A Nano-Enhanced Vaccine for Metastatic Melanoma Immunotherapy

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Individual Technical Project

On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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1. Abstract

Many metastatic melanoma patients do not respond to the immunotherapies currently available. This technical project aims to address the gap in available metastatic melanoma treatments offering both efficacy and an improved quality of life while undergoing therapy. This project develops and tests cancer vaccines, a method of immunotherapy, enhanced with nanotechnology for treatment of metastatic melanoma. Immunogenic, nano-sized liposomes formulated previously by the author are characterized and employed in encapsulation of tumor-associated antigens, peptides uniquely expressed on cancer cell surfaces, identified by Slingluff *et al*. for metastatic melanoma (2008). Several central aims are met in this research: 1) nanoliposome stability property characterization, 2) *in vitro* T cell activation nanoliposome testing, and 3) *in vivo* mouse testing for nanoliposome biodistribution and immunogenicity. The nanoliposome vaccine ("nanovaccine") is characterized for factors including stability of nanoparticle shape and dispersity in solution, peptide release kinetics, surface charge, and peptide encapsulation efficiency. The characterized nanoliposomes are tested *in vitro* in white blood cell cultures derived from human peripheral blood and lymph node samples from patients immunized with the peptides alone in a 2008 clinical trial. The T cell response is measured through flow cytometry following nanovaccine stimulation and incubation and is compared to the response from isolated peptide treatment, which does not use a nanotechnology approach. For *in vivo* assessment, the nanovaccine is tested in a BALB/c mouse model for nanovaccine biodistribution and immunogenicity. The in vitro studies demonstrate significant evidence of increased CD4 T cell proliferation in

response to the unique combination of the nanoliposome "container," the MHP held inside of the nanoliposomes, and the surface bound KDO2 immunogen. The results of the *in vivo* testing suggest that the KDO2-nanoliposomes enhance the antibody formation against the helper peptides in a mouse when compared to the free peptide administration for IV treatment injection. Finally, the stability of the combined nano-vaccine mixture is demonstrated over time by measures of resistance to nanoliposome aggregation and peptide leakiness.

2. Introduction

Melanoma of the skin makes the top ten list for most common cancers in the United States, and approximately one out of fifty Americans will develop this cancer in their lifetime (*About Melanoma*, 2018). Metastatic stages of melanoma are aggressive and pose significant risk to one's mortality, but recent immunotherapy approaches shown promising improvement in even the advanced stage metastatic malignant melanoma five year survival rate (Lim et al., 2019). Immunotherapy is a treatment approach that enhances one's own immune system to better recognize and mount a response against cancerous cells in the body, which normally evade detection (Abdel-Wahab et al., 2016; Alsaab et al., 2017).

Many metastatic melanoma patients, however, do not respond to the immunotherapies currently available (Dance, 2017 ; Lim *et al*., 2019). These patients do not get the additional protection against cancer recurrence that immunotherapies used in adjuvant therapy offer, nor the side effect reduction and improved quality of life (Rudmann, 2012). Therefore, there is a significant need for new, innovative forms of immunotherapy to help the large population of melanoma patients unresponsive to current treatments. An approach that can be applied to immunotherapy and address some of the reasons for failure of otherwise hopeful treatments is

that of nanomedicine. Specifically, cancer vaccines are a promising immunotherapy method that suffer from issues of low circulation half-life because of swift clearance by the body, and a lack of coordination between the peptide antigens that need to interact with dendritic cells and the TLR agonist that is needed to activate the dendritic cells. Use of nanoliposomes, which are nano-sized vesicles used for drug delivery and other nanomedical applications, for encapsulating the melanoma-signaling peptide antigen and delivering a stimulatory signal from a surface conjugated immunogen has properties that offer a solution to the challenges faced by free peptide cancer vaccines (Nakagawa & Ebara, 2018; Truskey, Yuan, & Katz, 2010).

3. Background

While melanoma has encouraging survival rates, this cancer becomes far more challenging for treatments to address once it becomes metastatic. The spread of melanoma to distant sites on the body characterizes metastasis, and this occurrence of disease progression marks the advanced stages and a 5-year survival rate drop by as much as 50% (*Survival Rates of Skin Cancer*, 2016). At the metastatic stages, surgery alone cannot treat the cancer (*Treatment of Skin Cancer*, 2018). Further, the painful and distressing symptoms of traditional cancer treatments like chemotherapy and radiation therapy motivate the development of a treatment using nanomedical approaches, which are shown to reduce the side effects experienced from off-target drug interactions (Gu *et al*., 2007; Rudmann, 2012; Shin, Song & Um, 2015). One nanomedical approach that has successfully made its way into the clinic for cancer treatment is the employment of nanoliposomes (Gabizon, Shmeeda & Barenholz, 2003). Use of nano-sized liposomes offers a host of flexibility and benefits to the development of a cancer treatment (Fan & Moon, 2015). For instance, the ability to chemically modify the exterior surface of a liposome

allows for the addition of targeting properties that enhance liposome delivery and stealth properties that increase biocompatibility and circulation time in the body (Cisterna *et al.*, 2016; Tassa *et al.*, 2010; Tiet & Berlin, 2017).

While the immunotherapies that are in the clinic currently for treatment of melanoma are checkpoint inhibiting antibodies and one oncolytic virus, the method of immunotherapy that this research used is that of a cancer vaccine. Cancer vaccines introduce peptides that have been identified as uniquely expressed in high concentrations on the cancer cells' surface to the body's immune system to aid in cancer cell recognition and immune response mounting (Klyushnenkova & Alexander, 2012; Kreuter, 1995). This is the idea behind the cancer vaccines developed by the Slingluff research group at UVA (Slingluff, *et al*., 2007, 2008, 2013a, 2013b). Their cancer vaccine incorporates six different melanoma helper peptides (6MHP), which are a combination of TAAs and CTAs (shown in Table 1). They have found that their free peptide injections are not immunogenic enough.

Each of the 6MHP has been successfully encapsulated by the author prior to the work performed for this project (see Tables 3-5 in the Appendix). Each helper peptide was encapsulated in one of three formulations according to the peptide's charge and polarity, and the final peptide encapsulation averages are shown in Table 1. The full KDO2-nanoliposome formulations with The TLR4 agonist KDO2 was also incorporated into each. The author also previously developed HPLC methods for use in medium-throughput screening of nanoliposome peptide encapsulation (see Figure 1). The HPLC method was used in addition to the liquid chromatography-mass spectrometry (LC-MS) method.

Table 1: Nanoliposome encapsulation of helper peptides.

Amino acid sequence (letter = 1 amino acid)	**Abbreviation**	**Epitope (protein, residue numbers)**	**Liposome formulation**	**Encapsulation (µg/mL)**
WNRQLYPEWTEAQRLD	WNR	gp100 44-59	Anionic	8.34
TSYVKVLHHMVKISG	TSY	MAGE-3 281-295	Anionic	94.71
LLKYRAREPVTKAE	LLK	MAGE-1,2,3,6121-134	Neutral	129.16
FLLHHAFVDSIFEQWLQRHRP	FLL	Tyrosinase 386-406	Neutral	139.56
RNGYRALMDKSLHVGTQCALTRR	RNG	Melan-A/MART-151-73	Neutral	70.52
AQNILLSNAPLGPQFP	AQN	Tyrosinase 56-70	Cationic	8.72

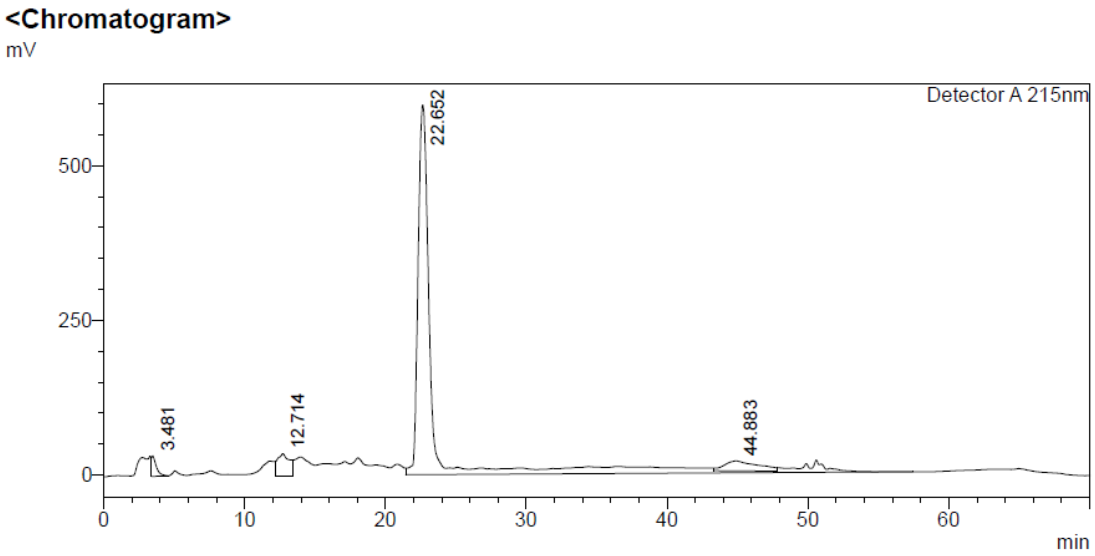

Figure 1: Example of HPLC Chromatogram for AQN levels.

4. Materials and Methods

4.1 Nanoliposome Stability and Release Kinetics

Release kinetics studies were performed to evaluate the amount of peptide released over time when the fully fabricated MHP-KDO2-nanoliposomes are stored in the refrigerator. The MHP-KDO2-nanoliposome formulations corresponding to the FLL, LLK, and WNR peptides

were tested in this experiment. The fabricated sample was split into fractions among Eppendorf tubes, and half had 10% FBS added by volume. The fractions were spun down and its supernatant tested at various time points to track the potential release of peptide from the liposomes over time.

The stability study was conducted in order to determine the aggregation tendency of the nanoliposome formulations in solution. The formulations were tested as separate solutions over time by dynamic light scattering for the measurements of polydispersity, average hydrodynamic diameter of particles in solution, and the number of peaks appearing in the particle size distribution.

4.2 *In Vitro* Lymphocyte Stimulation

The in vitro study purpose is to assess the capability of the mixture of MHP-KDO2-nanoliposomes to stimulate dendritic cell activation and subsequent induced helper peptide presentations to melanoma-reactive T cells *in vitro*. The *in vitro* studies used cells collected from the peripheral blood of two patient donors and from the sentinel immunized lymph node (SLN) of two different patient donors. All donors were previously immunized with the 6MHP in clinical trials Mel43 and Mel63 by Slingluff *et al.* (2007) and tested for a cellular immune response following separate stimulation by four treatments:

1. a single free peptide treatment of only free MAGE-3281-295 peptide (Table 2), denoted "TSY,"
2. a pooled free peptide treatment of the six MHP listed in Table 2, denoted "6MHP,"
3. a single MHP-KDO2-nanoliposome treatment of only TSY-containing KDO2-nanoliposomes, and

4. a pooled MHP-KDO2-nanoliposome treatment of a mixed solution of 5 different KDO2-nanoliposomes that separately encapsulate five MHP listed in Table 2, the WNR, TSY, FLL, LLK, and AQN peptides, denoted “5MHP.”

Controls for treatments 1 and 2 include a media-only negative control and an HIV GAG peptide positive control. Controls for treatment 3 include an empty nanoliposome without KDO2, an empty nanoliposome with surface bound KDO2, and a nanoliposome containing TSY peptide without KDO2. Additionally, a preliminary treatment group was performed with the mix of the 5MHP-KDO2-nanoliposomes. As such, two additional controls were included: a solution of empty nanoliposomes with five times greater (5X) of a final concentration of surface bound KDO2 and a solution of the 5 different nanoliposomes, each containing one of the 5MHP, without KDO2. The treatment was allowed an incubation period of five days in the cell culture to allow time for activation, cellular uptake, intracellular processing, and stimulation.

A CFSE proliferation assay was performed to evaluate the donor immune response on a flow cytometer. The standard CFSE proliferation assay protocol required modifications to avoid cell starvation from the high PBS content of the nanoliposomes’ solvent, relative to the necessary culture media nutrient content to sustain the culture over the course of the incubation period. The assay modifications included pulsing the cultures with TSY-KDO2-nanoliposomes or 5MHP-KDO2-nanoliposomes, single (TSY) or mixed (5MHP) free peptide, or a corresponding control for two hours. The plates were then washed with wash medium with a 10% FCS in RPMI1640 composition to remove non-internalized nanoliposomes or peptide. The pelleted cells were resuspended in culture medium. The culture medium was composed of AIM V and 5% filtered Human type AB blood serum.

4.3 *In Vivo* Murine Pilot Study

A pilot *in vivo* murine study was conducted using BALB/c female mice. The study was performed in two parts: 1) an 18-day study (treatment doses on days 0 & 11) to determine the immune response to the 6MHP-KDO2-nanoliposomes and 2) a 6-day study to determine nanoliposome biodistribution by daily live-mouse imaging using the IVIS Spectrum.

For part 1, each group of three mice received one of six treatments:

1. Mixed solution of the free 6MHP (Table 2), denoted in Figure 4 as "Peptides," delivered subcutaneously (SQ)
2. Empty nanoliposomes (lacking peptide and KDO2) delivered SQ
3. Nanoliposomes containing peptide (lacking KDO2) delivered SQ
4. Nanoliposomes containing surface bound KDO2 (lacking peptide) delivered SQ
5. Nanoliposomes containing peptide and surface bound KDO2 delivered SQ
6. Nanoliposomes containing peptide and surface bound KDO2 delivered intravenously (IV)

Serum samples were collected from the mice in part 1 on day 18 and tested by ELISA for antibody development against the 6MHP in response to each treatment.

For part 2, four mice received a DiR fluorescently labeled 6MHP-KDO2-nanoliposome injection: two mice were injected SQ and the other two were injected IV. The mice were given isoflurane anesthesia and IVIS live imaging was performed daily for six days. On the sixth day, the mice were euthanized, and their organs were harvested for follow-up *ex vivo* studies.

4.4 Ex Vivo Murine Studies

Following the completion of the *in vivo* mouse studies on day eighteen for the mice in part 1 and day six for the mice in part 2, the mice were euthanized, and their organs were harvested. The organs selected for harvest included the lungs, liver, spleen, kidneys, and the lymph nodes.

Organs from the *in vivo* study were transferred to 24-well plates, separated by organs harvested from part 1 versus part 2, and fluorescent imaging data were collected on the IVIS imaging system for qualitative determination of biodistribution. Single cell suspensions were made from a section of the harvested spleens after imaging. The cells were stimulated with 6MHP peptide, allowed time for any immune response proliferation to occur. Then, the cells were fluorescently labeled and analyzed by flow cytometry.

5. Results and Discussion

5.1 Nanoliposome Stability and Release Kinetics

The results of the release kinetics studies are shown in Table 2. Analysis by LC-MS did not detect a measurable amount of peptide present in the MHP-KDO2-nanoliposome solvent after a testing period of five weeks at the temperature condition of 4 ˚C. The first row of Table 2 ("PBS, Entire solution") shows the encapsulation data for peptide amount in the fabricated liposomes at the time immediately following fabrication completion. This row provides a positive control for the experiment, for the presence of peptide in the liposomes from time zero is confirmed, as well as the ability of the LC-MS to detect the presence of the helper peptides. These data suggest that the nanoliposomes did not have a significant release of encapsulated peptide from its core into the surrounding solvent for the tested period. Consequently, the shelf-

stability is determined to be at least greater than a month-long storage period, but additional testing to find the limit of fully maintained stability would be beneficial for future work. This result is promising for the logistical feasibility of this therapy because the nano-vaccine would need to be stable when stored in at refrigerated temperature conditions.

Table 2: LC-MS Determined Encapsulation Data from Release Kinetics Study.

Testing Condition (all +4 °C)	Amino Acid Abbreviation	Liposome Formulation	Amount of Peptide Encapsulated (µg/mL)	Dilution Correction Factor	Starting Conc. (mg/mL)	Encapsulation Efficiency
PBS, Entire solution	FLL	Neutral, 0.01 KDO2	69.79	78.04	1.90	4.11 %
PBS, Supernatant	LLK	Neutral, 0.01 KDO2	ND	N/A	0.454	N/A
FBS, Supernatant	LLK	Neutral, 0.01 KDO2	0.008	N/A	0.454	N/A
PBS, Supernatant	FLL	Neutral, 0.01 KDO2	ND	N/A	1.90	N/A
FBS, Supernatant	FLL	Neutral, 0.01 KDO2	ND	N/A	1.90	N/A
PBS, Supernatant	WNR	Anionic, 0.01 KDO2	ND	N/A	.454	N/A
FBS, Supernatant	WNR	Anionic, 0.01 KDO2	ND	N/A	.454	N/A

The polydispersity stability of the mixed solution was also critical to determine because of the varied overall charge of the MHP-KDO2-nanoliposome formulations. The formulations specific to the TSY and WNR peptides have an overall anionic charge, as determined by zeta potential measurements on the Malvern ZetaSizer. indicating all six liposome formulations to be highly stable. Dynamic light scattering analysis results on the Malvern ZetaSizer indicated stability of the mixed solution of variously charged liposomes for at least 5 days without aggregation occurring and an average nanoparticle diameter of 113.5 nm (Figure 2). This is an advantageous result for the application of this research in the clinical setting, for the mixed solution of all six MHP-KDO2-nanoliposomes would be able to be mixed together without issue and administered to the patient as a single subcutaneous injection of solution.

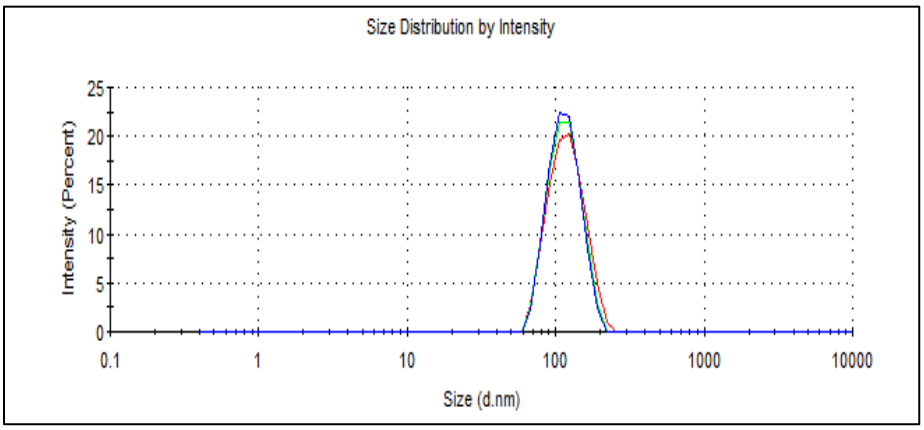

Figure 2: Size distribution of all 6 MHP-KDO2-nanoliposomes in solution 5 days after mixing.

5.2 In Vitro Lymphocyte Stimulation

A graph of lymphocyte overall population viabilities is shown before treatment (Control) and following the various treatments for each donor is shown in Figure 3. Cell viability was determined using a live-dead marker detectable by flow cytometry. A statistically significant difference in mean viability is seen between the SLN 2 donor group and every other donor group (*** $P < 0.001$). The control donor group consists of cells harvested from patient SLN prior to 6MHP immunization.

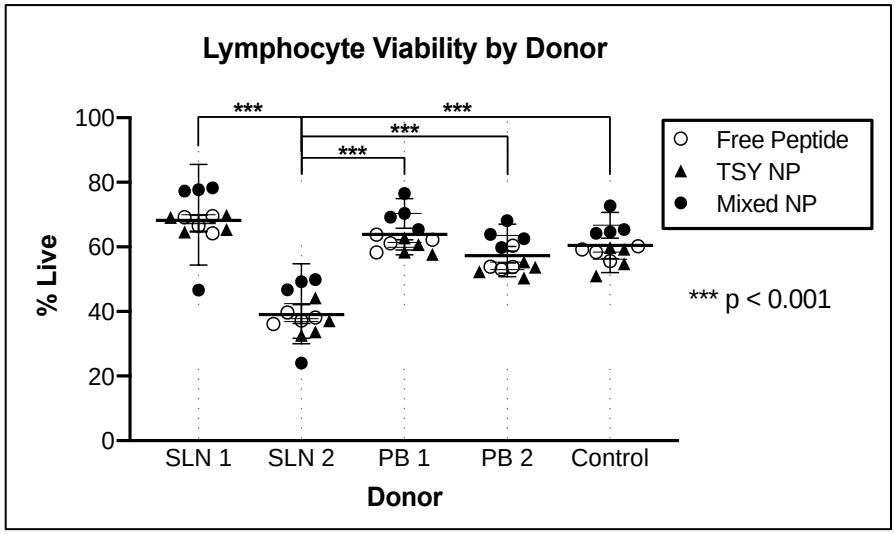

Figure 3: Graph of lymphocyte viabilities post-treatment by donor. Thick horizontal bars represent mean viability among treatments for a donor.

Figure 4 displays a graph of the average proliferation of CD4+, CD8- (Figure 4A.) and CD8+, CD4- (Figure 4B.) gated cell populations for various culture treatment conditions. Cell cultures were expanded from harvested sentinel lymph node (SLN) biopsy of each donor. Measure of cell proliferation was performed by CFSE dye dilution. Proliferation is reported as the percentage of CD3+ gated population that are dividing CD4+ cells (Figure 4A.) and CD8+ cells (Figure 4B.).

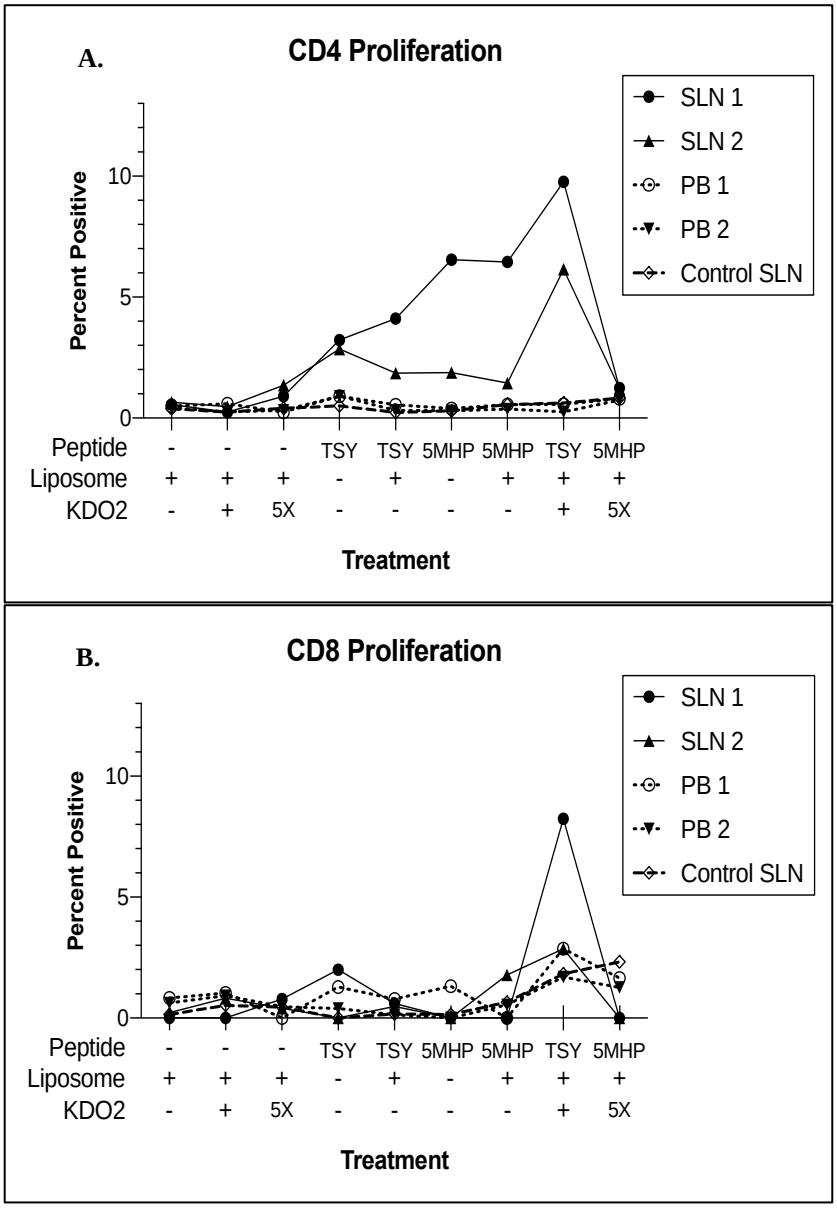

Figure 4: Graph of average proliferation of A) CD4 and B) CD8 cell populations in various culture treatment conditions.

Figure 4A shows the first donor's SLN-derived CD4 lymphocytes (SLN 1) responded significantly above Control LN (** $p < 0.005$) whereas the SLN 2 CD4 response was not significantly above Control LN CD4 response according to Dunnett's multiple comparisons test. Figure 4A shows CD4 T cells had a similar, moderate response to treatment with TSY-nanoliposomes without KDO2 as with free TSY peptide. When KDO2 was added to the MHP-nanoliposome surface, the CD4 cells showed an enhanced response that was greater than both free peptide treatment responses. Figure 4A also shows a recurring positive trend between the single peptide conditions and the mixed 5MHP conditions; the CD4 response increases when raising the peptide load from TSY to 5MHP in the free peptide treatment and non-immunogenic liposome treatment. The immunogenic TSY-KDO2-nanoliposome showed the greatest response of all treatments; however, increasing the peptide load from TSY- KDO2-nanoliposomes to 5MHP-KDO2-nanoliposomes did not follow the observed trend in CD4 proliferation and instead caused a dramatic decrease in CD4 proliferation for both donors. This lower response to the 5MHP-KDO2-nanoliposomes is believed to be due cytotoxicity caused by the 5-fold increased load of KDO2 on the cells in addition to the release of encapsulated peptide. A dose dependent experiment with MHP-KDO2-nanoliposomes on PBMCs and SLN cells is recommended for future work to avoid an apoptotic effect in future experiments. Figure 4B shows pronounced CD8 division above Control SLN in response to TSY- KDO2-nanoliposomes, particularly by SLN 1 lymphocytes. Further, the observed CD8 responses could be below their potential if the culture contained an insufficient amount of endogenous IL-2, for exogenous IL-2 was not added to the culture. Thus, there could be a peptide- nanoliposome mechanism of CD8 cell stimulation that is not possible by the free peptide alone. Although this spike in CD8 activity in Figure 4B

was not found significant using Dunnett's multiple comparisons test, this is an unexpected and interesting result that we plan to investigate further in future experiments.

The peripheral blood cell cultures (PB 1 & PB 2) did not show significant CD4+ or CD8+ cell proliferation (Figure 3). This result may be because of the greater difficulty in expanding PBMC derived T cells compared to T cells taken from the SLN, as discussed by Chianese-Bullock and others (2005), who used PB and SLN cells from the same clinical trials as these experiments. The greater response by SLN 1 lymphocytes than SLN 2 lymphocytes for nearly all treatment conditions is likely a result of SLN 2 having significantly lower cell viability than all other donor samples, as seen in Figure 3 (*** $P < 0.001$). Figures 4A and 4B use a "percent positive" measurement to show the percentage of live, CD3+ lymphocytes that are dividing CD4+ and CD8+ cells, respectively. An additional *in vitro* study will be completed to further investigate the CD8+ cell response to the full vaccine noted as well as the immune response to the mixed treatment of the 6MHP-KDO2-nanoliposomes once a KDO2-tolerant dose is determined. The current results are extremely promising, for a single MHP-KDO2-nanoliposome was able to stimulate a response far above treatment with a mix of the six free peptides.

One limitation of this study results from the necessary altering of the standard CFSE assay. This research opted to uses pulses of treatment rather than long-term contact because of the high concentration of PBS in the nanoliposomes' solvent. Therefore, the nanoliposome or peptide treatment was not in constant, direct contact with the cells in culture over the five-day incubation period, and ultimately the time of treatment exposure to the cells was reduced from that which is typically recommended in the company's assay user instructions. Another limitation of this study is that exogenous IL-2 was not added to the cell cultures. IL-2 is a key

cytokine in the activation response mechanism of cytotoxic T cells (CD8s). Consequently, it is unable to be determined if the low CD8 response observed in Figure 2A is a result of a lack of activation by the applied treatments, or if the changes to the CFSE assay protocol caused a mitigated immune response to be observed in these studies.

5.3 *In Vivo* Murine Pilot Study

The ELISA results for part 1 for antibody development against the 6MHP in response to each treatment from the serum samples are shown in Figure 5. The serum antibody content of the mice who had 6MHP-KDO2- nanoliposomes delivered IV was significantly above the antibody against 6MHP in the serum from the empty nanoliposome control, as expected, while the 6MHP-KDO2-nanoliposomes delivered SQ did not appear to have a lasting serum antibody response significantly above that of the empty nanoliposome control (Figure 5).

On day 18 peripheral blood and tissue samples were harvested from the mice in part 1 of the *in vivo* study, and PBMCs were isolated. These cells were studied for immune response by stimulation with the 6 melanoma helper peptides. T cell responses to the peptides were not detected by flow cytometry, which could be because they are human sequences. However, this may not be the reason for a lack of response, for the experimental controls suggested that all of the cells in the experiment were dead. If this were the case, then it is likely that too high of a concentration of peptide was used for the cell treatment or that cell handling from harvest to culture may have killed them. Therefore, future work includes performing a titration to determine concentrations that BALB/c mouse spleen cells can tolerate.

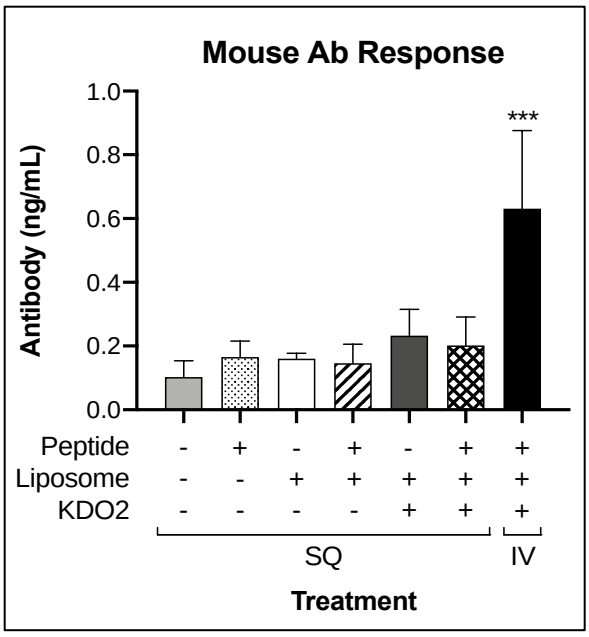

Figure 5: Anti-6MHP antibody concentrations from ELISAs of mouse serum.

The results for part 2 are shown in Figure 6. Figure 6 shows the biodistribution of the labeled nanoliposomes in mice in Part 2 of the study at days 0 and 6. Possible collection in the axillary lymph nodes (Figure 6a) and the superficial cervical lymph nodes (Figure 6h) was observed.

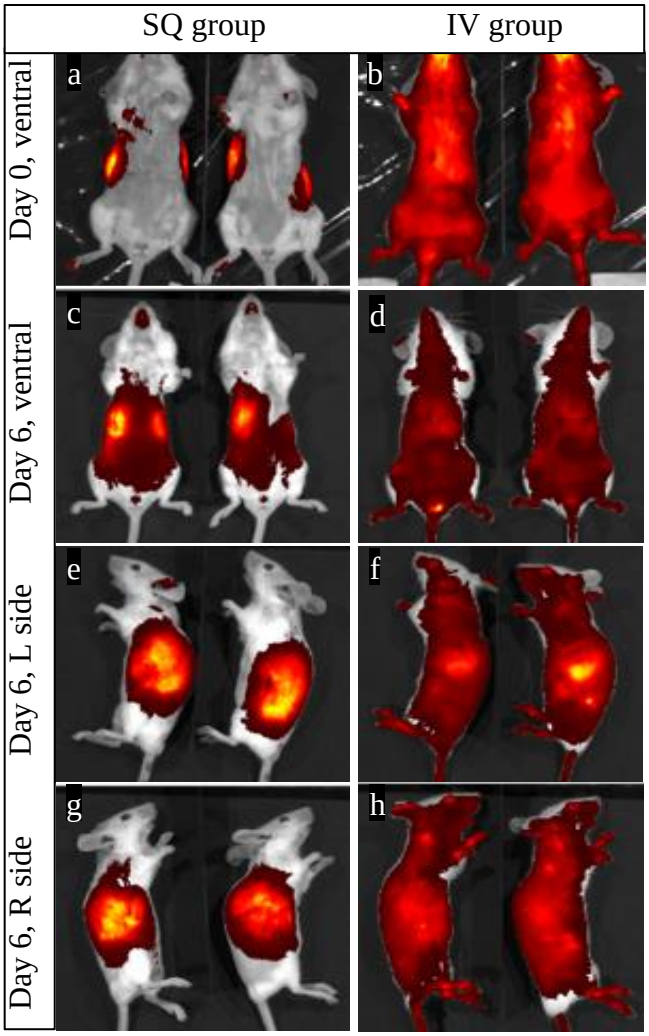

Figure 6: Fluorescent imaging of liposome biodistribution in mice on days 0 and 6.

A mouse tumor model was out of the scope of this research study, however, is intended to be performed in future work in this research area. Preliminary work in finding equivalent mouse melanoma helper peptides has been done, and mouse vaccine trial studies will need to be performed next, before a melanoma mouse model to characterize the behavior of these peptides and compare these results to the behavior of the human 6 melanoma helper peptides in human patients with melanoma.

5.4 Ex Vivo Murine Studies

A sample image from the IVIS imaging data that is representative of the data collected for all 18 mice. he fluorescence measurement results from the harvested organs of the 18 mice from part 1 is shown in Figure 7. plate of organs for each mouse had no detectable rhodamine fluorescence.

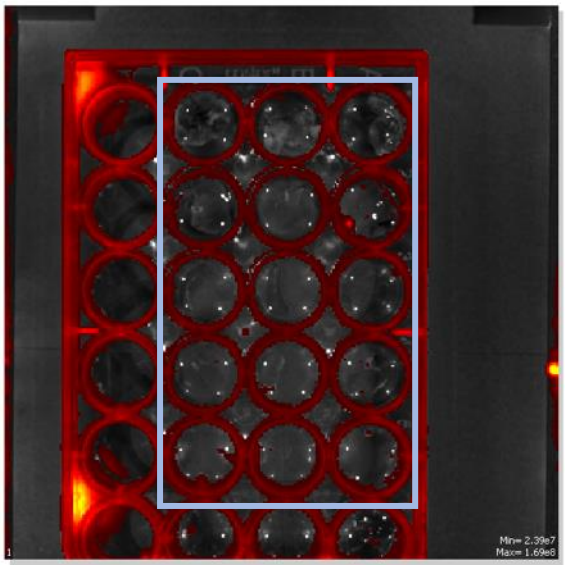

Figure 7: Sample IVIS image from part 1, Group 2 mice harvested organs.

Figure 8 shows the biodistribution of the DiR labeled MHP-KDO2-nanoliposomes in the harvested organs from the mice in Part 2 of the *in vivo* study. The harvested spleen of the IV injection group of Part 2 was found to be enlarged and highly fluorescent, indicating collection of DiR labeled 6MHP- KDO2-nanoliposomes as well as a large immune response (Figure 8). Cells were isolated from the harvested organs from Part 2, expanded in culture, and analysis by flow cytometry way performed in an attempt to detect a proliferation response that would suggest presence of immune cells that had interacted with, and been activated in a priming manner by the helper peptides contained within the nanoliposomes. However, the fluorescent

marker used to stain the harvested cells overlapped with the emission wavelength of the DiR nanoliposome label used in part 2, so no meaningful results were obtained from this analysis.

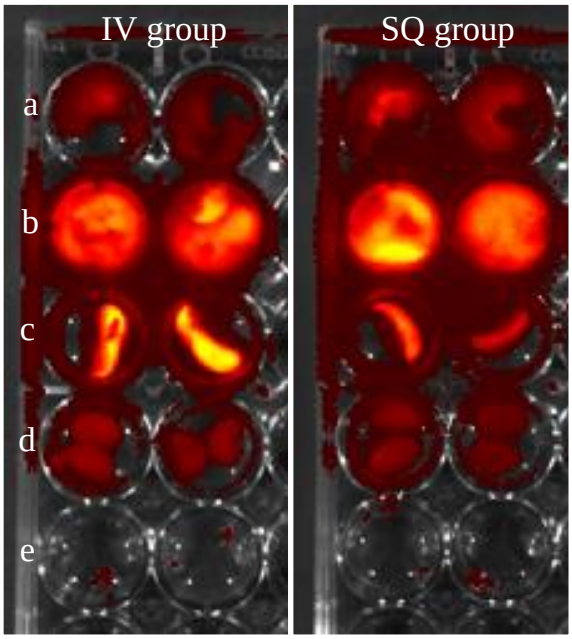

Figure 8: Fluorescent imaging of harvested organs by row: a) lungs, b) liver, c) spleen, d) kidneys, and e) lymph nodes.

6. Conclusions

The application of nanoliposome technology to immunotherapy as a strategy for the enhancement of cancer vaccines in this research has shown promising evidence suggesting that the engineered KDO2-nanoliposomes address the issues of biocompatibility, circulation half-life, and simultaneous delivery of antigen and stimulatory components to dendritic cells faced by traditional peptide cancer vaccine administrations. Aims for future work include development of a B16 melanoma mouse model for testing the 6MHP-KDO2-nanoliposomes in its cancer bio-environment. Additionally, work to create a protocol for covalently attaching dendritic cell-targeting antibodies to the surface of the current 6MHP-KDO2-nanoliposome formulations. The studies performed in this project establish an evaluation framework for testing and assessing the efficacy of the antibody-modified nanoliposomes.

7. Appendix

Table 3: Neutral nanoliposome formulation.

Lipid	Molar Ratio	µL Used
DSPC	**4.6**	**511.1**
DOPE	**2.14**	**223.9**
PEG	**0.25**	**98.63**
KDO2	**0.01**	**81.10**
Chol.	**3**	**163.1**

Table 4: Anionic nanoliposome formulation.

Lipid	Molar Ratio	µL Used
DSPC	**3.91**	**449.1**
DOPE	**1.83**	**197.5**
PEG	**0.25**	**101.7**
KDO2	**0.01**	**83.65**
DHP	**1.0**	**79.32**
Chol.	**3**	**168.2**

Table 5: Cationic nanoliposome formulation.

Lipid	Molar Ratio	µL Used
DOTAP	**0.7**	**63.92**
DSPC	**3.92**	**404.7**
DOPE	**1.83**	**178.0**
PEG	**0.542**	**198.8**
KDO2	**0.01**	**75.39**
Chol.	**3**	**151.6**

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