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RESEARCH PAPER

# Multi-Compartmental Vaccine Delivery System for Enhanced Immune Response to gp100 Peptide Antigen in Melanoma Immunotherapy

Mayurkumar Kalariya • Srinivas Ganta • Mansoor Amiji

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## ABSTRACT

***Purpose*** To develop a multi-compartmental vaccine delivery system for safe and efficient delivery of the gp100 peptide antigen in melanoma immunotherapy.

***Methods*** Water-in-oil-in-water (W/O/W) multiple emulsion-based multi-compartmental vaccine delivery system containing the gp100 peptide was prepared by a two-step emulsification method. *In vivo* prophylactic and active immunization effectiveness of the novel squalane oil-containing gp100 vaccine was evaluated in the murine B16 melanoma model and compared with that of an incomplete Freund's adjuvant (IFA)-based vaccine.

***Results*** Morphological evaluation of the W/O/W multiple emulsions showed that the oil-droplets were homogenously dispersed with the gp100 peptide encapsulated in an inner aqueous phase. Immunization with the gp100 peptide delivered in the W/O/W multiple emulsions-based vaccine resulted in increased protection against tumor challenge compared to IFA-based vaccine ($p < 0.05$, $n=8$) signifying induction of enhanced anti-tumor immunity. In addition, serum Th1 cytokine levels and immuno-histochemistry of excised tumor tissues indicated activation and local infiltration of antigen specific cytotoxic T-lymphocytes into and/or surrounding the tumor mass. Moreover, the newly developed vaccine formulation did not induce any overt systemic toxicity.

***Conclusion*** Novel W/O/W multiple emulsions-based vaccine efficiently delivers the gp100 peptide antigen to induce cell-mediated anti-tumor immunity and offers an alternate, safe vaccine delivery system.

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**KEY WORDS** anti-tumor immunity · cancer vaccine · gp100 peptide antigen · melanoma · W/O/W multiple emulsions

## ABBREVIATIONS

| | |
|---|---|
| APCs | antigen-presenting cells |
| CTLs | cytotoxic T-lymphocytes |
| IFA | incomplete Freund's adjuvant |
| PBS | phosphate-buffered saline |
| SME | squalane oil multiple emulsions |
| W/O/W | water-in-oil-in-water |

## INTRODUCTION

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, adjuvants are necessary to overcome various tolerance mechanisms and facilitate induction of cytotoxic T lymphocytes (CTLs) that can traffic to and lyse malignant cells (1). Particulate adjuvants as an alternative to immunostimulatory adjuvants have been investigated by several groups (2, 3). The particulate adjuvants have comparable dimensions to the pathogens; therefore, they are naturally targeted for uptake by antigen presenting cells (APCs) leading to potent immune responses. The multi-compartmental W/O/W multiple emulsions-based vaccine delivery systems have potential to enhance the immune responses due to: (1) the reservoir effect of an immunogen incorporated in the inner aqueous phase and (2) a particulate adjuvant effect of the oil-droplets (4–6). Squalene oil-based formulations are internalized by APCs at the site of injection (7) and demonstrated potent vaccine adjuvant effects in preclinical and clinical evaluations (8, 9). Squalane is a fully saturated analog prepared by hydrogenation of squalene (10), and

therefore, possesses increased stability against auto-oxidation. Consequently, biodegradable squalane oil-containing W/O/W multiple emulsions-based vaccine delivery system offer an efficient antigen delivery, improved tolerability, and notable ease of injection mainly due to low viscosity of the formulation.

Melanoma exhibits an inherent immunogenicity; consequently immunotherapy is suitable for prevention of tumor growth, metastasis, and relapse (11). 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 (12). A 9-amino acid epitope of gp100 protein, $gp100_{25-33}$ (KVPRNQDWL) is restricted by H-$2D^b$ (13, 14) and, therefore, stimulates potent cell-mediated anti-tumor immunity. Antigen-specific CTLs play an important role in anti-tumor immunity (15, 16) for destruction and eradication of a growing tumor. To accomplish this, the vaccine delivery system should: (1) recruit APCs such as dendritic cells and macrophages at the site of injection and (2) efficiently deliver the encapsulated pay load to APCs to induce an antigen-specific immune response. This can be achieved by the superior vaccine delivery systems containing efficient adjuvants to provoke APCs. Currently, gp100 peptide emulsified in incomplete Freund's adjuvant (IFA) is used in melanoma immunotherapy (17). It contains a mixture of mineral oil and surfactant, mannide monooleate that creates a W/O emulsion when emulsified with aqueous antigen solution. It 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 the melanoma immunotherapy.

In this study, we have investigated *in vivo* effectiveness of squalane oil-containing W/O/W multiple emulsions-based gp100 peptide vaccine following prophylactic and 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 vaccine 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.

## MATERIALS AND METHODS

### Materials

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.

### Preparation and Characterization of Vaccine Formulation

#### *gp100-Containing W/O/W Multiple Emulsion-Based Vaccine*

The W/O/W multiple emulsion-based gp100 peptide vaccine formulation was prepared by two-step emulsification method as previously described by Okochi (6, 18) for water soluble payload. Squalane oil was used as oil phase of the W/O/W multiple emulsions. 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 min. 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 min to produce the W/O/W multiple emulsions. Dispersed oil-droplets size and surface charge for three different preparations was determined using Zetasizer ZS instrument (Malvern Instruments Ltd, UK). To ascertain the W/O/W phase configuration, the internal aqueous-phase and the dispersed oil-phase were stained using a water-soluble dye, Evans blue and an oil-soluble dye, Sudan red 7B followed by morphological characterization by microscopy using an Olympus microscope (Olympus America, Inc., Chelmsford, MA).

#### *IFA-Containing W/O Emulsion-Based Vaccine*

The IFA-containing W/O emulsion-based vaccine formulation was prepared by homogenization method. 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.

#### *Determination of Vaccine Formulation Viscosity and Syringe Glide Force*

Viscosity of the vaccine formulations was measured using the Kinexus-pro® rotational rheometer (Malvern Instruments Ltd, UK), which was installed with a 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 syringe plunger to drive 25 mm distance at 100 mm/min speed while injecting the vaccine formulations through 1 mL syringe with 27 G needle was measured using an Instron (Model: 5565, Instron, Norwood, MA) fitted with T101320-1002 syringe testing fixture.

## Evaluation of *In Vivo* Immunization Effectiveness

### *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 prior to any experimental procedure.

### *Immunization and Tumor Challenge*

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 (17) 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 (19, 20) suspended in 0.1 mL PBS on the hind flank of mice. 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 IACUC guidance, mice were euthanized when tumor volume in control animals 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. Tumor masses were recorded; representative images were captured, and cryo-preserved for the immuno-histochemical analysis.

### *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 (19, 21): $V = 1/2\left[L \times (W)^2\right]$; 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 = (mean time for tumors to grow from 200 $mm^3$ to 800 $mm^3$)$_{\text{vaccine treated group}}$ - (mean time for tumors to grow from 200 $mm^3$ to 800 $mm^3$)$_{\text{saline treated control group}}$.

### *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 (22, 23) such as IL-2, IL-12, INF-γ, and TNF-α 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, Logan, UT). The Th1 cytokine levels in each sample were calculated using the standard curve prepared following the instructions.

### *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 (Olympus America, Inc., Chelmsford, MA).

#### *Preliminary Tolerability of Vaccine Formulations*

The preliminary tolerability of vaccine formulation was evaluated by monitoring body weight of the animals in all treatment groups every week. The mean ratio of body weight to the initial body weight of all animals in the vaccinated group was determined and compared with that of the animals in the control groups. An abrupt change or loss in the animal body weight may indicate overt systemic toxicity due to immunotherapy.

### 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.

## RESULTS

### Vaccine Formulation and Characterization

#### *Squalane Oil-Containing W/O/W Multiple Emulsions-Based Vaccine*

The two-step emulsification method shown in Fig. 1 created the W/O/W multiple emulsions comprised of distinct and stable three-phase system. Morphological evaluation of the W/O/W multiple emulsions, shown in Fig. 1, demonstrate that the oil-droplets were discrete, round, and dispersed in outer aqueous-phase. In addition, staining of the W/O/W multiple emulsions using water-soluble and oil-soluble dyes ascertained that it contained an internal aqueous-phase (1) encapsulating, dispersed oil-phase (2) stabilized in an outer aqueous phase (3). The dispersed oil-droplets hydrodynamic diameter was $1.6 \pm 0.2$ μm (mean ± SD, $n=3$) and coefficient of variance for polydispersity index value was 0.4 ($n=3$). The surface charge on the dispersed oil-droplets was $-37.9 \pm 1.1$ mV (mean ± SD, $n=3$).

#### *Vaccine Formulation 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 Fig. 2 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 W/O IFA based vaccine formulation. In addition, force required for vaccine formulations to inject through 1 mL syringe with 27 G needle (syringe glide force) is shown in Fig. 2. 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 W/O IFA based vaccine formulation.

### *In Vivo* Prophylactic and Active Immunization Effectiveness

In this study, we examined ability of the W/O/W multiple emulsions-based gp100 peptide vaccine to induce protective cell-mediated antitumor immunity and compared with that of IFA-based gp100 peptide vaccine using murine B16 melanoma model. *In vivo* prophylactic and active immunization protocols are illustrated in Fig. 3.

#### *Protection Against Tumor Challenge*

Mean tumor volume and tumor growth delay time were determined to evaluate protection against tumor challenge. Mean tumor volume growth curves for five treatment groups:

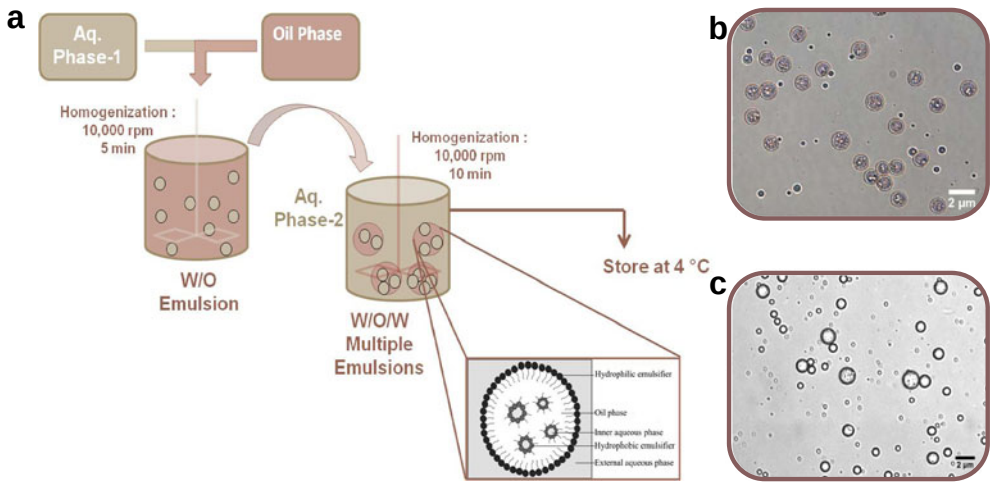


**Fig. 1** Two-step emulsification method and the bright-field images of the water-in-oil-in-water (W/O/W) multiple emulsion system. (**a**) The W/O/W multiple emulsions-based vaccine formulation was prepared by two-step emulsification method. The first emulsification step involved homogenization of 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 gp-100 peptide antigen.

(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 and active immunization are shown in Fig. 3. In addition, mean tumor mass and a representative picture of the excised tumor tissue from each group are shown in Fig. 4. The results demonstrated that prophylactic and active immunization using gp100 peptide vaccine formulations resulted in significant tumor growth suppression compared to immunization with control formulations. Moreover, 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. Similarly, 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 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)

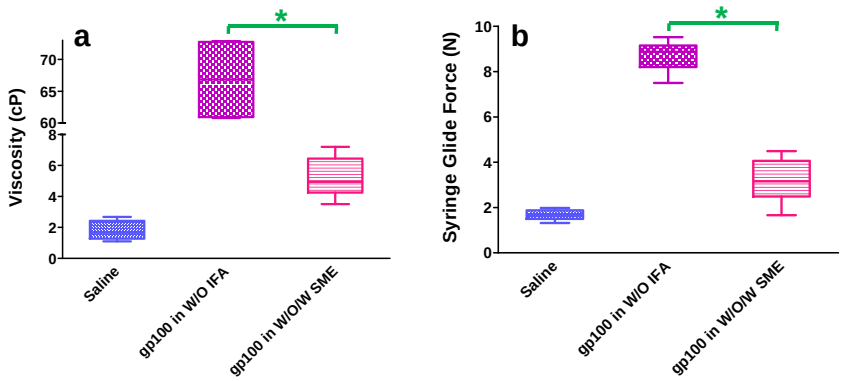


**Fig. 2** Vaccine formulations injectability comparison. (**a**) Viscosity and syringe glide force were used to compare injectability of the vaccine formulations: (1) saline (*blue*), (2) gp100 in W/O IFA emulsion (*purple*), and (3) gp100 in W/O/W SME (*pink*). Viscosity was measured at 25°C and shear 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 ± SD, $n=3$ (*$P < 0.05$). (**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 27 G needle was measured using Instron fitted with T101320-1002 syringe testing fixture. Results are presented as mean ± SD, $n=8$ (*$p < 0.05$).

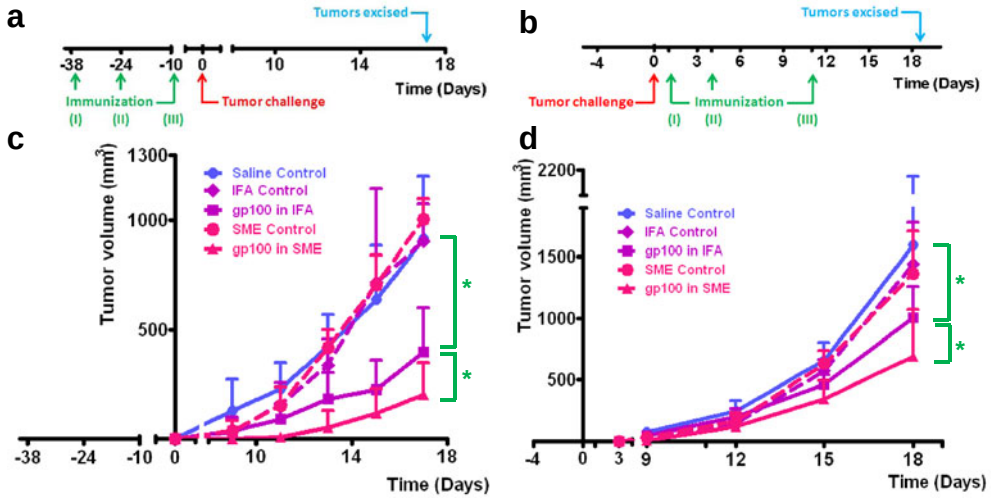


**Fig. 3** *In vivo* immunization protocol and vaccine formulation effectiveness. Five groups of mice were treated with: (1) saline control (●), (2) W/O IFA emulsion control (♦), (3) gp100 in W/O IFA emulsion (■), (4) W/O/W squalane oil multiple emulsions (SME) control (●), and (5) gp100 in W/O/W SME (▲) 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 10 days after the last injection tumor challenge was initiated. (**b**) 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: $V = 1/2\left[L \times (W)^2\right]$; where L is the longest dimension and W is the dimension perpendicular to the L. Tumor growth curves for prophylactic (**c**) and active (**d**) treatment modes. Results are presented as mean ± SD, $n=8$ ($^*p < 0.05$).

due to immunization were determined. Prophylactic and active immunization using W/O/W multiple emulsions-based gp100 peptide vaccine resulted in corresponding 6-fold and 2-fold increase in tumor growth delay time compared to IFA-based gp100 peptide vaccine. Thus, tumor growth delay times suggested immunotherapy using the W/O/W multiple emulsions-based gp100 peptide vaccine increased protection against melanoma tumor challenge in C57BL/6 mice.

### *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 determine induction of CD4+ and CD8+ 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

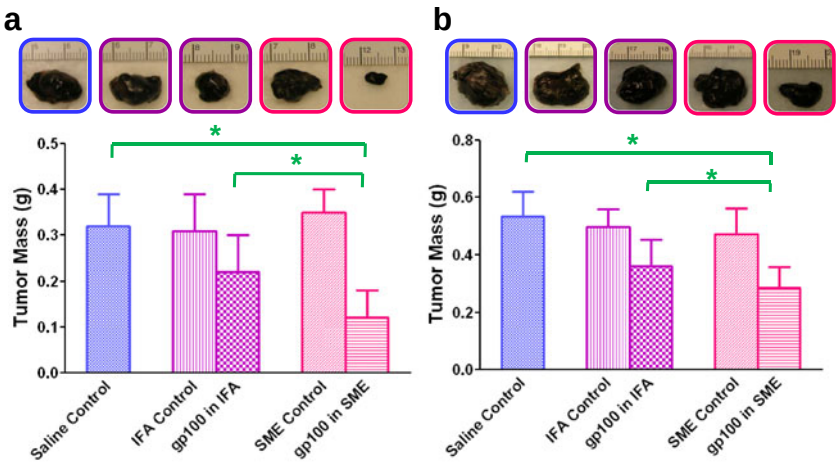


**Fig. 4** Tumor mass and a representative picture of excised tumors. Animals were euthanized and tumor tissues were excised when mean tumor volume for saline treated group of mice reached to 1,000 $mm^3$. Excised tumors were freed of skin remnants, rinsed in phosphate buffered saline and dried on blotting paper. The mass and a representative picture of excised tumors from five treatment groups: (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 (**a**) and active (**b**) treatment modes are shown. Results are presented as mean ± SD, $n=8$ ($^*p < 0.05$).

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 and active immunization are shown in Fig. 5. 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.

### *Immuno-Histochemical Staining of Tumor Tissue*

Immuno-histochemical staining on excised tumor tissue was performed to investigate the activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site. 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 Fig. 6. Immuno-staining revealed presence of increased number of both CD4+ and CD8+ T-cells in tumor surrounding and/or within tumor tissue from the mice immunized using gp100 peptide vaccine. Thus, tumor tissue immuno-histochemistry demonstrated activation and increased local infiltration of CD4+ and CD8+ T-cells surrounding tumor site providing evidence for involvement of cell-mediated antitumor immunity that might have protected mice against tumor challenge.

### Preliminary Tolerability of Vaccine Formulations

The preliminary tolerability of the W/O/W multiple emulsions-based gp100 peptide vaccine formulation was determined by comparing the mean ratio of body weight to the initial body weight of mice in immunized groups with that of mice in control formulation treated groups. An overt systemic toxicity due to immunotherapy may result in an abrupt change or loss in body weight. The mean ratios of body weights as a function of time for the mice in immunized and control formulation treated groups are shown in Fig. 7. The results demonstrated that the body weight of the animals in all treatment groups increased during immunization and tumor challenge phase. Thus, the results indicated that squalane oil-containing W/O/W multiple emulsions-based vaccine was well tolerated and did not cause noticeable overt systemic toxicity that might result in abrupt weight loss.

## DISCUSSION

The objectives of this vaccine delivery system development were 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 itself is not an immune-adjuvant, therefore 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 (24, 25). Consequently, squalane oil-

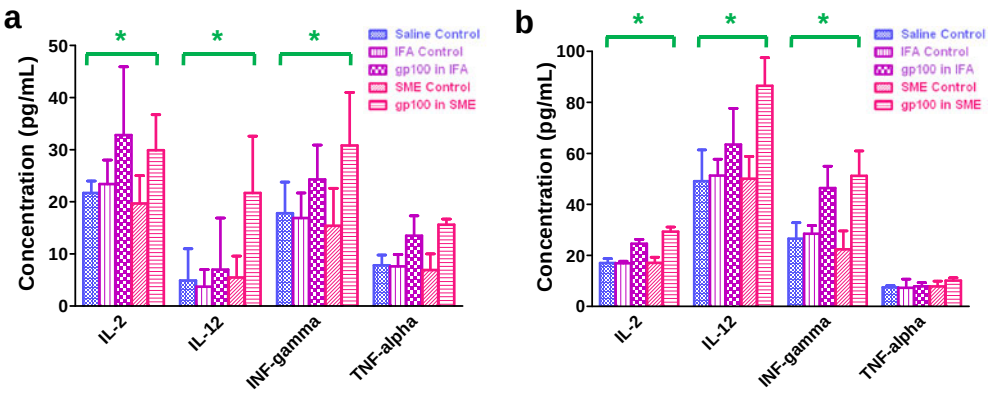


**Fig. 5** Th1 cytokine levels in the serum samples. Immediately after animal euthanasia the blood samples were collected and allowed to clot for about 30 min at 4°C followed by centrifugation to separate the serum. The Th1 cytokines such as IL-2, IL-12, INF-γ, and TNF-α level in the sera were quantitatively measured by enzyme-linked immunosorbent assay (ELISA) using Q-Plex™ array. The Th1 cytokine levels in the sera from five treatment groups: (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 (**a**) and active (**b**) treatment modes are shown. Results are presented as mean ± SD, $n=8$ (*$p<0.05$).

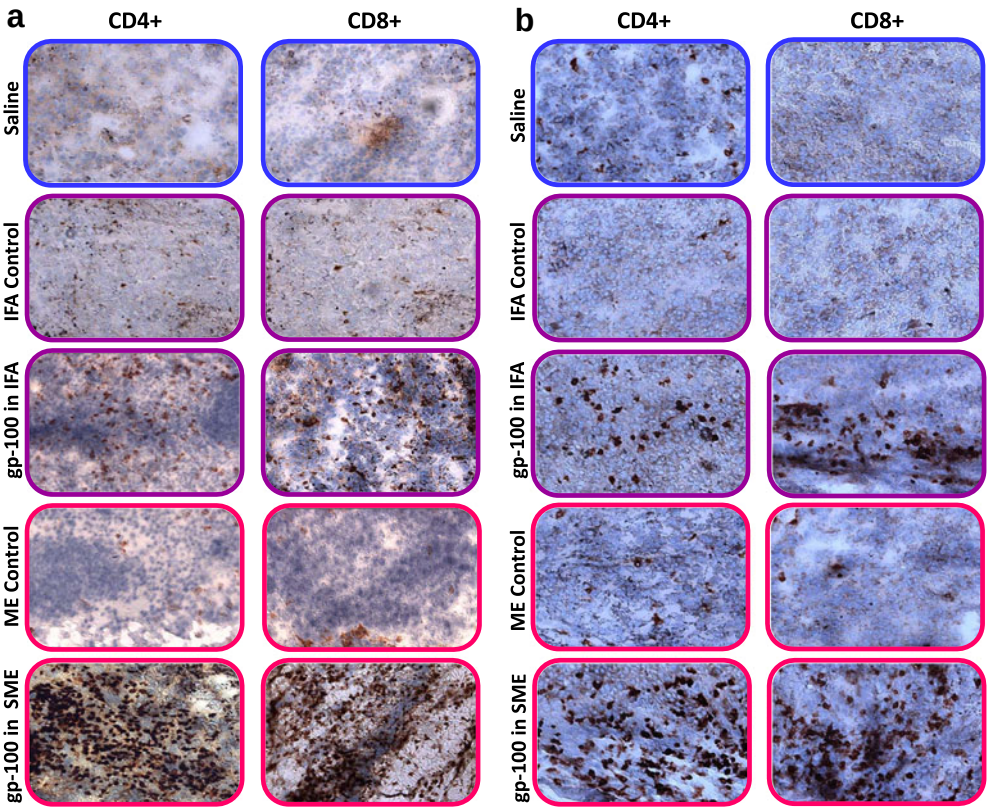


**Fig. 6** Tumor tissue immunohistochemistry. Activation and local infiltration of antigen-specific CD4+ and CD8+ T-cells at the tumor site was investigated by immuno-histochemical staining of excised tumor tissues. Tumor tissues were cryo-sectioned (6–8 $\mu$ thickness) and incubated with anti-CD4 or 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: (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 (**a**) and active (**b**) treatment modes are shown.

containing the W/O/W multiple emulsions-based multi-compartmental vaccine delivery systems offer a combined antigen reservoir effect and particulate adjuvant effect; as a result they have potential to enhance the immune responses and induce long-term immunity. In addition, due to their low viscosity the W/O/W multiple emulsions offer improved injectability.

The W/O/W multiple emulsions-based vaccine was prepared by two-step emulsification process (6, 18) wherein the first emulsification step involved homogenization of gp100 peptide 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 outer aqueous-phase containing Pluronic® to form the W/O/W multiple emulsions. Notably, the two-step emulsification method was reproducible as indicated by the low coefficient of variance for polydispersity index value and created stable W/O/W multiple emulsions. Staining of the inner aqueous-phase and the oil-phase using Evan's blue and Sudan red 7B dyes respectively and subsequent morphological characterization revealed blue-colored aqueous globules enclosed in

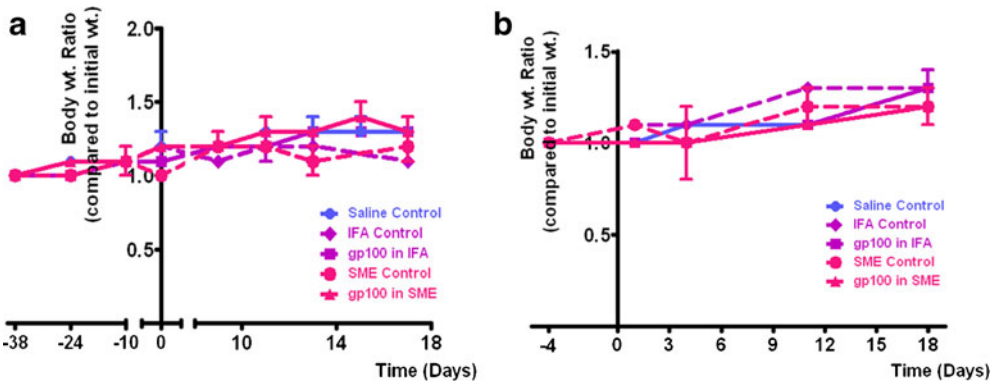


**Fig. 7** Preliminary tolerability of the gp-100 peptide vaccine formulation. The preliminary tolerability of vaccine formulations was evaluated by monitoring body weight of the animals in all groups every week. The mean ratio of body weight to the initial body weight of all animals in five treatment 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 (▲) are shown. Results are presented as mean ± SD, $n$=8.

pink-colored oil-droplets that were stabilized in clear continuous aqueous-phase. Thus, three-phase configuration of water-in-oil-in-water (W/O/W) multiple emulsions was ascertained. In addition, *in vitro* characterizations showed that the dispersed phase of the W/O/W multiple emulsions contain discrete, spherical oil-droplets with about 1–2 μm size and about −40 mV surface charges.

Cell-mediated immune responses such as CD4+ and CD8+ CTLs have a vital role in the immunologic rejection of growing tumors (26). Upon immune response stimulation primed CD8+ T-cells infiltrate into tumor stroma, identify tumor-antigens, and release cytotoxins such as perforin and granulysin that form pores in the target tumor cell membrane. The pore formation allows ions and water influx and cause cell burst or lysis (27). Also, CD8+ T-cells release granzyme, a serine protease that enters cells *via* pores to induce apoptosis (cell death) through Fas-Fas ligand (FasL) pathway (28–30). In addition, CD4+ T-cells secrete cytokines that aid to immune rejection of tumor cells (31–33). Consequently, it is critical to induce antigen-specific, anti-tumor T-cell functions for an effective cancer immunotherapy.

In this study, we explored activation of cell-mediated immunity and corresponding tumor growth suppression as a measure of the W/O/W multiple emulsions-based gp100 peptide vaccine *in vivo* effectiveness. The vaccine effectiveness in both prophylactic and active immunization treatments was investigated in murine B16 melanoma model. Moreover, 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 vaccine. Our study results demonstrated that *in vivo* prophylactic and active immunization of C57BL/6 mice using gp100 peptide vaccine formulations resulted in substantial protection against B16-F10 melanoma tumor challenge. More importantly, in both treatment modes gp100 peptide vaccination using the newly developed vaccine delivery system, 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 and 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.

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-cells (31) and TNF-α can induce DC-mediated tumor antigen recognition (34). In addition, IL-2 and IL-12 are key cytokine to induce Th1 cell differentiation and enhance Th1 function (35–37). To investigate CD4+ and CD8+ T-cell function, a panel of cytokines in sera from immunized mice was quantified and compared with that from control formulation treated mice. The levels of IL-2, IL-12, and INF-γ were significantly higher in the W/O/W multiple emulsions-based gp100 peptide vaccine treated mice than that from saline treated mice in prophylactic and active immunization. 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*.

Immunologic eradication of established tumors depends on three criteria (38): (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 (39, 40). Immuno-staining of the tumor tissues demonstrated that prophylactic and 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. Thus, tumor tissue immuno-histology indicated 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.

The gp100 peptide vaccine formulation preliminary tolerability assessment was made by monitoring animal body weight during immunization and tumor challenge phase. Vaccine formulation preliminary tolerability evaluation suggested that the W/O/W multiple emulsions prepared using squalane oil was well tolerated and did not cause overt systemic toxicity that may result in abrupt weight loss.

## CONCLUSIONS

Squalane oil-containing W/O/W multiple emulsions-based multi-compartmental vaccine delivery system is developed for safe and efficient delivery of the gp100 peptide in melanoma immunotherapy. The two-step emulsion method is capable to prepare stable and reproducible gp100 peptide encapsulated W/O/W multiple emulsions formulation. Prophylactic and active immunization of C57BL/5 mice in a murine B16 melanoma model, use of the newly developed vaccine formulation resulted in enhanced immune response to melanoma antigen, gp100 peptide, compared to conventional immunotherapy. Our study demonstrates that the W/O/W multiple emulsions-based vaccine delivery systems can efficiently

deliver the peptide antigens and induce enhanced cytotoxic T-cell mediated anti-tumor immunity. The future goal for this vaccine delivery system is to extend the delivery application to a variety of immunogens, including plasmid DNA- and tumor exosomes-based cancer vaccines.

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