatively investigated using fluorescent microscopy. The FITC labeled gp100 peptide was incorporated in the internal aqueous-phase of the W/O/W multiple emulsions. The digital pictures showing fluorescent light exited the W/O/W multiple emulsions are captured in Figure 6. Fluorescent microscopy pictures demonstrated that higher intensity of fluorescent light was emitted from the dispersed oil-droplets compared to the outer-aqueous phase. Thus, suggested FITC labeled gp100 peptide was accumulated within the dispersed oil-phase of the W/O/W multiple emulsions.

2.3.3 The W/O/W Multiple Emulsions Viscosity and Syringe Glide Force

Viscosity of the W/O/W multiple emulsions based- and the W/O IFA- based vaccines were measured and are compared with that of saline in Figure 7 to investigate formulation injectability. Viscosity of the W/O/W multiple emulsions based vaccine formulation was comparable to that of saline and about 13-fold lower than that of the W/O IFA based vaccine formulation. In addition, force required for vaccine formulations to inject through 1 mL syringe with 27G needle (syringe glide force) is shown in Figure 7. Syringe glide force values demonstrated that injection of the W/O/W multiple emulsions- based vaccine formulation required about 3-fold lower force than that needed for injection of the W/O IFA based vaccine formulation. Both viscosity and syringe glide force results demonstrated improved injectability of the W/O/W multiple emulsions.
Figure 6: Bright Field (a) and Fluorescent Light Excited (b) Images of the W/O/W Multiple Emulsions Containing FITC-gp100.
Fluorescent light excited the W/O/W multiple emulsions displayed higher intensity of fluorescent light (Cyan) emitted from the dispersed oil-droplets compared to the outer-aqueous phase. Fluorescent microscopy demonstrated accumulation of FITC labeled gp100 peptide within the dispersed oil-phase of the W/O/W multiple emulsions.
Figure 7: Vaccine Formulations Injectability Comparison.
(a) Viscosity and syringe glide force were used to compare injectability of the vaccine formulations: (1) saline (blue), (2) gp100 in W/O IFA emulsion (purple), and (3) gp100 in W/O/W SME (pink). Viscosity was measured at 25 °C and shear rate of 100 S-1 using kinexus pro rotational rheometer installed with 40 mm diameter parallel plate geometry. Mean viscosity from twelve data points measured in 1 min is reported as one measurement. Results are presented as mean ± S.D., n = 3 (*P < 0.05). (b) Force required for syringe plunger to inject the vaccine formulations: (1) saline (blue), (2) gp100 in W/O IFA emulsion (purple), and (3) gp100 in W/O/W SME (pink) through 1 mL syringe with 27G needle was measured using Instron fitted with T101320-1002 syringe testing fixture. Results are presented as mean ± SD, n = 8 (*p < 0.05).
2.3.4 Stability of the W/O/W Multiple Emulsions

Stability of the W/O/W multiple emulsions was evaluated at refrigerated condition (4 °C) by monitoring formulation attributes for up to 8 weeks. The oil-droplets size and surface charge values determined to monitor formulation stability are outlined in Table 4. In addition, any change in the W/O/W multiple emulsions morphology was examined by optical microscopy and digital pictures are shown in Figure 8. The results demonstrated that oil-droplets size and surface charge remained stable up to 8-week when at 4 °C. Moreover, microscopic pictures showing morphology revealed that discrete, round oil-droplets were dispersed in the outer aqueous-phase. Furthermore, visual inspection of the multiple emulsions during stability evaluation showed creaming of the dispersed oil-droplets that was easily re-dispersed by vortex mixing for 30-60 sec; however, there were no signs of phase separation. Thus, stability study results indicated that the W/O/W multiple emulsions were stable up to 8-week when stored at 4°C in dark.

### Table 4: Formulation Attributes for the W/O/W Multiple Emulsions Stored at 4 °C up to 8 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Oil-Droplets Size (µm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>4-week</td>
</tr>
<tr>
<td>Lot – 1</td>
<td>1.8 ± 0.2 [0.1]</td>
<td>--</td>
</tr>
<tr>
<td>Lot – 2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Lot – 3</td>
<td>1.6 ± 0.2 [0.2]</td>
<td>1.8 ± 0.2 [0.3]</td>
</tr>
</tbody>
</table>

The results are shown as mean ± SD, n = 3. Values in [ ] shows polydispersity index.
Figure 8: Morphology of the W/O/W Multiple Emulsions Stored at 4 °C up to 8 Weeks.

The W/O/W multiple emulsions morphology was examined by optical microscopy during stability evaluation for up to 8-week. The W/O/W multiple emulsions morphology investigation at initial (a) and after 8-week at 4 °C (b) showed that discrete, round oil-droplets were dispersed in the outer aqueous-phase indicating stability of the W/O/W multiple emulsions.
2.4 Conclusion

The two-step emulsification method was reproducible and produced the stable W/O/W multiple emulsions. The three-phase configuration was ascertained with color staining and subsequent morphological characterization of the W/O/W multiple emulsions. In addition, formulation attributes characterization demonstrated that the dispersed phase of W/O/W multiple emulsions contained discrete, spherical oil-droplets with about 2 µm diameter size and about -40 mV surface charges. More importantly, morphology and surface charge of the dispersed oil-droplets remains unchanged for up to 8 weeks when stored at 4°C, thus signifying the stability of the formulations. FITC labeled gp100 peptide encapsulation and subsequent fluorescent microscopy demonstrated that the peptide is retained in the internal aqueous-phase of the W/O/W multiple emulsions. The W/O/W multiple emulsions-based gp100 peptide vaccine offers improved syringeability compared to IFA-based vaccine that was demonstrated by viscosity and syringe glide force comparison.
CHAPTER 3
EVALUATION OF gp100 PEPTIDE VACCINE IN VIVO EFFECTIVENESS IN PROPHYLACTIC TREATMENT MODEL OF MURINE B16 MELANOMA

3.1 Introduction

Melanoma exhibits an inherent immunogenicity; consequently immunotherapy is suitable for prevention of tumor growth, metastasis, and relapse\textsuperscript{6}. The gp100 protein is a member of the melanocyte differentiation antigens family that is strongly expressed in most melanomas. It includes a variety of immunogenic epitopes that are recognized by CTLs\textsuperscript{35}. A 9-amino acid epitope of human gp100 protein (hgp100), gp100\textsubscript{25-33} (KVPRNQDWL) is restricted by H-2D\textsuperscript{b} and therefore stimulates potent cell-mediated anti-tumor immunity in murine B16 melanoma model\textsuperscript{78,79}. However, corresponding epitope of mouse gp100 protein (mgp100), gp100\textsubscript{25-33} (EGSRNQDWL) is non-immunogenic to induce specific CD8\textsuperscript{+} T cell response\textsuperscript{80}. Although the full-length hgp100 and mgp100 molecules are 76\% identical at the amino acid level, differences within the MHC class I-restricted epitope determine the ability to induce specific CTLs. The hgp100\textsubscript{25-33} peptide differed from its mouse counterpart in three NH\textsubscript{2}-terminal amino acids that dramatically increased affinity of the hgp100\textsubscript{25-33} peptide for the mouse MHC class I allele, H-2D\textsuperscript{b}, compared with the mgp100\textsubscript{25-33} peptide. Therefore, hgp100\textsubscript{25-33} (gp100) with significantly greater avidity for MHC class I to induce self-reactive T cells was selected for this study.
Cell-mediated immune responses, such as antigen-specific CTLs, play an important role in anti-tumor immunity for destruction and eradication of a growing tumor\textsuperscript{14,15}. To accomplish this, the vaccine deliver system should: (1) recruit antigen presenting cells (APCs) such as dendritic cells and macrophages at the site of injection and (2) efficiently deliver the encapsulated payload to APCs to induce an antigen-specific immune responses. Importantly, a massive challenge in cancer vaccination is activation of anti-tumor CTLs with long-term memory against the tumor antigens. This can be achieved by a superior vaccine delivery system containing efficient immune-adjuvants to provoke APCs. Currently, gp100 peptide emulsified in incomplete Freund’s adjuvant (IFA) is used for melanoma immunotherapy\textsuperscript{81}. It contains a mixture of mineral oil and surfactant, mannide monooleate that creates W/O emulsion when emulsified with antigen solution. The IFA is non-biodegradable and imposes enormous challenges during injection due to high viscosity of the formulation. Therefore development of alternate effective vaccine delivery system is warranted for melanoma immunotherapy.

It was hypothesized that biodegradable squalane oil-containing W/O/W multiple emulsions-based vaccine delivery systems will enhance the immune responses due to: (1) reservoir effect of an immunogen incorporated in the inner aqueous-phase\textsuperscript{82,83} and (2) particulate adjuvant effect of the dispersed oil-droplets\textsuperscript{60,72,82}. In addition, the W/O/W multiple emulsions-based vaccine will offer improved injectability and tolerability due to low viscosity of the formulations\textsuperscript{84,85}. In this study, we have investigated \textit{in vivo} effectiveness of squalane oil-containing W/O/W multiple emulsions-based gp100 peptide vaccine following prophylactic immunization approach in murine B16 melanoma model. Increase in the immune responses was determined by comparing protection against tumor
challenge produced by the W/O/W multiple emulsions-based vaccines with that produced by IFA-based vaccine in immunized C57BL/6 mice. Additionally, induction of cell-mediated anti-tumor immunity was confirmed by analyzing serum Th1 cytokine levels and local infiltration of CD4+ and CD8+ CTLs at tumor site.

3.2 Materials and Methods

3.2.1 Preparation of the W/O/W Multiple Emulsions Vaccine

The gp100 peptide-containing the W/O/W multiple emulsions were prepared as described in Section 2.2.1.

3.2.2 Preparation of Incomplete Freund’s Adjuvant W/O Emulsion Vaccine

The gp100 peptide-containing incomplete Freund’s adjuvant W/O emulsion vaccine was prepared as described in Section 2.2.3.

3.2.3 Determination of the Oil-Droplets Size, Surface Charge, and Morphology

The oil-droplets size, surface charge, and morphology characterization was performed as described in Section 2.2.4.

3.2.4 Melanoma Cell Line and Animal Model

B16-F10 (B16), a pigmented murine melanoma cell line of C57BL/6 origin was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento,
CA) and 50 IU/mL penicillin, 50 µg/mL streptomycin combined antibiotics (PenStrep; GIBCO). Female C57BL/6, inbred 4–6 weeks old mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed under pathogen-free conditions in the Division of Laboratory Animal Medicine (DLAM) facility and provided with food and water ad libitum. All studies involving mice were approved by and performed according to the guidelines established by the Northeastern University Institutional Animal Care and Use Committee (IACUC). The mice were allowed to acclimatize for at least 48 hours prior to any experimental procedure.

3.2.5 Immunization and Tumor Challenge Protocol

The mice were randomized into five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W squalane oil multiple emulsions (SME) control, and (5) gp100 in W/O/W SME containing eight animals in each. In prophylactic immunization mode anesthetized mice received three subcutaneous (s.c.) injections of 0.1 mL control or vaccine [50 µg dose\textsuperscript{81} per injection] formulations at 2-week intervals. Ten days after the last immunization, tumor challenge was initiated by s.c. implantation of 100,000 B16-F10 cells\textsuperscript{36,87} suspended in 0.1 mL PBS on the hind flank of mice. The mice were monitored daily for any pain or distress, and general health. According to Northeastern University’s IACUC guidance, mice were euthanized when tumor volume reached to 1,000 mm\textsuperscript{3}. Immediately after euthanasia, the blood sample was collected and from that serum was separated for Th1 cytokine levels measurement. In addition, tumor tissues were excised, washed in PBS, and blot dried. Excised tumor masses were recorded; representative images were captured, and cryo-preserved for the immuno-histochemical analysis.
3.2.6 Evaluation of Protection against Tumor Challenge

The mean tumor volume and tumor growth delay times were determined and compared among treatment groups to evaluate the relative rank order of vaccine treatment for protection against tumor challenge. Tumor volume growth was examined by measuring two perpendicular tumor dimensions (mm) using the calipers until tumors in control groups reached the end point (1,000 mm^3 volume) or until survival of the mice. The tumor volumes (V, mm^3) were calculated using the formula\(^86,88\): \[ V = \frac{1}{2}[L \times (W)^2]; \]
where L, length is the longest dimension and W, width is the dimension perpendicular to the length. Additionally, time required for an individual tumor volume to grow from 200 mm^3 to 800 mm^3 was determined using regression analysis of tumor mean volume growth curves. Difference between the mean value of this variable for the saline treated control group and vaccine treated groups was defined as the tumor growth delay time (t, days): \[ t = (\text{mean time for tumors to grow from 200 mm}^3 \text{ to 800 mm}^3)_{\text{vaccine treated group}} - (\text{mean time for tumors to grow from 200 mm}^3 \text{ to 800 mm}^3)_{\text{saline treated control group}}. \]

3.2.7 Serum Th1 Cytokine Analysis

Blood samples were allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines\(^89,90\) such as IL-2, IL-12, INF-\(\gamma\), and TNF-\(\alpha\) level in the sera were quantitatively analyzed by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array (Quansys Biosciences, Logan, UT) according to manufacturer’s instructions. The samples and the calibration standards were added into the wells of pre-coated 96-wells plate and incubated on a plate shaker for 1 h at room temperature. After rinsing the plate three times, appropriate biotinylated-detection antibody was added and incubated on a plate shaker for 1 h at room
temperature. Plate was washed three times and incubated with streptavidin-conjugated horseradish peroxidase (HRP) for 15 min at room temperature. Plate was washed six times and a mixture of chemiluminescent HRP substrate A and B was added. The digital images of the plate were captured using Kodak *In Vivo* FX imager (Carestream Health, Rochester, NY). Pixel-intensity for each multiplex array spot on the digital image was determined using Q-View Software (Quansys Biosciences). The Th1 cytokine levels in each sample were calculated using the standard curve prepared following the instructions.

### 3.2.8 Tumor Tissue Immuno-Histochemical Analysis

Excised tumor tissues were embedded in the Tissue-Tek OCT compound (Sakura Finetek USA, Inc. Torrance, CA) and stored frozen at -80 °C. The frozen samples were equilibrated to -20 °C and cut into 6-8 µm sections using the Microm HM550 cryostat (MICROM International GmbH, Germany). The sections were placed onto the pre-cleaned microscope slides (Fisher Scientific) and fixed in 200-proof ethanol (Acros Organics) at -20 °C for 2 min. The tumor sections were washed in PBS to remove residues of OCT compound and incubated in PBS containing 0.3% hydrogen peroxide (J. T. Baker Chemical Co., Phillipsburg, NJ) to eliminate endogenous peroxidase activity. The sections were washed in PBS and incubated with blocking serum (Vector Laboratories, Burlingame, CA) diluted in PBS for 30 min at room temperature. The sections were washed and incubated with rat anti-mouse CD4 or rat anti-mouse CD8a primary antibody (1:20 dilution; BD PharMingen, San Diego, CA) for 30 min at room temperature. After being washed in PBS, the sections were incubated with biotinylated-rabbit anti-rat IgG detection antibody (1:100 dilution; Vector Labs) for 30 min at room temperature. The sections were washed in PBS and incubated with Vectastatin® Elite
ABC reagent (Vector Laboratories) for 30 min at room temperature. After being washed in PBS, the sections were incubated with ImmPACT™ NovaRED™ peroxidase substrate (Vector Laboratories) at room temperature for 15 min or until desired color intensity was developed. The sections were washed in water, counterstained with hematoxylin (Fisher Scientific), and dehydrated in 200-proof ethanol. The slides were cleared in xylene substitute (Thermo Scientific Shandon, UK) and mounted using a mounting medium (Immu-Mount; Thermo Scientific Shandon). Optical microscopy was used to perform histological analysis. The digital images of the stained specimens were captured using an Olympus microscope (Model: IX51, Olympus America Inc., Chelmsford, MA).

3.2.9 Data analysis

The statistical analysis of the data sets was performed using the GraphPad PRISM software, version 5.01 (GraphPad Software Inc., La Jolla, CA). The unpaired student’s t-tests were performed between relevant groups. No multiple comparisons were performed to control for type I errors. The \( p \) value of \(< 0.05\) (95% confidence interval) was considered statistically significant.

3.3 Results and Discussion

The goal for the multi-compartmental, W/O/W multiple emulsions-based vaccine delivery system was to induce enhanced cell-mediated anti-tumor immunity by efficient delivery of melanoma antigen, gp100 peptide and to improve formulation injectability. The dispersed oil-droplets of the W/O/W multiple emulsions acts as reservoir for the
encapsulated antigen and have comparable dimensions to the pathogens; therefore naturally targeted for uptake by APCs. Squalane oil does not cause an inflammatory response and is well tolerated at the site of injection. The emulsions of squalene oil and its hydrogenated form, squalane oil, in conjunction with surfactants such as the Pluronic® have been used as adjuvant in vaccine formulations. Consequently, squalane oil-containing the W/O/W multiple emulsions-based vaccine delivery systems offer a combined antigen reservoir effect and particulate adjuvant effect; as a result they have potential to enhance the immune responses. In addition, the W/O/W multiple emulsions offer improved injectability mainly due to low viscosity of the formulation. Cell-mediated immune responses such as CD4+ and CD8+ CTLs have a vital role in the immunologic rejection of growing tumors. Upon immune response stimulation primed CD8+ T-cells infiltrate into tumor stroma, encounter tumor-antigens, and release cytotoxins thereby cause cell death. In addition, CD4+ T-cells secrete cytokines that aid to immune rejection of tumor cells. Consequently, it is critical to induce antigen-specific, anti-tumor T-cell functions for an effective cancer immunotherapy. In this study, we examined ability of the W/O/W multiple emulsions-based gp100 peptide vaccine to induce protective cell-mediated anti-tumor immunity and compared with that of the IFA-based gp100 peptide vaccine using murine B16 melanoma model. In vivo prophylactic immunization protocol is illustrated in Figure 9.
3.3.1 Protection against Tumor Challenge

Mean tumor volume and tumor growth delay time were determined to evaluate protection against tumor challenge. Additionally, increase in immune response stimulation was examined by comparing protection against tumor challenge produced by the W/O/W multiple emulsions-based gp100 peptide vaccine with that of IFA-based gp100 peptide vaccination. Mean tumor volume growth curves for five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W SME control, and (5) gp100 in W/O/W SME; following prophylactic immunization are shown in Figure 9. In addition, mean tumor mass and a representative picture of the excised tumor tissue from each group are shown in Figure 10. The results demonstrated that prophylactic immunization using the W/O/W multiple emulsions-based versus IFA-based vaccine resulted in about 3-fold versus 2-fold reduction in the mean tumor volume respectively and 2.6-fold versus 1.3-fold reduction in the mean excised tumor mass respectively, compared to treatment with saline. More importantly, differences between the mean tumor volume for gp100 peptide immunized and control formulation treated groups were statistically significant ($p < 0.05$) suggesting induction of enhanced immune responses against melanoma due to gp100 peptide vaccination. Furthermore, time delay for tumor volume to grow between two specific sizes (tumor growth delay times) due to immunization were determined. Prophylactic immunization using W/O/W multiple emulsions-based gp100 peptide vaccine resulted in 6-fold increase in tumor growth delay time compared to IFA-based gp100 peptide vaccination.
Figure 9: In Vivo Prophylactic Immunization Protocol and Vaccine Formulation Effectiveness.

Five groups of mice were treated with: (1) saline control (●), (2) W/O IFA emulsion control (♦), (3) gp100 in W/O IFA emulsion (■), (4) W/O/W squalane oil multiple emulsions (SME) control (●), and (5) gp100 in W/O/W SME (▲) containing placebo or 50 µg gp100 peptide per injection. (a) In prophylactic treatment mode mice were treated with control or vaccine formulation three-times at 2-week interval and ten days after the last injection tumor challenge was initiated by subcutaneous (s.c.) injection of 100,000 B16-F10 cells on the hind flank of the mice. Tumor growth was monitored by measuring two perpendicular tumor dimensions (mm) using the calipers and tumor volumes (V, mm³) were calculated using the formula: \( V = \frac{1}{2} [L \times (W)^2] \); where L is the longest dimension and W is the dimension perpendicular to the L. (b) Tumor growth curves for prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p<0.05).
Figure 10: Tumor Mass and a Representative Picture of Excised Tumors.

Animals were euthanized and tumor tissues were excised when mean tumor volume for saline treated group of mice reached to 1000 mm$^3$. Excised tumors were freed of skin remnants, rinsed in phosphate buffered saline and dried on blotting paper. A representative picture (a) and mass (b) of excised tumors from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p < 0.05).
The results demonstrated that in vivo prophylactic immunization of C57BL/6 mice using gp100 peptide vaccine formulations resulted in substantial protection against B16-F10 melanoma tumor challenge. More importantly, gp100 peptide vaccination using the newly developed vaccine delivery system, the W/O/W multiple emulsions formulation evoked significantly higher tumor growth suppression compared to IFA-based vaccine delivery; as evidenced by noticeable reduction in mean tumor volume. Additionally, prophylactic using the W/O/W multiple emulsions-based gp100 peptide vaccine resulted in increased tumor growth delay time compared to IFA-based vaccine suggesting increased protection against tumor challenge.

3.3.2 Serum Th1 Cytokine Levels

CD4+ T-cells (also called Th1 cells) secrete a panel of cytokines called Th1 cytokines including INF-γ and TNF-α and thereby offer a synergistic effect to induce anti-tumor immunity. For example, INF-γ can activate CD8+ T-cells22 and TNF-α can induce DC-mediated tumor antigen recognition93. In addition, IL-2 and IL-12 are key cytokine to induce Th1 cell differentiation and enhance Th1 function94-96. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in sera were quantitatively measured using ELISA method and compared among different treatment groups to investigate induction of CD4+ and CD8+ cytotoxic T-cell function due to immunization. The Th1 cytokine levels measured in sera collected from five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W SME control, and (5) gp100 in W/O/W SME, following prophylactic immunization are shown in Figure 11.
**Figure 11: Serum Th1 Cytokine Levels.**

Immediately after animal euthanasia the blood samples were collected and allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in the sera were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array. The Th1 cytokine levels in the sera from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
The results demonstrated increased levels of IL-2, IL-12, INF-γ, and TNF-α in the serum samples from the mice immunized using gp100 peptide vaccine compared to the mice treated with control formulations. Increased Th1 cytokine levels indicated immune response stimulation through Th1 pathway that activates CD8+ T-cell mediated antitumor immunity. Noticeably, immunization using the W/O/W multiple emulsions-based gp100 peptide vaccine resulted in significantly increased (p < 0.05) levels of IL-2, IL-12, and INF-γ compared to saline treatment. Thus, serum cytokine analysis revealed that the immunization using gp100 peptide vaccine induced secretion of IL-2, IL-12, and INF-γ that in turn might favor Th1 immune response and mediate tumor growth inhibition in vivo.

3.3.3 Immuno-Histochemical Staining of Excised Tumor Tissue

Immunologic eradication of established tumors depends on three criteria:\textsuperscript{16} [1] sufficient numbers of immune cells with highly avid recognition of tumor antigens must be generated in vivo, [2] these cells must traffic to and infiltrate the tumor stroma, and [3] the immune cells must be activated at the tumor site to manifest appropriate effector mechanisms causing tumor cell destruction. The antigen-specific CD4+ and CD8+ T-cell trafficking and infiltration into tumor stroma was examined by immuno-histochemically staining excised tumor tissues using anti-CD4 and anti-CD8a antibodies\textsuperscript{12,97}. Tumor tissue sections were incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the stained tumor sections are shown in Figure 12.
Figure 12: Tumor Tissue Immuno-Histochemistry.
Activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site was investigated by immuno-histochemical staining of excised tumor tissues. Tumor tissues were cryo-sectioned (6-8 μ thickness) and incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the immuno-stained tumor sections from five treatment groups top to bottom (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in prophylactic immunization are shown.
Immuno-staining of the excised tumor tissues demonstrated that prophylactic immunization of C57BL/6 mice using gp100 peptide vaccine resulted in increased infiltration of CD4+ and CD8+ T-cells into and/or tumor surroundings compared to control formulation treatment; thereby providing evidence for involvement of cell-mediated antitumor immunity that might have protected mice against tumor challenge. Thus, tumor tissue immuno-histology indicated prophylactic immunization using the W/O/W multiple emulsions-based gp100 peptide vaccine activates antigen-specific CD4+ and CD8+ T-cells that subsequently mediate anti-tumor immunity and protects immunized mice against melanoma tumor challenge.

3.4 Conclusions

*In vivo* prophylactic immunization of C57BL/6 mice using the multi-compartmental, W/O/W multiple emulsion-based gp100 peptide vaccine evokes cell-mediated antitumor immunity that provided increased protection against tumor challenge compared to IFA-based gp100 peptide vaccine.
CHAPTER 4
EVALUATION OF gp100 PEPTIDE VACCINE IN VIVO EFFECTIVENESS IN ACTIVE TREATMENT MODEL OF MURINE B16 MELANOMA

4.1 Introduction

Active vaccination treatment is intended to treat an existing cancer by strengthening the body’s natural defenses against the cancer. In this study, we have investigated in vivo effectiveness of squalane oil-containing W/O/W multiple emulsions-based gp100 peptide vaccine following active immunization approach in murine B16 melanoma model. Increase in the immune responses was determined by comparing protection against tumor challenge produced by the W/O/W multiple emulsions-based vaccines with that produced by IFA-based vaccine in immunized C57BL/6 mice. Additionally, induction of cell-mediated anti-tumor immunity was confirmed by analyzing serum Th1 cytokine levels and local infiltration of CD4+ and CD8+ CTLs at tumor site.

4.2 Materials and Methods

4.2.1 Preparation of the W/O/W Multiple Emulsions Vaccine

The gp100 peptide-containing the W/O/W multiple emulsions were prepared as described in Section 2.2.1.
4.2.2 Preparation of Incomplete Freund’s Adjuvant W/O Emulsion Vaccine

The gp100 peptide-containing incomplete Freund’s adjuvant W/O emulsion vaccine was prepared as described in Section 2.2.3.

4.2.3 Determination of the Oil-Droplets Size, Surface Charge, and Morphology

The oil-droplets size, surface charge, and morphology characterization was performed as described in Section 2.2.4.

4.2.4 Melanoma Cell Line and Animal Model

B16-F10 murine melanoma cell line and C57BL/6 mice used for in vivo gp100 vaccine effectiveness evaluation in active treatment mode are described in Section 3.2.4.

4.2.5 Immunization and Tumor Challenge Protocol

The mice were randomized into five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W squalane oil multiple emulsions (SME) control, and (5) gp100 in W/O/W SME containing eight animals in each. In active immunization mode tumor challenge was initiated by s.c. implantation of 100,000 B16-F10 cells suspended in 0.1 mL PBS on day 0. On day 1, 4, and 11 anesthetized mice received s.c. injections of 0.1 mL control or vaccine formulations [50 µg dose per injection]. The mice were monitored daily for any pain or distress, and general health. According to Northeastern University’s IACUC guidance, mice were euthanized when tumor volume reached to 1,000 mm³. Immediately after euthanasia, the blood sample was collected and from that serum was separated for Th1 cytokine levels measurement. In addition, tumor tissues were excised, washed in PBS, and blot dried.
Tumor masses were recorded; representative images were captured, and cryo-preserved for the immuno-histochemical analysis.

4.2.6 Evaluation of Protection against Tumor Challenge

The method used to evaluate protection against tumor challenge is described in Section 3.2.6.

4.2.7 Serum Th1 Cytokine Analysis

The method used to analyze serum Th1 cytokine levels is described in Section 3.2.7.

4.2.8 Tumor Tissue Immuno-Histochemical Analysis

The method used to determine activation and local infiltration of CD4+ and CD8+ CTLs by immuno-histochemical staining of excised tumor tissues is described in Section 3.2.8.

4.2.9 Data analysis

The statistical analysis of the data sets was performed as described in Section 3.2.9.
4.3 Results and Discussion

The goal for active immunization using the multi-compartmental, W/O/W multiple emulsions-based gp100 vaccine was to induce cell-mediated anti-tumor immunity and examine its ability to protect mice against tumor challenge in B16 melanoma model. In addition protective anti-tumor immunity induced by the W/O/W multiple emulsions-based vaccine was compared with that of the IFA-based vaccine. In vivo active immunization protocol is illustrated in Figure 13.

4.3.1 Protection against Tumor Challenge

Mean tumor volume and tumor growth delay time were determined to evaluate protection against tumor challenge. Additionally, increase in immune response stimulation was examined by comparing protection against tumor challenge produced by the W/O/W multiple emulsions-based gp100 peptide vaccine with that of IFA-based gp100 peptide vaccination. Mean tumor volume growth curves for five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W SME control, and (5) gp100 in W/O/W SME; following active immunization are shown in Figure 13. In addition, mean tumor mass and a representative picture of the excised tumor tissue from each group are shown in Figure 14. The results demonstrated that active immunization using W/O/W multiple emulsions-based versus IFA-based vaccine resulted in about 2-fold versus 1.5-fold reduction in the mean tumor volume respectively and 1.8-fold versus 1.4-fold reduction in the mean excised tumor mass respectively, compared to treatment with saline. More importantly, differences between
Figure 13: *In Vivo* Active Immunization Protocol and Vaccine Formulation Effectiveness.

Five groups of mice were treated with: (1) saline control (●), (2) W/O IFA emulsion control (♦), (3) gp100 in W/O IFA emulsion (■), (4) W/O/W squalane oil multiple emulsions (SME) control (●), and (5) gp100 in W/O/W SME (▲) containing placebo or 50 µg gp100 peptide per injection. (a) In active treatment mode tumor challenge was initiated on day 0 followed by the control or vaccine formulation injection on day 1, 4, and 11. Tumor challenge was initiated by subcutaneous (s.c.) injection of 100,000 B16-F10 cells on the hind flank of mice. Tumor growth was monitored by measuring two perpendicular tumor dimensions (mm) using the calipers and tumor volumes (V, mm³) were calculated using the formula: \( V = \frac{1}{2}[L \times (W)^2] \); where L is the longest dimension and W is the dimension perpendicular to the L. (b) Tumor growth curves for active immunization are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
Figure 14: Tumor Mass and a Representative Picture of Excised Tumors.

Animals were euthanized and tumor tissues were excised when mean tumor volume for saline treated group of mice reached to 1000 mm$^3$. Excised tumors were freed of skin remnants, rinsed in phosphate buffered saline and dried on blotting paper. A representative picture (a) and mass (b) of excised tumors from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in active immunization are shown. Results are presented as mean ± SD, n = 8 (*p < 0.05).
the mean tumor volume for gp100 peptide immunized and control formulation treated groups were statistically significant \( p < 0.05 \) suggesting induction of enhanced immune responses against melanoma due to gp100 peptide vaccination. Furthermore, time delay for tumor volume to grow between two specific sizes (tumor growth delay times) due to immunization were determined. Active immunization using W/O/W multiple emulsions-based gp100 peptide vaccine resulted in 2-fold increase in tumor growth delay time compared to IFA-based gp100 peptide vaccination.

The results demonstrated that in vivo active immunization of C57BL/6 mice using gp100 peptide vaccine formulations resulted in protection against B16-F10 melanoma tumor challenge. More importantly, gp100 peptide vaccination using the newly developed vaccine delivery system, the W/O/W multiple emulsions formulation increased tumor growth suppression compared to IFA-based vaccine delivery; as evidenced by decrease in mean tumor volume. Additionally, active immunization using the W/O/W multiple emulsions-based gp100 peptide vaccine resulted in increased tumor growth delay time compared to IFA-based vaccine suggesting increased protection against tumor challenge. However, increase in tumor growth delay time in active immunization (2-fold) was lower than that observed in prophylactic immunization (6-fold). That may be due to the aggressive nature of melanoma tumor model providing insufficient time for immune stimulation and subsequent decrease in malignant cells burden.

### 4.3.2 Serum Th1 Cytokine Levels

The Th1 cytokines such as IL-2, IL-12, INF-\( \gamma \), and TNF-\( \alpha \) level in sera were quantitatively measured using ELISA method and compared among different treatment
groups to investigate induction of CD4+ and CD8+ cytotoxic T-cell function due to immunization. The Th1 cytokine levels measured in sera collected from five treatment groups: (1) saline control, (2) W/O IFA emulsion control, (3) gp100 in W/O IFA emulsion, (4) W/O/W SME control, and (5) gp100 in W/O/W SME, following active immunization are shown in Figure 15.

The results demonstrated increased levels of IL-2, IL-12, and INF-γ in the serum samples from the mice immunized using gp100 peptide vaccine compared to the mice treated with control formulations. Increased Th1 cytokine levels indicated immune response stimulation through Th1 pathway that activates CD8+ T-cell mediated antitumor immunity. Noticeably, immunization using the W/O/W multiple emulsions-based gp100 peptide vaccine resulted in significantly increased (p < 0.05) levels of IL-2, IL-12, and INF-γ compared to saline treatment. The Th1 cytokines levels observed in active immunization are higher than that in prophylactic immunization. That may be due to differences in time lapse after the last vaccine injection causing surge in the Th1 cytokines secretion.

4.3.3 Immuno-Histochemical Staining of Excised Tumor Tissue

The antigen-specific CD4+ and CD8+ T-cell trafficking and infiltration into tumor stroma was examined by immuno-histochemically staining excised tumor tissues using anti-CD4 and anti-CD8a antibodies. Tumor tissue sections were incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the stained tumor sections are shown in Figure 16.
Figure 15: Serum Th1 Cytokine Levels.
Immediately after animal euthanasia the blood samples were collected and allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in the sera were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array. The Th1 cytokine levels in the sera from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in active immunization are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
**Figure 16: Tumor Tissue Immuno-Histochemistry.**
Activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site was investigated by immuno-histochemical staining of excised tumor tissues. Tumor tissues were cryo-sectioned (6-8 μ thickness) and incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the immuno-stained tumor sections from five treatment groups top to bottom (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) W/O IFA emulsion control (purple), (3) gp100 in W/O IFA emulsion (purple), (4) W/O/W squalane oil multiple emulsions (SME) control (pink), and (5) gp100 in W/O/W SME (pink) in active immunization are shown.
Immuno-staining of the excised tumor tissues demonstrated that active immunization of C57BL/6 mice using gp100 peptide vaccine resulted in increased infiltration of CD4+ and CD8+ T-cells into and/or tumor surroundings compared to control formulation treatment; thereby providing evidence for involvement of cell-mediated antitumor immunity that might have protected mice against tumor challenge. Thus, tumor tissue immuno-histology indicated active immunization using the W/O/W multiple emulsions-based gp100 peptide vaccine activates antigen-specific CD4+ and CD8+ T-cells that subsequently mediates anti-tumor immunity and protects immunized mice against melanoma tumor challenge.

4.4 Conclusions

*In vivo* active immunization of C57BL/6 mice using the multi-compartmental, W/O/W multiple emulsion-based gp100 peptide vaccine evokes cell-mediated antitumor immunity that provided increased protection against tumor challenge compared to IFA-based gp100 peptide vaccine.
CHAPTER 5

IN VITRO EVALUATION OF PLASMID DNA DELIVERY AND TRANSFECTION USING WATER-in-OIL-in-WATER MULTIPLE EMULSIONS

5.1 Introduction

DNA vaccines typically contain plasmid DNA molecules that encode an antigen(s) derived from a pathogen or tumor cell. DNA vaccination is an emerging technology and offer distinct advantages over traditional vaccines (killed or attenuated pathogens) and the more recently developed subunit vaccines. Unlike most subunit vaccines, DNA vaccines induce both the humoral and the cellular immune responses\(^{98}\). DNA vaccination has rapidly become a preferred strategy for anti-tumor immunity induction. A large amount of data has been generated in preclinical model systems, and more sustained cellular responses and more consistent antibody responses are being observed in the clinic\(^ {99} \). The molecular precision offered by gene-based vaccines, together with the flexibility to include additional genes to direct and amplify immunity, has always been attractive\(^ {100} \). CD4+ T-cell help is a key step to bypass immune tolerance and to activate high levels of anti-tumor antibody or cytotoxic T cells. Sequences derived from microbial antigens can be incorporated into anti-tumor DNA vaccines to mobilize help for anti-tumor responses from the large non-tolerized anti-microbial repertoire. Moreover, DNA vaccines eliminate the risk of pathogen reversion to the virulent state that is associated with attenuated vaccine.
The mechanisms by which DNA vaccines produce antigen-specific immunity in vivo are illustrated in Figure 17 and Figure 18. The optimized gene sequence encoding target antigen is injected to the skin, subcutaneum or muscle. The plasmid enters the nucleus of transfected local cells, including resident antigen presenting cells (APCs) at the site of injection. Expression of plasmid-encoded genes results in synthesis of foreign antigen protein. The host-synthesized antigens then become the subject of immune surveillance in the context of both major istocompatibility complex (MHC) class I and class II molecules of APCs in the vaccinated host. Antigen- loaded APCs travel to the draining lymph nodes and ‘present’ antigenic peptide-MHC complexes in combination with signaling by co-stimulatory molecules to naive T cells. This interaction provides the necessary secondary signals to initiate an immune response and to activate and expand T-cells and activate B-cells followed by antibody production; thereby both humoral and cellular immune responses are provoked.

Pharmacological activity of the DNA vaccines is dependent on their ability to be targeted to appropriate cell types, to be transported across the cell membrane, to have some degree of nuclease resistance and to maintain steady-state concentrations in the appropriate intracellular compartments. Polar and anionic nature of the plasmid molecules do not readily allow transfer across the biological membranes therefore one of the major challenges in DNA vaccination is optimum gene delivery to target immune-cells. An ideal delivery system should: (1) prevent degradation of the payload during transit, (2) increase and sustain antigen expression, and (3) increase vaccine immunogenicity. The multi-compartmental, W/O/W multiple emulsions are particulate delivery systems and are internalized by immune cells thereby transfecting them with
**Figure 17: Induction of Cellular and Humoral Immunity by DNA Vaccines.**

Plasmid DNA encoding target antigen is injected followed by transfection of local cells, including resident antigen presenting cells (APCs) at the site of injection. Plasmid-encoded target antigen protein becomes the subject of immune surveillance in the context of MHC complex class I and class II molecules of APCs. Antigen-loaded APCs travel to the draining lymph nodes and ‘present’ antigenic peptide-MHC complexes to naive T cells. This interaction activates and expands T cells resulting in cellular immune response and B cells resulting in antibody production. Together, both humoral and cellular immune responses are provoked.

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Figure 18: Routes of Antigen Presentation.
DNA vaccines are generally injected into muscle or skin. Encoded antigen is then expressed in myocytes or keratinocytes. For activation of T cells, antigen must be transferred to a ‘professional’ antigen-presenting cell, usually a dendritic cell (DC). This indirect process of transfer of antigenic material, possibly as apoptotic vesicles, is termed cross-presentation. A small proportion of DNA is also taken up directly by DCs and the encoded antigen can then be processed and presented endogenously.
encapsulated plasmid DNA\textsuperscript{59}. Thus, W/O/W multiple emulsions-based vaccines have potential to induce protective immunity against protein antigen encoded by encapsulated plasmid DNA. The W/O/W multiple emulsions-based plasmid DNA delivery systems offer several advantages including: (1) physical protection from enzymatic degradation, (2) physically confer gene delivery into immune cells by non-viral means, (3) alter or modify transgene expression levels over time by multiple gene delivery regimen, (4) modification of oil-droplets surface chemistry for immune-cells specific delivery; and (5) simultaneous delivery of multiple candidate therapeutic agents.

In this study we examined the plasmid DNA encapsulation into the inner aqueous-phase of the W/O/W multiple emulsion and protection of encapsulated plasmid DNA from enzymatic degradation. In addition, \textit{in vitro} transfection ability of the W/O/W multiple emulsions encapsulated plasmid DNA encoding enhanced green fluorescent protein (EGFP) or human gp100 (SILV) protein was investigated in J774A.1 murine adherent alveolar macrophage and murine dendritic cell lines.

\section*{5.2 Materials and Methods}

\subsection*{5.2.1 Plasmid DNA Construction}

The EGFP and human gp100 (SILV) encoding plasmid DNA transformed E.coli bacterial cultures were purchased from Genecopoeia Inc. (Rockville, MD). The OmicsLink\textsuperscript{TM} Expression Clone is a mammalian vector (pReceiver-M02) that contains human cytomegalovirus (CMV) promoter gene and ampicillin resistance gene as
selection marker. The EGFP encoding plasmid DNA was constructed by ligating EGFP cDNA fragment into the multiple cloning site of the expression clone (EX-EGFP-M02, Genecopoeia Inc.). The human gp100 (hgp100) plasmid was constructed by ligating hgp100 cDNA fragment into the multiple cloning site of the expression clone (EX-Z0375-M02, Genecopoeia Inc.). The empty plasmid was constructed by ligating scrambled sequence fragment into the multiple cloning site of the expression clone (EX-NEG-M02, Genecopoeia Inc.).

5.2.2 Amplification, Purification, and Isolation of Plasmid DNA

The frozen bacterial culture was streaked on a solid Luria Agar (Sigma-Aldrich, St. Louis, MO) medium containing 10 µg/ml ampicillin (Roche Diagnostics Corporation, Indianapolis, IN) using a sterile inoculation loop (Fisher Scientific, Fair Lawn, NJ) and incubated for 12 hours at 37°C. From that a single colony was picked and suspended in 1 mL of Luria Broth (Sigma-Aldrich) containing 10 µg/ml ampicillin for preferential selection of transformed E.coli. The bacterial culture was incubated for 8 h at 37°C on a shaker. Resulting bacterial culture was diluted (1:1,000) with Luria Broth containing 10 µg/ml ampicillin and continued to incubate for 16 h at 37°C on a shaker. Subsequently, amplified the plasmid DNA was extracted, purified, and isolated from E.coli bacterial culture using QIAGEN Plasmid Mega kit (Qiagen Inc., Valencia, CA) according to manufacturer’s instructions. Concentration and purity of the plasmid DNA extracts were determined by measuring absorbance at 260 and 280 nm using NanoDrop 2000 (Thermo Scientific, Wilmington, DE).
5.2.3 Characterization of EGFP and Human gp100 Encoding Plasmid DNA

The EGFP and hgp100 encoding plasmid DNAs were characterized for cDNA and expression vector backbone size by restriction enzyme digestion followed by agarose gel-electrophoresis. The EGFP encoding cDNA was cut from expression clone using combination of EcoRI/NotI and EcoRI/XhoI restriction enzymes (New England BioLabs Inc., Ipswich, MA). Similarly, hgp100 encoding cDNA was cut from expression clone using combination of XmnI/XhoI and NspV/XhoI restriction enzymes (NspV: Takara Bio Inc., Shiga, Japan). About 1 µg of plasmid DNA was incubated with 10 U of restriction enzyme in 50 µL of reaction buffer containing bovine serum albumin (BSA, 100 µg/mL) at 37°C for 1 hr. The resulting single (using one restriction enzyme) or double (using two restriction enzymes) digested linear DNA fragments were electrophoresed on a 0.8% agarose gel (0.8% E-Gel, Invitrogen, Carlsbad, CA) pre-stained with ethidium bromide, and visualized under ultraviolet radiation according to manufacturer’s instructions. The 1Kb Plus and a 100bp DNA Ladders (both from Invitrogen) were used as a molecular ruler.

5.2.4 Preparation of the Plasmid DNA-Encapsulated the W/O/W Multiple Emulsions

The W/O/W multiple emulsions encapsulating the plasmid DNA were prepared by two-step emulsion method described in Section 2.2.1. Plasmid DNA was dissolved in the internal aqueous-phase (3 mg/mL) of the W/O/W multiple emulsions followed by emulsification with oil-phase (squalane oil: Span 80TM, 9:1) using a homogenizer (Silverson’s Model: L4RT-A; Silverson Machines) at 10,000 rpm for 5 minutes to form the primary W/O emulsion. The resulting primary emulsion was further emulsified with
outer aqueous-phase (0.5% w/v Pluronic® F127 solution in water) using the homogenizer at 10,000 rpm for 10 minutes to create the W/O/W multiple emulsions.

5.2.5 Determination of the Oil-Droplets Size and Surface Charge

The oil-droplets size and surface charge characterization was performed as described in Section 2.2.4.

5.2.6 Quantitative Determination of the Plasmid DNA Encapsulation

The amount of plasmid DNA retained in the dispersed oil-droplets and excreted in the outer aqueous-phase of the W/O/W multiple emulsions was quantitatively measured to determine encapsulation efficiency. About 0.5 mL of the W/O/W multiple emulsions was centrifuged in centrifuge filter (ULTRAFREE®- MC 0.1 µm, Millipore, Billerica, MA) at 10,000 rpm for 30 min to separate dispersed oil-droplets from the outer continuous-phase. The retained oil-droplets were dissolved in isopropyl alcohol (Fisher Scientific) to extract and precipitate encapsulated plasmid DNA followed by centrifugation at 13,000 rpm for 30 min. Resulting supernatant was discarded and DNA pellet was dissolved in DNAnse free water (HyClone HyPure Molecular Biology Grade Water, Thermo Scientific). The amount of DNA extracted from oil-droplets and in separated outer aqueous-phase was measured using the NanoDrop 2000 (Thermo Scientific). In addition, integrity of the encapsulated plasmid DNA was examined by agarose gel-electrophoresis.
5.2.7 Determination of Encapsulated Plasmid DNA Protection from DNase Activity

The W/O/W multiple emulsion encapsulated plasmid DNA was incubated with DNase I to evaluate protection from DNase activity. About 10 µg of naked plasmid DNA dissolved in water or encapsulated in the W/O/W multiple emulsions were incubated with 10 µg of DNase I (Sigma-Aldrich) at 37 °C for 30 min. Following incubation plasmid DNA was extracted from the dispersed oil-droplets as described in Section 5.2.5. Integrity of the plasmid DNA incubated with DNase I was determined by agarose gel-electrophoresis as described in Section 5.2.3.

5.2.8 Cell Lines and Culture Methods

J774A.1 murine adherent alveolar macrophage cell line was purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 50 IU/mL penicillin, 50 µg/mL streptomycin combined antibiotics (PenStrep; GIBCO).

Murine dendritic cell line was purchased from Astarte Biologics LLC (Redmond, WA). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (GIBCO) supplemented with 10% fetal bovine serum (Gemini Bio-Products) and 5 ng/mL recombinant murine GM-CSF (PeproTech Inc., Rocky Hill, NJ).
5.2.9 Determination of \textit{In Vitro} Transfection Ability of the W/O/W Multiple Emulsion Encapsulated Plasmid DNA

\textit{In vitro} transfection ability of the W/O/W multiple emulsions encapsulated plasmid DNA encoding EGFP reporter gene was investigated in J774A.1 murine adherent alveolar macrophage (ATCC, Manassas, VA) and murine dendritic (Astarte Biologics LLC., Redmond, WA) cell lines. Qualitative and Quantitative assessment of transgene expression was performed by fluorescence microscopy and by EGFP ELISA methods respectively.

Approximately 200,000 adherent alveolar macrophage or murine dendritic cells were cultured in DMEM supplemented with 10% FBS (and 5 ng/mL recombinant murine GM-CSF for dendritic cell line) in each well of a 6-well plate (BD Biosciences, San Jose, CA) for 12 hours. After this period serum containing medium was replaced with 20 µg per well plasmid DNA containing formulation diluted in serum free medium. Incubation was continued at 37°C for 6 hours to allow sufficient uptake of formulation by the cells. The five different treatment groups included: (1) Serum free medium (negative Control), (2) Naked EGFP plasmid, (3) W/O/W SME control, (4) EGFP plasmid:Lipofectin® (Invitrogen) complex (positive control), and (5) EGFP plasmid encapsulated in W/O/W SME. After 6 hours formulation containing medium was replaced with DMEM supplemented with 10% FBS- and 50 IU/mL penicillin, 50 µg/mL streptomycin combined antibiotics (PenStrep) for J774A.1 murine macrophage cell line or 5 ng/mL recombinant murine GM-CSF for murine dendritic cell line. The cell lines were continued to incubate at 37°C followed by transgene expression analysis at 24 hours, 48 hours, 72 hours, and 96 hours.
For qualitative determination of EGFP transgene expression cells were treated with Hoechst 33342 nucleic acid stain (Invitrogen) and fluorescent light excited microscopic pictures were captured using an Olympus microscope (Model: IX51, Olympus America Inc., Chelmsford, MA). For quantitative determination of EGFP transgene expression, total cytosolic proteins were extracted by cell lysis using NP40 Cell Lysis Buffer (Invitrogen) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, MP BioMedicals) and Complete, Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics Corporation) at 4°C. Total protein content in the cell extracts was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific) according to manufacturer’s instructions. The GFP levels in the cell extracts were measured by ELISA method. 96-well plate was coated with primary anti-EGFP monoclonal antibodies (Novus Biologics, Littleton, CO) followed by blocking non-specific binding sites using SuperBlock® blocking buffer (Thermo Scientific, Milwaukee, WI). The cell extract samples and the calibration standards were added into the 96-wells plate and incubated for 12 h at 4°C. The 96-well plate was washed and alkaline phosphatase enzyme-linked, polyclonal anti-EGFP detection antibodies (Novus Biologics) were added followed by incubation for 2 hours at room temperature. The 96-well plate was washed and alkaline phosphate substrate (Thermo Scientific) was added followed by incubation for 30 min at room temperature. The reaction was stopped by adding 0.5 N NaOH solution and absorbance was measured at 408 nm using Bio-Tek® Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT) with KC4 software (BioTek Instruments, Inc.). The GFP amount in each sample was calculated using the calibration curve and presented in nanograms per milligrams of total protein in the cell extract.
5.2.10 *In Vitro* Transfection of Human gp100 Plasmid DNA and Determination of Transgene Expression

*In vitro* transfection ability of the hgp100 plasmid encapsulated in the W/O/W multiple emulsions was examined in J774A.1 murine adherent alveolar macrophage (ATCC, Manassas, VA) cell line. Qualitative assessment of transgene expression was performed by SDS-PAGE followed by Western Blot methods. *In vitro* transfection and cell extracts collection were performed as described in Section 5.2.9. The five different treatment groups included: (1) serum free medium (negative Control), (2) hgp100 plasmid encapsulated in the W/O/W SME, (3) hgp100 plasmid:Lipofectin® (Invitrogen) complex (positive control), (4) Naked hgp100 plasmid, and (5) Placebo the W/O/W multiple emulsions. Total cytosolic proteins were extracted by cell lysis using NP40 Cell Lysis Buffer (Invitrogen) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, MP BioMedicals) and cOmplete, Mini, EDTA-free protease inhibitor cocktail (Roche Diagnostics Corporation) at 4°C for determination of hgp100 transgene expression. Total protein content in the cell extracts was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific) according to manufacturer’s instructions. The proteins were separated on 1.7% SDS-PAGE gels (Bio-Rad Laboratories Inc., Waltham, MA). The separated proteins were transferred on the nitrocellulose membranes (iBlot® Transfer Stack, Invitrogen) using the dry blotting system (iBlot®, Invitrogen) according to manufacturer’s instructions. The membranes were blocked with StartingBlock™ buffer (Thermo Scientific) for 30 minutes at room temperature followed by incubation with anti-hgp100 (LifeSpan BioSciences Inc., Seattle, WA) or anti-β actin (Cell Signaling Technology, Inc., Danvers, MA) primary antibodies for 3 hours at room temperature. The
membranes were then washed with TBST (Hoefer® Inc., Holliston, MA) and subsequently incubated with a horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Inc.) for 2 hours at room temperature. The membranes were again washed with TBST; flash rinsed with purified water, and incubated with an enhanced chemiluminescence substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) for 2-10 min. The digital images of the membranes were captured using the Kodak In Vivo FX imager (Carestream Health, Rochester, NY).

5.2.11 Data Analysis

The statistical analysis of the data sets was performed as described in Section 3.2.9.

5.3 Results and Discussion

The EGFP and hgp100 (SILV) encoding plasmid DNA were constructed by ligating respective cDNA fragments into multiple cloning sites of the OmicsLink™ Expression Clone (Figure 19), a mammalian vector (pReceiver-M02) containing CMV gene promoter and ampicillin resistance gene as selection marker for E.coli culture. The plasmid DNA transformed bacteria were cultured in Luria Broth and amplified plasmid DNA was extracted, purified, and isolated using QIAGEN Mega Plasmid Kit. The ratio of plasmid DNA extracts absorbance measured at 260 nm and 280 nm was greater than 1.8 indicating acceptable purity of the extracted plasmids.
5.3.1 EGFP and Human gp100 Encoding Plasmid DNA Characterization

The EGFP and hgp100 cDNA fragment size was examined by restriction enzyme digestion followed by agarose gel-electrophoresis. A combination of EcoRI/NotI and EcoRI/XhoI restriction enzymes was used to slice the EGFP cDNA fragment from the expression clone, EX-EGFP-M02. Similarly, a combination of XmnI/XhoI and NspV/XhoI restriction enzymes was used to cut the hgp100 cDNA fragment from the expression clone, EX-Z0375-M02. The cDNA fragments were separated from vector backbone using agarose gel-electrophoresis and size was determined by comparing against molecular ruler. A digital image showing EGFP and hgp100 cDNA fragments separated from vector backbone is shown in Figure 20 and Figure 21 respectively.

Gel-electrophoresis results demonstrated that the size of the EGFP cDNA fragment and expression clone were about 700 bp and 6.5 Kbp respectively. Whereas the size of the hgp100 cDNA and expression clone were about 2 Kbp and 8 Kbp respectively. Thus, restriction enzyme digestion followed by agarose gel-electrophoresis exhibited that the EGFP and the hgp100 cDNA size in the respective expression clones are in accordance with that declared by the manufacturer.
Figure 19: The OmicsLink™ Expression Clone.
The expression clone contains a mammalian vector (pReceiver-M02) that contains human cytomegalovirus (CMV) promoter gene and ampicillin resistance gene as selection marker. The EGFP and hgp100 (SILV) encoding plasmid DNA were constructed by ligating respective cDNA fragments into multiple cloning sites (ORF) of the OmicsLink™ Expression Clone. ORF regions of the EGFP encoding plasmid DNA (a) and the hgp100 encoding plasmid DNA (b) illustrating restriction sites are shown.
Figure 20: Characterization of the EGFP cDNA Fragment by Gel-Electrophoresis.
The cDNA fragment from the EGFP encoding plasmid DNA was cut by double digestion using a combination of EcoRI/NotI and EcoRI/XhoI restriction enzymes. Subsequent agarose gel-electrophoresis demonstrated that the EGFP cDNA fragment size was about 700 bp in accordance with that declared by the manufacturer.

Lane 1: Intact plasmid
Lane 2: Plasmid cut once (EcoRI)
Lane 3: Plasmid cut once (NotI)
Lane 4: Plasmid cut once (XhoI)
Lane 5: Plasmid cut twice (EcoRI+NotI)
Lane 6: Plasmid cut twice (EcoRI+XhoI)
Lane 7: 100 bp DNA ladder
Lane 8: 1 Kb Plus DNA ladder

Figure 21: Characterization of the hgp100 cDNA Fragments by Gel-Electrophoresis.
The cDNA fragment from the hgp100 encoding plasmid DNA was cut by double digestion using a combination of XmnI/XhoI and NspV/XhoI restriction enzymes. Subsequent agarose gel-electrophoresis demonstrated that the hgp100 cDNA fragment size was about 2 Kbp in accordance with that declared by the manufacturer.

Lane 1: Intact plasmid
Lane 2: Plasmid cut once (XmnI)
Lane 3: Plasmid cut once (NspV)
Lane 4: Plasmid cut once (XhoI)
Lane 5: Plasmid cut twice (XmnI+XhoI)
Lane 6: Plasmid cut twice (NspV+XhoI)
Lane 7: 100 bp DNA ladder
Lane 8: 1 Kb Plus DNA ladder
5.3.2 Plasmid DNA Encapsulated the W/O/W Multiple Emulsions

The plasmid DNA encapsulating the W/O/W multiple emulsions were prepared by two-step emulsification method previously optimized for gp100 peptide antigen. The first emulsification step involved homogenization of hgp100 plasmid-containing internal aqueous-phase with Span™ 80 containing squalane oil-phase to form W/O primary emulsion. The second emulsification step involved homogenization of the resulting W/O primary emulsion with an outer aqueous-phase containing Pluronic® F127 to form the W/O/W multiple emulsions. Morphological investigation of the plasmid DNA containing the W/O/W multiple emulsions exhibited similar observations that are described in Section 2.3.1. The formulation attributes for three independent the W/O/W multiple emulsions-based hgp100 plasmid DNA vaccines are outlined in Table 5.

| Table 5: hgp100 Plasmid-Containing the W/O/W Multiple Emulsions Formulation Attributes |
| --- | --- | --- |
| Formulation | Characterization of the Multiple Emulsion Formulations |
| | Oil-Droplets Size |
| | Mean Diameter (µm) | Polydispersity Index | Zeta Potential (mV) |
| Lot - 1 | 1.6 ± 0.1 | 0.2 | -28.6 ± 0.5 |
| Lot - 2 | 1.2 ± 0.3 | 0.2 | -25.2 ± 0.5 |
| Lot - 3 | 1.6 ± 0.2 | 0.1 | -28.3 ± 0.4 |

The results are shown as Mean ± SD, n = 3.
The oil-droplets mean hydrodynamic diameters were about 1.5 µm and surface charges were about -30 mV. The coefficient of variance for polydispersity index values was 0.4 (n = 3). Importantly, the two-step emulsification method was reproducible as indicated by coefficient of variance for polydispersity index value and created stable hgp100 plasmid DNA-containing the W/O/W multiple emulsions.

5.3.3 Plasmid DNA Encapsulation

The hgp100 plasmid DNA encapsulation into the inner-aqueous phase of the W/O/W multiple emulsions was quantitatively investigated as illustrated in Figure 22. The payload encapsulation was determined by measuring DNA concentration in the dispersed oil-droplets and outer continuous-phase. The dispersed oil-droplets were separated from the outer continuous-phase by centrifuge filtration. The plasmid DNA encapsulated within dispersed oil-droplets was extracted by dissolving oil-droplets in isopropyl alcohol followed by centrifugation to pellet out the precipitated plasmid DNA. The plasmid DNA pellet was dissolved in DNase free water and concentration was measured. Amount (percent by weight of target load) of plasmid DNA encapsulated in the dispersed oil-droplets and leached into outer continuous-phase during preparation of the W/O/W multiple emulsions is outlined in Table 6.

Table 6: Plasmid DNA Encapsulation in the Dispersed Oil-Droplets of the W/O/W Multiple Emulsions.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean Percent by Weight of Target Load</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dispersed Oil-Droplets</td>
<td>Outer Continuous- Phase</td>
</tr>
<tr>
<td>Lot - 1</td>
<td>96.5 ± 1.9</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Lot - 2</td>
<td>94.3 ± 1.3</td>
<td>4.4 ± 0.5</td>
</tr>
</tbody>
</table>

The results are shown as Mean ± SD, n = 3.
Figure 22: Schematics Showing Quantitative Determination of Plasmid DNA Encapsulation.
The dispersed oil-droplets were separated from the outer continuous-phase by centrifuge filtration. The plasmid DNA encapsulated within dispersed oil-droplets was extracted followed by centrifugation to pellet out the precipitated plasmid DNA. Amount of plasmid DNA in pellet was determined by measuring absorbance using NanoDrop 2000.
Results demonstrated that about 95% by weight of target payload was encapsulated within the dispersed oil-droplets. In addition, stability of the encapsulated plasmid DNA was determined by agarose gel-electrophoresis. A comparison of distance migrated by control plasmid DNA and that was extracted from the dispersed oil-droplets is shown in Figure 23. Gel-electrophoresis results demonstrated that integrity of the plasmid DNA encapsulated within dispersed-oil droplets was maintained during the W/O/W multiple emulsions preparation.

5.3.4 Protection of Encapsulated Plasmid DNA from DNAse I Activity

Protection of the W/O/W multiple emulsions encapsulated plasmid DNA from enzymatic degradation was examined. The naked plasmid DNA dissolved in water and the W/O/W multiple emulsions containing encapsulated plasmid DNA were incubated with DNAse I at 37 °C for 30 min. Following incubation plasmid DNA was extracted from the dispersed oil-droplets as illustrated in Section 5.3.3. Integrity of the plasmid DNA incubated with DNAse I was determined by gel-electrophoresis and results are shown in Figure 23.

Gel-electrophoresis results demonstrated that distance migrated by the plasmid DNA extracted from the W/O/W multiple emulsions with or without DNAse I treatment is comparable to that of the control plasmid DNA. In contrast, the naked plasmid DNA incubated with DNAse I may have been completely digested and therefore was not visible on the agarose gel. Thus, it was concluded that the W/O/W multiple emulsions encapsulated plasmid DNA is being protected from enzymatic degradation by DNAse I.
Figure 23: Protection of the Encapsulated Plasmid DNA from DNase Activity.
The naked and W/O/W multiple emulsions encapsulated plasmid DNA were incubated with DNase I at 37 °C for 30 min. After incubation the encapsulated plasmid DNA was extracted from the dispersed oil-droplets and integrity was examined by gel-electrophoresis. Distance migrated by the plasmid DNA extracted from the W/O/W multiple emulsions with or without DNase I treatment is comparable to that of the control plasmid DNA indicating integrity of the plasmid DNA.

**Lane 1**: 1 Kb Plus DNA Ladder  
**Lane 2**: Control plasmid DNA  
**Lane 3**: Plasmid DNA extracted from multiple emulsions (ME)  
**Lane 4**: Plasmid DNA extracted from ME after DNAse I treatment  
**Lane 5**: Naked plasmid DNA incubated with DNAse I  
**Lane 6**: Plasmid DNA in outer continuous-phase after DNAse I treat.  
**Lane 7**: Plasmid DNA in outer continuous-phase
5.3.5 *In vitro* Transfection Ability of the W/O/W Multiple Emulsions Encapsulated Plasmid DNA

*In vitro* transfection ability of the W/O/W multiple emulsions encapsulated plasmid DNA encoding EGFP reporter gene was investigated. Qualitative assessment of transgene expression in J774A.1 murine macrophage and murine dendritic cell lines was performed by fluorescence microscopy and digital images captured at 24 hr, 48 hr, 72 hr, and 96 hr are shown in Figure 24 and Figure 25 respectively. Quantitative assessment of transgene expression in J774A.1 murine macrophage and murine dendritic cell lines was performed by ELISA method and amount of GFP expressed in nanograms per milligrams of total cell protein at 24 hr, 48 hr, 72 hr, and 96 hr is outlined in Figure 24 and Figure 25 respectively. Serum free medium treated and placebo the W/O/W multiple emulsions treated cells were utilized as negative control, whereas plasmid complexed with Lipofectin®, a commercially available positively charged lipid transfection reagent treated cells were utilized as positive control.

Both qualitative and quantitative examination demonstrated EGFP transgene expression in the cells treated with either EGFP plasmid:Lipofectin® complex or EGFP plasmid encapsulated the W/O/W multiple emulsions in J774A.1 murine macrophage and murine dendritic cell lines. The amount of GFP per milligrams of total protein increased up to 72 hours followed by gradual decline indicating transient expression of the EGFP transgene. More importantly, amount of GFP expressed in the EGFP plasmid encapsulated the W/O/W multiple emulsions treated cells was significantly higher than that expressed in control cells indicating ability of the W/O/W multiple emulsions
encapsulated plasmid DNA to transfect the murine immune cells and subsequently to express the transgene encoded protein *in vitro*.

Qualitative Assessment of the EGFP Transgene Expression in J774A.1 Murine Macrophage Cell Line.
Figure 24: Qualitative and Quantitative Assessment of the EGFP Transgene Expression in J774A.1 Murine Macrophage Cell Line.

Fluorescence microscopy (a) and GFP ELISA method (b) were used for qualitative and quantitative assessment of the EGFP transgene expression. *In vitro* transfection treatment groups included: (1) Control: cells treated with serum free medium, (2) Naked plasmid: cells treated with EGFP plasmid dissolved in serum free medium, (3) Control SME: cells treated with placebo the W/O/W multiple emulsions, (4) Plasmid:Lipofectin® complex: cells treated with EGFP plasmid complexed with Lipofectin®, and (5) EGFP plasmid in SME: cells treated with the EGFP plasmid encapsulated the W/O/W multiple emulsions. Both assessment demonstrated transient expression of EGFP transgene in cells treated with either EGFP plasmid:Lipofectin® complex or EGFP plasmid encapsulated the W/O/W multiple emulsions in J774A.1 murine macrophage cell line.
Qualitative Assessment of the EGFP Transgene Expression in Murine Dendritic Cell Line.
Figure 25: Qualitative and Quantitative Assessment of the EGFP Transgene Expression in Murine Dendritic Cell Line.

Fluorescence microscopy (a) and GFP ELISA method (b) were used for qualitative and quantitative assessment of the EGFP transgene expression. In vitro transfection treatment groups included: (1) Control: cells treated with serum free medium, (2) Naked plasmid: cells treated with EGFP plasmid dissolved in serum free medium, (3) Control SME: cells treated with placebo the W/O/W multiple emulsions, (4) Plasmid:Lipofectin® complex: cells treated with EGFP plasmid complexed with Lipofectin®, and (5) EGFP plasmid in SME: cells treated with the EGFP plasmid encapsulated the W/O/W multiple emulsions. Both assessment demonstrated transient expression of EGFP transgene in cells treated with either EGFP plasmid:Lipofectin® complex or EGFP plasmid encapsulated the W/O/W multiple emulsions in murine dendritic cell line.
5.3.6 *In Vitro* Human gp100 Plasmid DNA Transfection and Transgene Expression

The W/O/W multiple emulsions encapsulated the hgp10 plasmid DNA *in vitro* transfection and transgene expression in J774A.1 murine macrophage cell line was investigated by SDS-PAGE followed by western blot analysis of the cell extracts. The five different treatment groups included: (1) Serum free medium (negative Control), (2) hgp10 plasmid encapsulated in the W/O/W SME, (3) hgp100 plasmid:Lipofectin® (Invitrogen) complex (positive control), (4) Naked hgp100 plasmid, and (5) Placebo the W/O/W multiple emulsions. The proteins in the cell extracts were separated by SDS-PAGE followed by blotting on the nitrocellulose membrane. The human gp100 and mouse β-actin proteins transferred on nitrocellulose membrane were stained and visualized using protein-specific antibodies. The digital images of the western blot are shown in Figure 26.

The western blot images demonstrated presence of the human gp100 peptide in the extracts from cells treated with the hgp100 plasmid DNA encapsulated in the W/O/W multiple emulsions, the hgp10 plasmid DNA complexed with Lipofectin®, and naked the hgp10 plasmid DNA. However, amount of human gp100 peptide in the extract from the naked hgp100 plasmid DNA treated cells was considerably lower than that in the W/O/W multiple emulsions treated cell extract as indicated by relative intensities of human gp100 and mouse β-actin bands. Thus, SDS-PAGE and western blot analysis exhibited *in vitro* transfection and transgene expression ability of the hgp100 plasmid DNA encapsulated in the W/O/W multiple emulsions.
Figure 26: Human gp100 Plasmid DNA *In Vitro* Transfection and Transgene Expression.

*In vitro* transfection and transgene expression of the W/O/W multiple emulsions encapsulated the hgp100 plasmid DNA was investigated in J774A.1 murine macrophage cell line. The proteins in the cell extracts were separated by SDS-PAGE followed by western blot analysis using human gp100- and mouse β-actin- specific antibodies.

**Lane 1:** Control cells  
**Lane 2:** hgp100 plasmid DNA in the W/O/W multiple emulsions treated cells  
**Lane 3:** hgp100 plasmid DNA:Lipofectin® complex treated cells  
**Lane 4:** Naked hgp100 plasmid DNA treated cells  
**Lane 5:** Placebo the W/O/W multiple emulsions treated cells
5.4 Conclusions

The plasmid DNA encapsulating the W/O/W multiple emulsions were prepared by two-step emulsification method with about 95% by weight of target payload was encapsulated within the dispersed oil-droplets. The mean hydrodynamic diameter and surface charge for the plasmid DNA encapsulated dispersed oil-droplets were comparable to gp100 peptide encapsulated dispersed oil-droplets in previous studies. Agarose gel-electrophoresis of the hgp100 plasmid DNA extracted from the W/O/W multiple emulsions with or without treatment with DNAse I indicated that the encapsulated plasmid is protected during preparation and from enzymatic degradation. More importantly, in vitro transfection and transgene expression analysis demonstrated that the EGFP and the hgp100 plasmid DNA encapsulated in the W/O/W multiple emulsions were able to transfect and express transgene encoded proteins in the murine immune cells.
CHAPTER 6
EVALUATION OF Human gp100 EXPRESSING PLASMID DNA VACCINE IN VIVO EFFECTIVENESS IN PROPHYLACTIC TREATMENT MODEL OF MURINE B16 MELANOMA

6.1 Introduction

gp100 glycoprotein, a melanoma differentiation antigen is expressed on most melanoma cells and recognized by immune cells including melanoma-derived tumor-infiltrating lymphocytes. Therefore, gp100 protein is considered as a candidate tumor associated-antigen (TAA) to induce an effective immune response in melanoma. In fact, initial studies have reported that immunization with gp100-derived peptides can induce a measurable antitumor immune response in some cancer patients. Immunization using plasmid DNA that codes for TAAs is an evolving strategy for cancer immunotherapy. This immunization strategy is devised on the premise that sustained local expression of the transgenic TAAs may confer a greater immunogenic presentation of tumor antigens to enhance in vivo sensitization and activation of T cells that are capable of recognizing the TAA-associated peptides on the tumor cell surface. Several studies have demonstrated induction of T-cell dependent immune response against TAA by genetic immunization in murine models. However, the limitations of delivery systems and the concerns on biosafety of the viral vectors based gene therapy have motivated attempts to develop alternative, non-viral means of gene delivery into somatic cells in vivo.
The W/O/W multiple emulsions based vaccine delivery system being a particulate delivery system holds potential to transfect immune cells with encapsulated plasmid DNA followed by transgene expression of encoded antigen to induce antigen-specific immune response. In this study, we have investigated in vivo effectiveness of the W/O/W multiple emulsions-based hgp100 plasmid DNA vaccine following prophylactic immunization approach in murine B16 melanoma model. Protection against tumor challenge was examined in the immunized C57BL/6 mice to determine induction of immune responses. Additionally, involvement of cell-mediated anti-tumor immunity was confirmed by analyzing serum Th1 cytokine levels and local infiltration of CD4+ and CD8+ CTLs at tumor site.

6.2 Materials and Methods

6.2.1 Preparation of Plasmid DNA-Encapsulated the W/O/W Multiple Emulsions Vaccine

The hgp100 plasmid DNA-containing the W/O/W multiple emulsions vaccine and empty vector-containing the W/O/W multiple emulsions control formulation were prepared as described in Section 5.2.4.

6.2.2 Determination of the Oil-Droplets Size and Surface Charge

The oil-droplets size and surface charge characterization was performed as described in Section 2.2.4.
6.2.3 Melanoma Cell Line and Animal Model

B16-F10 melanoma cell line and murine B16 melanoma tumor model used in the W/O/W multiple emulsions encapsulated the hgp100 plasmid DNA vaccine in vivo effectiveness investigation is described in Section 3.2.4.

6.2.4 Immunization and Tumor Challenge Protocol

The mice were randomized into four treatment groups: (1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME) containing eight animals in each. In prophylactic immunization mode anesthetized mice received three subcutaneous (s.c.) injections of 0.1 mL control or vaccine [50 µg dose\(^{105}\) per injection] formulations at 2-week intervals. Ten days after the last immunization, tumor challenge was initiated by s.c. implantation of 100,000 B16-F10 cells\(^{86,87}\) suspended in 0.1 mL PBS on the hind flank of mice. The mice were monitored daily for any pain or distress, and general health. According to Northeastern University’s IACUC guidance, mice were euthanized when tumor volume reached to 1,000 mm\(^3\). Immediately after euthanasia, the blood sample was collected and from that serum was separated for Th1 cytokine levels measurement. In addition, tumor tissues were excised, washed in PBS, and blot dried. Excised tumor masses were recorded; representative images were captured, and cryo-preserved for the immuno-histochemical analysis.
6.2.5 Evaluation of Protection against Tumor Challenge

The method used to evaluate protection against a tumor challenge is described in Section 3.2.6.

6.2.6 Serum Th1 Cytokine Analysis

The method to analyze serum Th1 cytokine levels is described in Section 3.2.7.

6.2.7 Tumor Tissue Immuno-Histochemical Analysis

The method used to determine activation and local infiltration of CD4+ and CD8+ CTLs by immuno-histochemical staining of excised tumor tissues is described in Section 3.2.8.

6.2.8 Data analysis

The statistical analysis was performed as described in Section 3.2.9.

6.3 Results and Discussion

The goal for immunization using the multi-compartmental, W/O/W multiple emulsions-based hgp100 plasmid DNA vaccine was to induce cell-mediated anti-tumor immunity and examine its ability to protect the mice against tumor challenge in B16 melanoma model. The immunization protocol used to evaluate the hgp100 plasmid DNA vaccine in vivo effectiveness is illustrated in Figure 27.
6.3.1 Protection against Tumor Challenge

Mean tumor volume growth was determined to evaluate protection against tumor challenge. Mean tumor volume growth curves for four treatment groups: (1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME); following immunization are shown in Figure 27. In addition, mean tumor mass and a representative picture of the excised tumor tissue from each group are shown in Figure 28. The results demonstrated that immunization using W/O/W multiple emulsions-based hgp100 plasmid DNA vaccine resulted in about 2.5-fold decrease in the mean tumor volume and about 2-fold decrease in the mean excised tumor mass, compared to treatment with saline or control formulations. More importantly, differences between the mean tumor volume for hgp100 plasmid DNA vaccine immunized and control formulation treated groups were statistically significant ($p < 0.05$) suggesting induction of enhanced immune responses against melanoma due to hgp100 plasmid DNA vaccination.

The results demonstrated that immunization of C57BL/6 mice using the W/O/W multiple emulsions encapsulated hgp100 plasmid DNA vaccine resulted in tumor growth suppression as evidenced by decrease in mean tumor volume. Thus, results demonstrated that C57BL/6 immunization using hgp100 plasmid DNA vaccine offers protection against B16-F10 melanoma tumor challenge.
Figure 27: *In Vivo* Prophylactic Immunization Protocol and hgp100 Plasmid DNA Vaccine Formulation Effectiveness.

Four groups of mice were treated with: (1) saline control (●), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (♦), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME) (■), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME) (▲) containing placebo or 50 µg hgp100 plasmid DNA per injection. (a) In prophylactic treatment mode mice were treated with control or vaccine formulation three-times at 2-week interval and ten days after the last injection tumor challenge was initiated by subcutaneous (s.c.) injection of 100,000 B16-F10 cells on the hind flank of the mice. Tumor growth was monitored by measuring two perpendicular tumor dimensions (mm) using the calipers and tumor volumes (V, mm³) were calculated using the formula: \( V = \frac{1}{2} [L \times (W)^2] \); where L is the longest dimension and W is the dimension perpendicular to the L. (b) Tumor growth curves for prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
Figure 28: Tumor Mass and a Representative Picture of Excised Tumors.

Animals were euthanized and tumor tissues were excised when mean tumor volume for saline treated group of mice reached to 1000 mm$^3$. Excised tumors were freed of skin remnants, rinsed in phosphate buffered saline and dried on blotting paper. A representative picture (a) and mass (b) of excised tumors from four treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (purple), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME) (purple), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME) (pink) following prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p < 0.05).
6.3.2 Serum Th1 Cytokine Levels

The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in sera were quantitatively measured using ELISA method and compared among different treatment groups to investigate induction of CD4+ and CD8+ cytotoxic T-cell function due to immunization. The Th1 cytokine levels measured in sera collected from four treatment groups: (1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME), following immunization are shown in Figure 29.

The results demonstrated increased levels of IL-2, IL-12, INF-γ, and TNF-α in the serum samples from the mice immunized using hgp100 plasmid DNA vaccine compared to the mice treated with control formulations. Increased Th1 cytokine levels indicated immune response stimulation through Th1 pathway that activates CD8+ T-cell mediated antitumor immunity. Noticeably, immunization using the W/O/W multiple emulsions-based hgp100 plasmid DNA vaccine resulted in significantly increased (p < 0.05) levels of IL-2, IL-12, INF-γ, and TNF-α compared to saline treatment.

6.3.3 Immuno-Histochemical Staining of Excised Tumor Tissue

The antigen-specific CD4+ and CD8+ T-cell trafficking and infiltration into tumor stroma was examined by immuno-histochemically staining excised tumor tissues using anti-CD4 and anti-CD8a antibodies^{12,97}. Tumor tissue sections were incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies.
Figure 29: Serum Th1 Cytokine Levels.
Immediately after animal euthanasia the blood samples were collected and allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in the sera were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array. The Th1 cytokine levels in the sera from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (purple), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME) (purple), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME) (pink) are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
and histochemical staining. The digital pictures of the stained tumor sections are shown in Figure 30.

Immuno-staining of the excised tumor tissues demonstrated that immunization of C57BL/6 mice using hgp100 plasmid DNA vaccine resulted in increased infiltration of CD4+ and CD8+ T-cells into and/or tumor surroundings compared to control formulation treatment; thereby providing evidence for involvement of cell-mediated antitumor immunity that might have protected mice against B16-F10 melanoma tumor challenge. Thus, tumor tissue immuno-histology indicated immunization using the W/O/W multiple emulsions-based hgp100 plasmid DNA vaccine activates antigen-specific CD4+ and CD8+ T-cells that subsequently mediates anti-tumor immunity and protects immunized mice against melanoma tumor challenge.

6.4 Conclusions

Immunization of C57BL/6 mice using the multi-compartmental, W/O/W multiple emulsion-based hgp100 plasmid DNA vaccine evokes cell-mediated antitumor immunity that provided protection against tumor challenge. Thus, the W/O/W multiple emulsions based vaccine delivery systems are capable to deliver the encapsulated genetic payload to the immune cells and subsequently induce transgene encoded antigen-specific anti-tumor immunity.
Figure 30: Tumor Tissue Immuno-Histochemistry.
Activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site was investigated by immuno-histochemical staining of excised tumor tissues. Tumor tissues were cryo-sectioned (6-8 µ thickness) and incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the immuno-stained tumor sections from five treatment groups top to bottom (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (purple), (3) empty vector in the W/O/W multiple emulsions (Vector in W/O/W ME) (purple), and (4) the hgp100 plasmid encapsulated the W/O/W multiple emulsions (hgp100 in W/O/W ME) (pink) are shown.
CHAPTER 7
EVALUATION OF THE EFFECTS OF CAFFEINE CO-ADMINISTRATION WITH gp100 PEPTIDE AND DNA VACCINATION IN PROPHYLACTIC TREATMENT MODEL OF MURINE B16 MELANOMA

7.1 Introduction

Adenosine is a ubiquitous signaling molecule whose physiological functions are mediated by its interaction with four G-protein-coupled receptor subtypes, termed as A₁, A₂A, A₂B and A₃. Adenosine concentrations increase under metabolically unfavorable conditions such as in cancer tissues and also appear to be implicated in growth of tumors¹⁰⁷. In particular, activation of A₂A receptors leads to immunosuppressive effects, which decreases anti-tumoral immunity and thereby encourages tumor growth. Adenosine inhibits dendritic cell function; T-cell activation and differentiation; and T-cell effector function through A₂A AR activity¹⁰⁸. Additionally, the A₂A AR selectively enhance anti-inflammatory cytokines promoting up regulation of PD-1 and CTLA-4 that generates LAG-3 and Foxp3+ regulatory T cells and thereby mediates the inhibition of regulatory T cells¹⁰⁹-¹¹¹. Consequently, these combined immunosuppressive properties are identified as mechanisms by which tumors evade host immune surveillance. Initial in vivo studies demonstrated that genetic and pharmacological inhibition of the A₂A AR leads to robust antitumor immunity stimulation. These results suggested that adenosine is at least partially responsible for promoting the tumor defense mechanisms against immune response¹¹². Therefore, pharmacological inhibition of A₂A AR activation by specific antagonists may enhance the effect of cancer immunotherapy¹¹²-¹¹⁴. The safety of
A2A AR antagonists has been demonstrated in clinical trials for the treatment of Parkinson’s disease115.

It was hypothesized that co-administration of A2A AR antagonists with tumor-specific vaccination may enhance the effectiveness of immunotherapy by: (1) blocking up regulation of regulatory T cells, (2) blocking adenosine-mediated CTLs suppression, (3) enhanced activity of the APCs, and (4) enhanced effector function of CTLs. In this study we explored co-administration of caffeine, the A2A AR antagonist in gp100 peptide and hgp100 plasmid DNA prophylactic vaccination to augment the melanoma immunotherapy effectiveness.

7.2 Materials and Methods

7.2.1 Preparation of the W/O/W Multiple Emulsions Vaccine

The gp100 peptide and the hgp100 plasmid DNA-containing the W/O/W multiple emulsions vaccines were prepared as described in Section 2.2.1 and 5.2.4.

7.2.2 Determination of the Oil-Droplets Size and Surface Charge

The oil-droplets size and surface charge characterization was performed as described in Section 2.2.4.
7.2.3 Melanoma Cell Line and Animal Model

B16-F10 melanoma cell line and murine B16 melanoma tumor model used in this *in vivo* vaccination effectiveness investigation is described in Section 3.2.4.

7.2.4 Immunization and Tumor Challenge Protocol

The mice were randomized into six treatment groups: (1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) containing eight animals in each. In prophylactic immunization mode anesthetized mice received three subcutaneous (s.c.) injections of 0.1 mL control or vaccine [50 µg dose,81,105 per injection] formulations at 2-week intervals. In addition, 0.07% w/v caffeine (Sigma-Aldrich) was included in drinking water for co-administration during both immunization and tumor challenge phases of the study. Ten days after the last immunization; tumor challenge was initiated by s.c. implantation of 100,000 B16-F10 cells86,87 suspended in 0.1 mL PBS on the hind flank of mice. The mice were monitored daily for any pain or distress, and general health. According to Northeastern University’s IACUC guidance, mice were euthanized when tumor volume reached to 1,000 mm³. Immediately after euthanasia, the blood sample was collected and from that serum was separated for Th1 cytokine levels measurement. In
addition, tumor tissues were excised, washed in PBS, and blot dried. Excised tumor masses were recorded; representative images were captured, and cryo-preserved for the immuno-histochemical analysis.

7.2.5 Evaluation of Protection against Tumor Challenge

The method used to evaluate protection against a tumor challenge is described in Section 3.2.6.

7.2.6 Serum Th1 Cytokine Analysis

The method used to analyze serum Th1 cytokine levels is described in Section 3.2.7.

7.2.7 Tumor Tissue Immuno-Histochemical Analysis

The method used to determine activation and local infiltration of CD4+ and CD8+ CTLs by immuno-histochemical staining of excised tumor tissues is described in Section 3.2.8.

7.2.8 Data analysis

The statistical analysis of the data sets was performed as described in Section 3.2.9.
7.3 Results and Discussion

The goal for caffeine, an A2A adenosine receptor antagonist co-administration during immunization and tumor challenge phase of this study was to enhance immunotherapeutic effectiveness of the vaccination by adenosine mediated: (1) blocking of regulatory T-cell up regulation, (2) blocking of CTLs suppression, (3) enhanced activity of the APCs, and (4) enhanced effector function of CTLs. The immunization protocol including caffeine co-administration used to evaluate the effect of caffeine on vaccine in vivo effectiveness is illustrated in Figure 31.

7.3.1 Protection against Tumor Challenge

Mean tumor volume growth was determined to evaluate protection against tumor challenge. Mean tumor volume growth curves for six treatment groups: (1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine); following immunization are shown in Figure 31. In addition, mean tumor mass and a representative picture of the excised tumor tissue from each group are shown in Figure 32. The results demonstrated that immunization using W/O/W multiple emulsions-based gp100 peptide or hgp100 plasmid DNA vaccine resulted in about 2.5-fold decrease in the mean tumor volume and about 2-
Figure 31: In Vivo Prophylactic Immunization Protocol and Effect of Caffeine Co-administration on Vaccine Effectiveness.

Six groups of mice were treated with: (1) saline control (●), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (○), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (♦), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (◊), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (▲), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (Δ) containing placebo or 50 µg gp100 peptide or hgp100 plasmid DNA per injection. (a) In prophylactic treatment mode mice were treated with control or vaccine formulation three-times at 2-week interval and ten days after the last injection tumor challenge was initiated by subcutaneous (s.c.) injection of 100,000 B16-F10 cells on the hind flank of the mice. Tumor growth was monitored by measuring two perpendicular tumor dimensions (mm) using the calipers and tumor volumes (V, mm³) were calculated using the formula: V =1/2[L x (W)²]; where L is the longest dimension and W is the dimension perpendicular to the L. (b) Tumor growth curves for prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
Figure 32: Tumor Mass and a Representative Picture of Excised Tumors. Animals were euthanized and tumor tissues were excised when mean tumor volume for saline treated group of mice reached to 1000 mm$^3$. Excised tumors were freed of skin remnants, rinsed in phosphate buffered saline and dried on blotting paper. A representative picture (a) and mass (b) of excised tumors from six treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (pink) following prophylactic immunization are shown. Results are presented as mean ± SD, n = 8 (*p < 0.05).
fold decrease in the mean excised tumor mass, compared to treatment with saline or control formulations. Co-administration of caffeine during immunization and tumor challenge phase resulted in similar tumor growth suppression, however further increase in immune responses were not observed as indicated by mean tumor volume.

The results demonstrated that immunization of C57BL/6 mice using the W/O/W multiple emulsions encapsulated gp100 peptide of hgp100 plasmid DNA vaccine resulted in tumor growth suppression as evidenced by decrease in mean tumor volume. However, substantial increase in vaccination in vivo effectiveness and protection against tumor challenge was not observed with caffeine co-administration during immunization and tumor challenge phase. This may be due to weak antagonism offered by caffeine at A2A AR may not be able to differentiate the effects in an aggressive tumor model such as murine B16 melanoma.

7.3.2 Serum Th1 Cytokine Levels

The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in sera were quantitatively measured using ELISA method and compared among different treatment groups to investigate increased cytotoxic function of CD4+ and CD8+ CTLs due to immunization. The Th1 cytokine levels measured in sera collected from six treatment groups: 1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with
caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine), following immunization are shown in Figure 33.

The results demonstrated increased levels of IL-2, IL-12, INF-γ, and TNF-α in the serum samples from the mice immunized using gp100 peptide of hgp100 plasmid DNA vaccine compared to the mice treated with control formulations. Increased Th1 cytokine levels indicated immune response stimulation through Th1 pathway that activates CD8+ T-cell mediated antitumor immunity. However, considerable increase in serum Th1 cytokine levels due to caffeine co-administration during immunization and tumor challenge phase was not observed.

7.3.3 Immuno-Histochemical Staining of Excised Tumor Tissue

The antigen-specific CD4+ and CD8+ T-cell trafficking and infiltration into tumor stroma was examined by immuno-histochemically staining excised tumor tissues using anti-CD4 and anti-CD8a antibodies. Tumor tissue sections were incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the stained tumor sections are shown in Figure 34.

Immuno-staining of the excised tumor tissues demonstrated that immunization of C57BL/6 mice using gp100 peptide or hgp100 plasmid DNA vaccine with/without caffeine co-administration resulted in comparable increase in CD4+ and CD8+ T-cells infiltration into and/or tumor surroundings compared to control formulation treatment; thereby providing evidence for involvement of cell-mediated antitumor immunity. that might have protected mice against B16-F10 melanoma tumor challenge.
Figure 33: Serum Th1 Cytokine Levels.
Immediately after animal euthanasia the blood samples were collected and allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in the sera were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array. The Th1 cytokine levels in the sera from five treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (pink) are shown. Results are presented as mean ± SD, n = 8 (*p< 0.05).
Figure 34: Tumor Tissue Immuno-Histochemistry.
Activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site was investigated by immuno-histochemical staining of excised tumor tissues. Tumor tissues were cryo-sectioned (6-8 µ thickness) and incubated with anti-CD4 or anti-CD8a antibodies followed by visualization using detection antibodies and histochemical staining. The digital pictures of the immuno-stained tumor sections from five treatment groups top to bottom (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (pink) are shown.
7.4 Conclusions

Immunization of C57BL/6 mice using the multi-compartmental, W/O/W multiple emulsion-based gp100 peptide or hgp100 plasmid DNA vaccine evokes cell-mediated antitumor immunity that provided protection against subsequent tumor challenge. However, considerable increase in vaccination \textit{in vivo} effectiveness and protection against tumor challenge was not observed with caffeine co-administration during immunization and tumor challenge phase. Therefore, adjunct therapy using potent A2A AR antagonists such as ZM-241385 or Istradefylline (KW-6002) is warranted to enhance \textit{in vivo} effectiveness of cancer immunotherapeutics.
8.1 Introduction

Safety and tolerability are critical regulatory concerns that pose the greatest hurdle in new adjuvant approvals. In addition to preclinical studies on the adjuvant itself, the combined antigen–adjuvant formulation must pass animal toxicology screens in at least two species at a dose and frequency covering anticipated human dose range, or higher, using the same route of administration, to assess safety and tolerability before human clinical trials. Therefore, Vaccine adjuvant benefits must be balanced against any increased reactogenicity or risk of adverse reactions. Unfortunately, in most cases, increased adjuvant potency is associated with increased reactogenicity and toxicity. Vaccine induced adverse effects can be divided into two types: (1) local and (2) systemic reactions. Local reactions range from injection site pain, inflammation, and swelling, to granulomas, sterile abscess formation, lymphadenopathy, and ulceration. Systemic vaccine reactions may include nausea, fever, adjuvant arthritis, eosinophilia, allergic reactions, organ-specific toxicity, anaphylaxis, or immunotoxicity mediated by liberation of cytokines, immunosuppression, and induction of autoimmune diseases. While some systemic reactions such as allergy and anaphylaxis are clearly due to the antigen, others, such as adjuvant arthritis, may be caused directly by or exacerbated by the adjuvant.
Lactate dehydrogenase (LDH) is an enzyme involved in conversion of pyruvate into lactate. It is present in many vital organs such as heart, liver, kidney, muscles, brain, blood cells, and lungs and therefore it is most often measured in serum samples to examine damage to these organs. Similarly, a very high level of alanine transaminase (ALT) enzyme is present in the liver. Consequently, an increased level of serum ALT levels indicates liver damage. In this study we investigated the W/O/W multiple emulsions-based gp100 peptide and hgp100 plasmid DNA vaccine systemic tolerability by monitoring animal body weight, serum LDH levels, and serum ALT levels pre and post immunization. An abrupt change or loss in the animal body weight and increase in serum LDH or ALT levels may indicate overt systemic toxicity due to immunotherapy. In addition, histo-pathological characterization of excised liver tissue may help determine changes in structure and integrity due to overt systemic toxicity.

8.2 Materials and Methods

8.2.1 Preparation of the W/O/W Multiple Emulsions Vaccine

The gp100 peptide and the hgp100 plasmid DNA-containing the W/O/W multiple emulsions vaccines were prepared as described in Section 2.2.1 and 5.2.4.

8.2.2 Animal Model

C57Bl/6 mice and experimental conditions used in vaccine formulation preliminary tolerability evaluation is described in Section 3.2.4.
8.2.3 Immunization Protocol

The immunization protocols for the vaccine formulations preliminary stability evaluation are described in Section(s) 3.2.5, 4.2.5, and 7.2.4.

8.2.4 Determination of Change in Animal Body Weight

The mean ratio of body weight to the initial body weight of all animals in the vaccinated group was determined during and after immunization and compared with that of the animals in the control groups.

8.2.5 Determination of Serum Lactate Dehydrogenase Levels

Blood samples from vaccinated and control animals were collected before and after immunization and from that serum was separated. The LDH levels in the serum samples were measured using the QuantiChrom™ Lactate Dehydrogenase Kit (BioAssay Systems, Hayward, CA) according to manufacturer’s instructions.

8.2.6 Determination of Serum Alanine Transaminase Levels

Blood samples from vaccinated and control animals were collected before and after immunization and from that serum was separated. The ALT levels in the serum samples were measured using ALT (SGPT) reagent set (Pointe Scientific Inc., Canton, MI) according to manufacturer’s instructions.
8.2.7  Histo-Pathological Characterization of Liver Tissue

Histo-pathological evaluation of excised liver tissue following treatment with vaccine formulations was performed at the Cummings School of Veterinary Medicine at Tufts University, Grafton MA. Excised liver tissues were embedded in OCT tissue freezing medium and stored at -80°C. Tissues were cleared of freezing medium and fixed in 10% neutral buffered (NB) formalin for 4 hours followed by embedding into molten paraffin to prepare sections. Paraffin embedded tissues were cut into 5 µm sections and mounted on a glass slides. Tissue sections were dried and de-paraffinized using xylene substitute followed by decreasing concentrations of ethanol to finally running purified water. Sections were incubated in hematoxylin, rinsed with water, and incubated with 1% acid alcohol (clearing reagent). Sections were rinsed and incubated with 4% ammonia solution (bluing reagent). Sections were then incubated with Eosin followed by dehydration by two changes each in 95% ethanol and 100% ethanol followed by final change of xylene substitute. Tissues were coverslipped and digital image was captured using a light microscopy. Analysis of tissue damage, if any, was done by Dr. Jerry Lyon, a certified veterinary pathologist.

8.2.8  Data analysis

The statistical analysis of the data sets was performed as described in Section 3.2.9.
8.3 Results and Discussion

Preliminary tolerability of the W/O/W multiple emulsions-based gp100 peptide and hgp100 plasmid DNA vaccine formulations was evaluated by monitoring animal body weight, serum LDH levels, serum ALT levels, and histo-pathological characterization of excised liver tissues from mice in the test group and comparing with that of mice in the control groups. An overt systemic toxicity due to immunotherapy may result in an abrupt change or loss in body weight and increased serum LDH or ALT levels.

8.3.1 Change in Animal Body Weight

This parameter was measured during each in vivo immunization effectiveness evaluation. The mean ratios of body weights as a function of time for the mice in test and the control groups are shown in Figure 35 and Figure 36. The results demonstrated that the body weight of the animals in all treatment groups increased during immunization and during tumor challenge phase. Thus, the results demonstrated that squalane oil-containing W/O/W multiple emulsions-based vaccine was well tolerated and does not cause noticeable overt systemic toxicity that results in abrupt weight loss.

8.3.2 Serum LDH Levels

The lactate dehydrogenase (LDH) is an enzyme involved in conversion of pyruvate into lactate. It is present in many vital organs such as heart, liver, kidney, muscles, brain, blood cells, and lungs and therefore it is most often measured to examine damage to these organs. Serum LDH levels in the blood samples from gp100 peptide and
Figure 35: Preliminary tolerability of the gp100 peptide vaccine formulation.
The preliminary tolerability of vaccine formulations was evaluated by monitoring body weight of the animals in all groups every week. The mean ratio of body weight to the initial body weight of all animals in five treatment groups: (1) saline control (●), (2) W/O IFA emulsion control (♦), (3) gp100 in W/O IFA emulsion (■), (4) W/O/W squalane oil multiple emulsions (SME) control (●), and (5) gp100 in W/O/W SME (▲) in prophylactic (a) and active (b) treatment modes are shown. Results are presented as mean ± SD, n = 8.
The preliminary tolerability of vaccine formulations was evaluated by monitoring body weight of the animals in all groups every week. The mean ratio of body weight to the initial body weight of all animals in six treatment groups: (1) saline control (●), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (○), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (♦), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (◊), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (▲), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (Δ) are shown. Results are presented as mean ± SD, n = 8.
hgp100 plasmid DNA vaccinated and control mice were measured using the QuantiChrom™ Lactate Dehydrogenase Kit and are shown in Figure 37.

Results demonstrated similar levels of serum LDH before and after immunization indicating minimal organ damage due to vaccination. However, a remarkable elevation in the serum LDH levels was observed in tumor-bearing mice\textsuperscript{116} that are expected due to nature of pathophysiology of the cancer causing damage to several organs. More importantly, difference between the serum LDH levels among vaccinated and control mice were similar across different time points. Thus, the serum LDH levels analysis indicated that the W/O/W multiple emulsions-based the gp100 peptide and hgp100 plasmid DNA vaccine is well tolerated and does not cause systemic organ damage that results in increased blood LDH levels.

8.3.3 Serum ALT Levels

The alanine transaminase (ALT) is an enzyme that catalyzes transfer of the amino group from L-alanine to α-ketoglutarate resulting in the formation of pyruvate and L-glutamate. The highest amounts of ALT is found in the liver. Therefore, injury to the liver results in release of the enzyme into the blood. Serum ALT levels in the blood samples from gp100 peptide and hgp100 plasmid DNA vaccinated and control mice were measured using the ALT (SGPT) reagent set and are shown in Figure 38.
Figure 37: Serum LDH Activity Levels in the gp100 Peptide and hgp100 Plasmid DNA vaccinated mice.

The serum LDH levels were quantitatively measured by the QuantiChrom™ Lactate Dehydrogenase Kit. The LDH levels in the sera from animals in six treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (pink) are shown. Results are presented as mean ± SD, n = 8.
Results demonstrated similar levels of serum ALT before and after immunization indicating minimal liver damage due to vaccination. However, a remarkable elevation in the serum ALT levels was observed in tumor-bearing mice that are expected due to nature of pathophysiology of the cancer causing damage to several organs. More importantly, difference between the serum ALT levels among vaccinated and control mice were similar across different time points. Thus, the serum ALT levels analysis indicated that the W/O/W multiple emulsions-based the gp100 peptide and hgp100 plasmid DNA vaccine is well tolerated and does not cause liver damage that results in increased blood ALT levels.

8.3.4 Liver Histo-Pathology

Excised liver tissues were cut into 5 µm sections and stained with hematoxylin/Eosin followed by histo-pathological characterization to investigate liver damage due to immunization using the W/O/W multiple emulsions based vaccine delivery. Digital images of the liver sections from five treatment groups: 1) saline control, (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine), and (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) were captured and are shown Figure 39.
Figure 38: Serum ALT Activity Levels in the gp100 Peptide and hgp100 Plasmid DNA vaccinated mice.

The serum ALT levels were quantitatively measured by the ALT (SGPT) reagent set. The ALT levels in the sera from animals in six treatment groups left to right (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink), and (6) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions with caffeine co-administration (hgp100 plasmid in W/O/W ME + Caffeine) (pink) are shown. Results are presented as mean ± SD, n = 8.
Figure 39: Histo-Pathological Characterization of Excised Liver Tissues
Structural integrity of the excised liver tissues were investigated by staining with hematoxylin/Eosin followed by histo-pathological analysis to determine liver damage due to vaccination. The liver tissue sections from animals in five treatment groups top to bottom (color mentioned in the parenthesis refers to the frame color in the figure): (1) saline control (blue), (2) placebo the W/O/W multiple emulsions (Placebo W/O/W ME) (blue), (3) gp100 peptide encapsulated the W/O/W multiple emulsions (gp100 peptide in W/O/W ME) (purple), (4) gp100 peptide encapsulated the W/O/W multiple emulsions with caffeine co-administration (gp100 peptide in W/O/W ME + Caffeine) (purple), and (5) hgp100 plasmid DNA encapsulated the W/O/W multiple emulsions (hgp100 plasmid in W/O/W ME) (pink) are shown.
The liver tissues from the all treatment groups demonstrated moderately diffuse vacuolation of hepatocytes that may be due to freeze-thaw cycle artifact. Moderate multifocal and random extramedullary hematopoiesis (back arrow heads in the images) was observed in livers from all the treatment groups. In addition, multifocal periportal and random aggregates of neutrophils, band neutrophils and round cells were observed that is due to extramedullary hematopoiesis. The extramedullary hematopoiesis observed is considered as an incidental finding and is not due to potential toxicity of the vaccine formulations. Histo-pathological characterization indicated that tissues examined in this study are regarded as within normal limits of structural integrity. Thus, excised liver histo-pathological characterization demonstrated that the W/O/W multiple emulsions based gp100 peptide and hgp100 plasmid DNA vaccines are well tolerated in vivo.

8.4 Conclusions

The W/O/W multiple emulsions-based gp100 peptide and hgp100 plasmid DNA vaccines are well tolerated and does not cause overt systemic toxicity that result in an abrupt change or loss in body weight or increased serum LDH or increased serum ALT levels or damage to structural integrity of livers.
CONCLUDING REMARKS

A significant challenge amidst therapeutic strategies against cancer is to precisely target tumor cells while minimizing collateral damage to normal tissues. Immunotherapy in cancer treatment has potential to accomplish this clear and critical need due to inherent biologic specificity of the immune system. Identification and characterization of melanoma-associated antigens have shifted a treatment paradigm from conventional therapy to immunotherapy for treatment and prevention of melanoma. The vaccine delivery systems containing adjuvants modulate magnitude, breadth, quality, and longevity of immune responses to antigen vaccinations aimed to induce cell-mediated immunity. A current approach to melanoma immunotherapy involves administration of the tumor specific antigens emulsified in the IFA to enhance an immune response. Despite its effectiveness, use of IFA in vaccine formulations imposes additional challenges such as severe adverse reactions and poor injectability. Therefore, it is desirable to develop a safe and effective delivery system for melanoma vaccines to induce enhanced anti-tumor immunity. The W/O/W multiple emulsions have potential to enhance an immune response due to reservoir effect of an antigenic peptide incorporated in the inner aqueous-phase. In addition, being a particulate delivery system it is internalized by immune cells thereby transfecting them with encapsulated hgp100 plasmid DNA to produce encoded protein antigen endogenously. Moreover, multiple emulsions offer notable advantages of improved tolerability, low viscosity, and ease of injection. The objective of this doctoral thesis project was to develop the W/O/W multiple emulsions-based multi-compartmental vaccine delivery system for safe and
efficient delivery of variety of melanoma antigens (peptide and hgp100 plasmid DNA) for immunotherapy.

The W/O/W multiple emulsions-based vaccine delivery system containing the melanoma antigens was prepared by a two-step emulsification method. The two-step emulsification method was reproducible and created stable, three-phase system comprised of the melanoma antigens encapsulated in homogenously dispersed oil-droplets. The dispersed oil-droplets were discrete and spherical with about 2 µm diameter size and about -40 mV surface charges. More importantly, the newly developed vaccine delivery system was stable for up to 8 weeks when stored at refrigerated condition. The W/O/W multiple emulsions-based melanoma vaccine offers improved injectability compared to IFA-based vaccine. Immunization of C57BL/6 mice using the novel W/O/W multiple emulsions encapsulated gp100 peptide or hgp100 plasmid DNA antigen vaccines evoked antigen-specific cell-mediated antitumor immunity that subsequently provided protection against melanoma tumor challenge. In particular, immunization with the W/O/W multiple emulsions encapsulated gp100 peptide antigen vaccine resulted in substantially increased protection against tumor challenge compared to IFA encapsulated gp100 peptide vaccine. Thus, it was concluded that the W/O/W multiple emulsions-based gp100 peptide vaccine enhanced immune responses against antigen compared to currently used IFA. Specific comparison of immune responses provoked by gp100 peptide and hgp100 plasmid DNA was not performed. However, gp100 peptide or hgp100 plasmid DNA vaccination using the W/O/W multiple emulsions resulted in significantly increased protection against tumor challenge signifying that the novel vaccine formulation efficiently delivers variety of melanoma antigens. Moreover,
squalane oil-containing the W/O/W multiple emulsions-based vaccine formulations were well tolerated \textit{in vivo}. Further enhancement of immune responses by preventing adenosine-mediated immune-suppression was investigated by concurrent administration of adenosine $A_2A$ receptor antagonist such as caffeine. A considerable increase in protection against tumor challenge due to co-administration of caffeine with gp100 peptide or hgp100 plasmid DNA immunization was not observed. Therefore, adjunct therapy using potent adenosine $A_2A$ receptor antagonists such as ZM-241385 or Istradefylline (KW-6002) is recommended to validate the hypothesis.

In summary, the W/O/W multiple emulsions-based vaccine formulation efficiently delivers the gp100 peptide and hgp100 plasmid DNA antigens to evoke cell-mediated anti-tumor immunity and protects C57BL/6 mice against subsequent tumor challenge. Thus, the novel multi-compartmental vaccine delivery system offers an alternate, safe antigen delivery for melanoma immunotherapy.
REFERENCES


