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Entitled

Activation of CD8+ Cytotoxic T Lymphocytes against Tumor Cells using a TLRL-MUC1-Tn Cancer Vaccine

By

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Medicinal Chemistry

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December, 2013
An Abstract of

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The immune generation of cytotoxic T lymphocytes (CTLs) for killing tumor cells has a critical step of cross-presentation, in which the tumor cells or tumor antigens are taken up by professional antigen presenting cells (APCs), processed and presented to naïve CD8+ T cells. Some professional APCs may express B7 co-stimulator to provide secondary signals for the differentiation of the naïve CD8+ T cells and may stimulate naïve CD4+ T cells to be helper T cells for CTL development. The differentiated CD8+ cytotoxic effector T cells are specific to some tumor-associated peptide antigens. These effector CD8+ T cells may effectively kill the tumor cells presenting tumor-associated peptide antigens without the need for co-stimulator B7 molecules. Hence, an immunogenic glycopeptide in complex with MHC class I has been targeted for activation of anti-tumor CTLs. A liposome based cancer vaccine consisting of a 20-amino-acid MUC1 peptide containing MUC1 glycopeptide GalNAc-O-Thr (Tn) has been decorated with L-rhamnose-cholesterol and conjugated to a functionalized Toll-like receptor ligand (TLRL), Pam3Cys. In the presence of anti-rhamnose antibody, this induces Fc-FcγR interaction to enhance antigen uptake. CD4+ helper T cells were activated by the liposomal vaccination in Rha-Ficoll immunized BALB/c mice, but CD8+ CTLs were not
stimulated effectively by the initial liposomal vaccine. I have tested a different peptide proposed to contain a CD8$^+$ T cell epitope, which is a small peptide that contains 8 amino-acids of the MUC1 sequence. The small 8 amino-acid glycopeptide has GalNAc-O-Thr (Tn) at a different position than in the CD4$^+$ T cell epitope. The GalNAc-O-Thr (Tn) in complex with the MHC class I of APCs works effectively as an anchor residue for positive bonding within the pocket of the MHC class I molecule. The successful cross-presentation process causes proliferation of cytotoxic T lymphocytes which can kill specific tumor cells. A JAM assay shows the capability to kill the tumor cells. Induction of CD8$^+$ T cells was measured by the level of intracellular or extracellular type II interferon (IFN-γ), which is a cytokine for tumor control. An intracellular staining method was used to fix the intracellular IFN-γ inside of the cell and analyze the level of IFN-γ with fluorescence. An IFN-γ ELISA assay was used to measure extracellular IFN-γ in the cell supernatant. It was shown that the use of anti-rhamnose antibody to enhance vaccine uptake caused an increase in CD8$^+$ T cell response in addition to an increased antibody response.
I once used the TARTA bus to get to the main campus from the Kroger on Glendale Ave., but the bus ran toward downtown Toledo. My destination was not there so I took another bus from the downtown Toledo transportation center. Many passengers had diverse clothing and I felt like a stranger. Several people spread food donations to the passengers. Then, I realized I had lived in a small village isolated from downtown since I came to Toledo from South Korea. I observed the eyes of many passengers. There are deep holes which I have to fill with my privilege and compassion. I have been loved by many people without any reason. I cannot express the favors with my short English words but I wish to respond to their love and favor for me. I present this paper for my neighbors in downtown Toledo. As much as I have been loved for 40 years, I would like to dedicate myself for them as a sister, a mother and a teacher.
Acknowledgements

“As iron sharpens iron, so one person sharpens another” (Proverbs 27:17) My research has been the process of sharpening. Dr. Steven Sucheck opened the door of his lab to me who had not been disciplined in a research lab. In his lab, undergraduate student David Long helped me to do experiments. Partha Karmarkar and Sourav Sarkar have collaborated in this cancer project. They supported me with their experience but also with data in this paper. Dr. Katherine Wall gave an opportunity to work in an immunology lab. I was really naïve in the immunology area. She explained immunology knowledge whenever I asked, so that I could learn and do research for this cancer project. Also, Dr. Slama and Dr. Grafenstein were glad to teach me with pleasure. Ramadan Ali, Ali Aldieri and Brandon Slotterbeck helped me a lot. Timnit Asfaha and Anusha Madineni encouraged me as a friend. Lisa, who has cleaned my lab as a customer service worker, cleaned the lab but also did favors for me. I was sharpened by their scientific knowledge or their heart. Sincerely, I thank them.
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List of Abbreviations

ADCC………….. Antibody-Dependent Cell-mediated Cytotoxicity
APCs…………… Antigen Presenting Cells

CTL……………..Cytotoxic T Lymphocyte

DCs…………… Dendritic Cells
DPPC…………….Dipalmitoylphosphatidylcholine Liposome or 1,2-dipalmitoyl- \( sn\)-

EBV……………...Epstein-Barr virus
glycero-3-phosphocholine

mAb………………Monoclonal Antibody
MHC……………...Major Histocompatibility Complex

Rha………………L-rhamnose

SD………………Standard Deviation
STn………………\( \alpha \text{Neu5Ac-(2,6)-}\alpha\text{GalNAc-Thr} \)

TACAs…………...Tumor-associated carbohydrate antigens
TEG………………Tetra ethylene glycol
TF………………..Thomsen-Friedenreich, \( \beta\text{Gal-(1,3)-}\alpha\text{GalNAc-Thr} \)
TLRL…………….Toll-like receptor ligand
Tn………………...\( \alpha\text{GalNAc-Thr} \)

VNTR……………MUC1 variable number tandem repeats
Chapter 1

Introduction

After chemotherapy, cancer patients often have a deficiency in anti-tumor immunity. In order to stimulate the immune system to attack reoccurring tumor cells in breast cancer, a peptide cancer vaccine can be used to enhance the immune response.

1.1 Tumor Antigens

Transformed cells, being tumor cells, express diverse types of molecules as foreign antigens. The antigens should be recognized as non-self by the immune system to prevent the cells from developing into harmful tumors. Chemical carcinogens and radiation may induce experimental tumors. The tumor antigens may be mutants of normal cellular proteins because the chemicals and radiation may mutate the normal cellular genes to cause transformed cells, tumor cells. Also, products of oncogenes or mutated tumor suppressor genes are presumably tumor antigens. The gene products cause malignant transformation of tumor cells. In contrast to mutants, normal proteins which are either over expressed or aberrantly expressed as self-protein are also tumor antigens. Oncogenic viruses may produce tumor antigens such as human papillomavirus E6, E7 proteins in
cervical carcinoma or Epstein-Barr virus (EBNA) proteins in EBV-induced lymphoma, which is a malignant tumor of B or T lymphocytes. Those antigens may be recognized by tumor-specific CD8+ T cells but also be recognized by CD4+ T cells.

1.2 Immune Mechanism of Tumor Eradication

The main concern is to kill tumor cells by cytotoxic T lymphocytes (CTLs) which are stimulated by class I MHC-associated peptides of the host antigen presenting cell. The class I MHC-associated peptides are cytosolic proteins which are endogenously synthesized and displayed by the class I MHC molecule. Usually CTL responses may be initiated by recognition of tumor antigens on host antigen presenting cells (APCs) because the APCs are able to ingest tumor cells or tumor antigens and present the antigens to T cells along with B7 co-stimulator and the help of class MHC II-restricted CD4+ helper T cells. It is known that all nucleated cells express class I MHC molecules, therefore tumor cells also are able to display class I MHC-associated peptides. But tumor cells do not often express B7 co-stimulator and/or class II molecule which can differentiate naïve CD4+ T cells to be helper CD4+ T cells. Hence, CD8+ T cell responses to tumors may be induced by cross-presentation or a cross-primed process. The cross-presentation occurs from one cell type (tumor cells) through another cell type (APCs) to CD8+ T cells. The tumor cells or tumor antigens are taken up by professional APCs, are broken down, and then presented to naïve CD8+ T cells. There are some limitations because only some APCs can express B7 co-stimulator which provides the second signal for differentiation of the naïve CD8+ T cells. Also, a limited number of APCs can stimulate CD4+ helper T cells which provide help for development of active cytotoxic T
lymphocytes (CTLs). Because of those limitations, the immune response to tumors can fail to protect from malignant tumors because tumors grow rapidly and many tumor antigens are weakly immunogenic. Often, the tumor growth overtakes the killing of tumor cells by CTLs. In addition, some tumors stop expressing class I MHC molecules. Because of the many barriers to tumor eradication, the concept of cross-presentation has been investigated to explore new vaccination against cancers.

1.3 Cancer Immunotherapy

Cancer immunotherapy increases anti-tumor activity such as antibodies and active T cells. Until now the treatment of disseminated cancers which cannot be removed surgically uses chemotherapy or irradiation even though the treatment harms normal cells. Thus, it is important to develop tumor specific immunity which eradicates tumor cells selectively.

1.3.1 Passive Immunization

In passive immunization, immune active cells are transplanted from donors to cancer patients. Monoclonal antibodies bind to tumor antigens. Antibodies containing potent toxins may transfer the toxins to the tumor cells and activate the host immune system. For example, some breast cancer cells overexpress the products of the HEB2/neu oncogene; therefore antibodies against the products of the HEB2/neu oncogene are useful to treat some breast cancer patients. The antibodies may bind to tumor antigens
caused by the *HEB2/neu* oncogene and may be targeted for phagocytosis or the complement system.\(^2\) Consequently, the immune system is activated against tumor cells. Another example is CD20 antibody which is used to treat B cell tumors and is used in combination with chemotherapy.\(^3\) CD20 is expressed on B cells but is not expressed on hematopoietic stem cells. Transformed B cells and normal B cells are eradicated by the immune system which detects bound anti-CD20 antibodies on B cells, but normal B cell can be replaced through hematopoiesis of stem cells.\(^4\)

### 1.3.2 Active Immunization

Vaccination of a patient involves boosting the host’s own immune responses against tumors. One way is to vaccinate patients with their tumor cells or their tumor antigens. The approach makes the immune system recognize tumor antigens and boost immunity against their own tumor cells. Usually the vaccine is formulated of recombinant proteins and adjuvant. Recently, dendritic cells (DCs), which are potent APCs, are used as a cancer vaccine. Dendritic cells are cultured from patients’ stem cells with growth factor, isolated from culture and pulsed with their tumor cells or tumor antigens in vitro. The in vivo DC vaccine is expected to demonstrate cross-presentation to naïve T cells for activation of cytotoxic T lymphocytes (CTLs) against tumor cells. As a molecular biology strategy, a plasmid containing complementary DNA (cDNA) encoding tumor antigens can be a cancer vaccine. The complementary DNA (cDNA) is injected in the patient and tumor antigens are synthesized in host cells. The antigen presenting cells (APCs) also take up the cDNA and produce tumor antigen to stimulate specific T cell
responses. In addition, vaccination with transgenic tumor cells containing plasmids expressing lymphocyte co-stimulator (B7) or a T cell growth factor, IL-2, enhances patients’ immune system activity. Interleukin 2 (IL-2) enhances proliferation and differentiation of tumor-specific T cells. Lymphocyte co-stimulator, B7-expressing tumor cells stimulate tumor-specific T cells. But IL-2 may cause serious side effects depending on the doses. One new approach which boosts the host’s immune system is to eliminate inhibitory signals in lymphocytes. Blocking the inhibitory T cell receptor, CTLA-4 induces forceful T cell responses without regulation. All of the strategies in enhancing the host’s own immune system manipulate immune mechanisms.4

1.4 Antibody-Mediated APC Targeting Liposomal Vaccine Components

1.4.1 MUC1 Glycopeptide Containing a GalNAc-O-Thr (Tn) TACA

Mucins are O-glycosylated glycopeptides that are associated with carcinomas when overexpressed or when they contain aberrant glycosylation. Thus, the MUC1 gene expresses a family of membrane-bound proteins. The diversity is caused by different expression patterns of the MUC1 gene by a genetic polymorphism. MUC1 variable number tandem repeats (VNTRs) conjugated to tumor-associated carbohydrate antigens (TACAs) have been evaluated to be immunogenic against tumors in wild type and humanized MUC1 transgenic mice.5 On cancer cell surfaces, an abnormal quantity of carbohydrates is conjugated to MUC1 glycopeptide. Table 1 shows TACAs based on the cancer type.6
Table 1. Major Carbohydrates Tumor Antigens in Specific Tumor Tissues

<table>
<thead>
<tr>
<th>Tumor Antigens/Cancer Type</th>
<th>GM2</th>
<th>GM3</th>
<th>GD2</th>
<th>GD3</th>
<th>TF</th>
<th>Tn</th>
<th>STn</th>
<th>Globo H</th>
<th>Leα</th>
<th>Poly sialic acid</th>
<th>Leα</th>
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<tr>
<td>Melanoma</td>
<td>V</td>
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<tr>
<td>Sarcoma</td>
<td>V</td>
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<td>B cell lymphoma</td>
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<td>V</td>
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<tr>
<td>Neuroblastoma</td>
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<td>Stomach</td>
<td>V</td>
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<td>Breast</td>
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<td>Prostate</td>
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<td>Ovary</td>
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Many kinds of tumor associated carbohydrate antigens (TACAs) are identified from MUC1 glycopeptide obtained from epithelial cancer cell lines. The Thomsen-Friedenreich (TF) and Tn are expressed in the breast at lactation and in malignancy. Hence, MUC1 tumor-associated carbohydrates (TACAs) are a serum marker for breast cancer.

1.4.2 Toll-Like Receptor Ligand (TLRL)

An obstacle to the use of TACAs as cancer vaccines is their weak immunogenicity. The generation of high affinity immunoglobulin G (IgG) antibodies against cancer cells is dependent on the interaction of B cells and helper T cells, which requires the antigen to
be presented by the major histocompatibility complex (MHC) class II molecules on the surface of antigen-presenting cells (APCs). Carbohydrate epitopes do not activate the helper T cells enough to produce high affinity IgG and IgM antibodies to control tumor cells; therefore immunogenic carrier proteins are conjugated to carbohydrate antigens for activation of helper T cells. Activated helper T cells generate high affinity immunoglobulin G (IgG).\(^7\) Toll-like receptors on APCs stimulates activation and antigen presentation when they bind their ligands; therefore a Toll-like receptors ligand (TLRL) such as monophosphoryl lipid A (MPLA)\(^8\) and Pam\(_3\)Cys have been conjugated to MUC1 Tn for improved immunogenicity. The combination of toll-like receptor ligand induces optimal activation of GM-CSF/IL-4 DCs in vitro.\(^9\)

### 1.4.3 DPPC (Dipalmitoylphosphatidylcholine) Liposome

A liposome is composed of lipids that exist naturally in the cell membrane such as phosphatidyl choline (PC) and cholesterol. Liposomes are safe and completely degradable in vivo. First of all, the advantage of a liposome based vaccine is the versatility. Lipid constituents and methods of vesicle preparation can be determined by the target vaccine’s physicochemical properties. Hydrophilic molecules can be encapsulated by the aqueous interior or conjugated to the aqueous surface, whereas hydrophobic compounds can be intercalated into the lipid bilayer. The versatility allows antigens of all types such as peptides, proteins, carbohydrate, nucleic acids, and small molecule haptens, to be used.
Table 2. Selected liposome and lipid-based vaccines approved for human use or in clinical trials.\textsuperscript{10}

<table>
<thead>
<tr>
<th>Applied Disease</th>
<th>Liposome Vaccine Components</th>
<th>Recent Status</th>
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<tr>
<td>Influenza</td>
<td>Virosomes-reconstituted influenza viral membranes (phospholipids, haemagglutinin, and neuraminidase) supplemented with PC</td>
<td>Inflexal in market</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Formalin-inactivated Hepatitis A virus adsorbed to virosomes</td>
<td>Epaxal in market</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>BLP25 (palmitoylated MUC1), MPL, DPPC, DMPG, Chol</td>
<td>Phase3 in reviewing</td>
</tr>
<tr>
<td>Malaria</td>
<td>Recombinant fusion of \textit{P. falciparum} circumsporozoite} protein and Hepatitis B surface antigen, PC, Chol, MPL, QS21</td>
<td>Phase3 in reviewing</td>
</tr>
</tbody>
</table>

Despite the abundant versatility of the potential liposomal vaccines, the lack of availability of liposomal vaccines is caused by their weak physical and chemical stability in solution with antigens. This may be resolved by the development of a stable, sterile, freeze-dried formulation process which is capable of maintaining physical properties post hydration and gives a chemically stable product.\textsuperscript{11} The liposome facilitates induction of intracellular processing of liposomal antigens. The liposome particle is digested by endocytosis. The liposome and antigens are internalized, after which antigens are presented on the surface through two tracks. Antigen may be fused with MHC II-
containing organelles and loaded onto MHC II molecules for antigen presentation on the cell surface. Antigens may escape from the endosome to the cytosol and be degraded by proteasomes. The degraded antigens are transferred to the endoplasmic reticulum and loaded onto MHC I molecules for antigen presentation on the cell surface. My project is concerned with this internalizing process of cross presentation.

1.4.4  L-Rhamnose (Xenoantigen)

In our vaccine design, we are incorporating the xenoantigen rhamnose onto the surface of the liposome. The purpose of the xenoantigen is to make complexes with naturally generated antibodies. The binding between xenoantigen and the antibody facilitates antigen presenting cells (APCs) to take up the target antigens. Our group has previously shown that rhamnose targeting in the presence of anti-rhamnose antibodies increases antigen presentation and the humoral immune response.\textsuperscript{12} Natural antibodies have previously been used to target vaccines to APCs. The $\alpha$-Gal epitope on cancer vaccine structures boosts immune responses because the human body has natural anti-$\alpha$-Gal antibodies.\textsuperscript{13} Human serum naturally has more anti-Rha antibodies which bind against the xenoantigen L-rhamnose (Rha) rather than anti-$\alpha$-Gal antibodies.\textsuperscript{14} The Rha epitope which is linked non-covalently to a liposomal vaccine can enhance the immune responses against a tumor-associated glycopeptide fragment of MUC1 in mice containing anti-Rha antibodies.\textsuperscript{12}

1.5  Molecular Strategy for Activation of Cytotoxic T Lymphocytes (CTL)
1.5.1 Glycopeptide-Binding in Complex with MHC class I of MUC1-8-5 GalNAc

In the cross-presentation process, antigen-presenting cells, primarily dendritic cells (DC), take up and process antigens and present peptides on MHC molecules. MHC class I consist of one α1, α2, or α3 heavy chain non-covalently associated with β2-microglobulin (light chain). The amino acid side chains of peptides (anchor residues) fit into pockets that extend along the floor of the groove of the α-chain. Crystallographic studies of MHC I–peptide complexes have demonstrated that the peptide–binding grooves form into various pockets. The MHC molecules have specific preferences for hydrophobic amino acids at certain positions along the peptide. For MHC I (H-2Kb), 8-mer peptides are compatible in three positions. P2 binds into the B pocket, P5 into the C pocket, and P8 into the F pocket. The preferred amino acid residues are Phe/Tyr in the central C pocket and Leu/Met in the F pocket. SAPDTRPA, MUC1 VNTR (MUC1-8) is usually associated with high affinity binding to H-2Kb, which has two small non-polar Ala residues at P2 and P8 and a small polar Thr residue at P5. Thus, at the central C pocket, a large cavity exists which allows GalNAc linked to P5-Thr to fit. Molecular modeling demonstrates that P5-Thr-GalNAc in the C pocket forms hydrogen bonds and van der Waals interactions. Thus, the interactions increase the affinity of MUC1-8-5GalNAc to more than MUC1-8. Figures 1 and 2 show the crystal structure of MUC1-8 and MUC1-8-5GalNAc bound to H-2Kb.
Figure 1. Solvent-accessible surface$^{15}$

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(A) MUC1-8 complex and (B) MUC1-8-5GalNAc complex with H-2K$^b$. Surfaces of the complexes are contributed by the residues of the H-2K$^b$ protein (α-domain, pink) and the residues of MUC1-8/MUC1-8-5GalNAc peptide (green). The white arrow in (A) complex indicates the cavity in the central pocket C. (B) complex does not have the cavity in C pocket.
Figure 2. Space-filling representation of peptides in complexes

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MUC1-8 (A and C) and MUC1-8-5GalNAc (B and D) are viewed from above the MHC class I, and C and D are viewed from the side. The domains of MHC are shown in pink and the sheet floor in cyan. The position of the C pocket is indicated with capital letter C inside of drawing C and D. Peptides are shown in Corey Pauling Kultun representations (yellow), the central P5 Thr is in red, and the GalNAc group is in green.
VSV8 and OVA8 are epitopes which have high affinity for murine MHC class I, H-2K\(^b\) (Table 3). Thus the affinity of MUC1-8-5GalNAc is similar to OVA8 or VSV8 using the value of \(K_D\) (nM).

**Table 3. Affinity measurements of peptides binding to H-2K\(^b\)**\(^{15}\)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>23°C (K_D), nM</th>
<th>37°C (K_D), nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1–8</td>
<td>SAPDTRPA</td>
<td>877</td>
<td>37,000</td>
</tr>
<tr>
<td>MUC1–8-5F8L</td>
<td>SAPDFRPL</td>
<td>60</td>
<td>300</td>
</tr>
<tr>
<td>MUC1–8-5GalNAc</td>
<td>SAPD (GalNAc) RPA</td>
<td>13</td>
<td>380</td>
</tr>
<tr>
<td>OVA8</td>
<td>SIINFEKL</td>
<td>10</td>
<td>82</td>
</tr>
<tr>
<td>VSV8</td>
<td>RGYVYQGL</td>
<td>27</td>
<td>163</td>
</tr>
</tbody>
</table>

The affinity of the nonglycosylated epitope, MUC1-8 is much lower than that of the glycosylated epitope, MUC1-8-5 GalNAc. MUC1-8-5F8L has Phe at P5 and Leu at P8 in the MUC1-8 peptide sequence. The Table 3 data gives the difference of affinity for binding to H-2K\(^b\). A lower value of \(K_D\) implies the dissociation of peptide-MHC1 complex is less likely than a higher value of \(K_D\) for the peptide-MHC1 complex. Therefore we chose the glycosylated peptide TLR2 ligand-HGVTSAPDT(α-D-
GalNAc)RPAPGSTAPPA as our immunogen, as it would be expected to generate the CD8 epitope SAPDT(α-D-GalNAc)RPA during processing.

1.5.2 Aberrantly Glycosylated MUC1 Tripartite Vaccine

In addition to the MUC1-8 glycopeptide epitope for activation of cytotoxic T lymphocytes (CTLs), aberrantly glycosylated MUC1 protein on tumor cells can be recognized by antibodies that bind to natural killer cells (NK), macrophages and neutrophils through binding between Fcγ receptor of NK or other cells and Fc portion of anti-MUC-1 antibodies. Consequently the NK cells lyse the target tumor cells and macrophages or neutrophils phagocytose the tumor cells. The antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-dependent phagocytosis (ADC) enhances the immune response against tumor cells along with the cytotoxicity of CTLs. Dr. Vani Lakshminarayanan’s team identified three components of a cancer vaccine to generate cytotoxic T lymphocytes (CTLs) and ADCC mediator antibodies against specific MUC1 tumor cells. The vaccine was composed of the immune adjuvant Pam₃CysSK₄, a peptide T helper epitope and an aberrantly glycosylated MUC1 peptide. The three components were linked covalently according to antigen design. The cytotoxicity of vaccines were tested in a mouse model of mammary cancer.¹⁶

\[
\text{T helper epitope} \\
\text{TLR2 ligand-SKKKKGCKLFAVWKITYKDTGTSAPDT(α-D-GalNAc)RPAP} \\
\text{TLR2 agonist} \\
\text{MUC1 epitope}
\]

Figure 3. Chemical Structure of Cancer Vaccines¹⁶
The cancer vaccine candidate had a tumor-associated glycopeptide derived from MUC1, the murine MHC class II restricted T\textsubscript{helper} epitope KLFAVWKITYKDT derived from polio virus and the lipopeptide Pam\textsubscript{3}CysSK\textsubscript{3} as an agonist of Toll-like receptor 2 (TLR2). Tumor cells, Yae-1.MUC1 and C57mg.MUC1 were used as a measure of antibody-dependent cell-mediated cytotoxicity (ADCC). The vaccine was shown to induce CTL and ADCC and specific CD8\textsuperscript{+} effector cells that produce interferon \(\gamma\), as measured by ELISA and intracellular cytokine staining (ICC).

### 1.5.3 Vaccine Design

My liposomal anticancer vaccine contained four kinds of components (Figure 4). Glycosylated MUC1 variable number tandem repeat (VNTR), in pink, is conjugated to tumor-associated carbohydrate antigens (TACAs) in red. The L-rhamnose (Rha) epitope was displayed on the surface of the liposome to take advantage of a natural antibody-dependent antigen uptake mechanism. A 20 amino acid sequence of the MUC1 glycopeptide contained a GalNAc-\(\text{O-}\)Thr (Tn) TACA and was conjugated with Pam\textsubscript{3}Cys, Toll-like receptor ligand (TLRL) in blue. Tetraethylene glycol (TEG) is a linker between L-rhamnose (Rha) and cholesterol in green as Rha-TEG-cholesterol. 1,2-dipalmitoyl-\(sn\)-glycero-3-phosphocholine (DPPC) in blue and yellow made up the body of the liposome.
This vaccine was shown to generate a strong anti-MUC1 Tn antibody response that was enhanced by addition of liposomal Rha when recipient mice were making anti-Rha antibodies. The goal of this research was to determine if the CD8$^+$ response could also be increased with the use of Rha-mediated uptake.
Chapter 2

Materials and Methods

2.1 Vaccine Production

The liposome was formulated with Rha-TEG-cholesterol (10%), Pam$_3$Cys-MUC1-Tn (0.69 μM), DPPC (80%), and cholesterol (10%). The first experiment used the peptide Pam$_3$Cys-PDTRPAPGST(α-D-GalNAc)APPAHGVTSA in BALB/c mice and Pam$_3$Cys-HGVTSAPDT(α-D-GalNAc)RPAPGSTAPPA in B6 mice. The liposome was prepared with a total lipid concentration of 30 mM. The stability, homogeneity, and size of the liposome were evaluated by scanning electron microscope (SEM) and dynamic light scattering (DLS) measurements. SEM images at 5 kV acceleration voltages demonstrate the liposome which was conjugated vaccine with Rha-TEG-cholesterol (10%), Pam$_3$Cys-MUC1-Tn (0.69 μM), DPPC (80%), and cholesterol (10%) bind with anti-Rha and mouse anti-human MUC1 antibodies in vitro.\textsuperscript{17}

2.2 Mouse Model
Groups of female BALB/c and C57BL/6 mice (6-8 weeks old), purchased from Jackson Laboratory, Bar Harbor Maine, were primed and boosted with 100 µg per mouse of Ficoll or rhamnose-Ficoll (Rha-Ficoll) in an adjuvant, Imject® Alum 100 µl (Thermo, Rockford, IL), for generation of anti-Rha antibodies in mice. Mice models were ready to be injected with vaccines when anti-Rha antibody titers showed enough anti-Rha antibody level in the serum of mice models compared with non-immunized mice, usually after the second boost. Each BALB/c or C57BL/6 was primed (day 0) and boosted three times (day14, 28, and 42) with 100 µL subcutaneous injections of each vaccine. The MUC1-Tn conjugate vaccines were formulated in phosphate buffer saline (PBS) and brought to 50 µg of antigen peptide per mouse for each injection. The mice were grouped according to Tables 5 and 8.

2.3 DCs Preparation

2.3.1 Isolation of Bones from a Mouse

A non-immunized mouse was killed by cervical dislocation after using a CO₂ gas-chamber. The skin of the mouse was pulled back and dissected to expose the femur and tibia. The knee joints were cut and the muscle along the femur and tibia was removed from the bone. The femur bones were severed from the hip joint. The four bones were rinsed twice in 70% ethanol and sterile PBS, then placed in T cell medium, Dulbecco’s modified Eagle medium (Mediatech, Inc., Manassas, VA) which included 10% fetal calf serum inactivated by heating, 1% HEPES (10mM) pH 7.4, 0.5% 2-mercaptoethanol (3 ×
10^{-5} M), 1% glutamine (2 × 10^{-3} M), 1% penicillin / streptomycin (100 U/ml Penn and 100 µg/ml Strep) (designated T cell medium). The muscle tissues surrounding the femurs and tibias were taken to expose the red lumen.

2.3.2 Extraction of Mononuclear Cells from Bones

Using a 1ml syringe (BD, Franklin Lakes, NJ) and 0.25 mm needle (BD), mononuclear cells from bone marrow were flushed out into T cell medium in a 60×15 mm sterile tissue culture Petri dish (CytoOne). The bone marrow cell clusters were disrupted by pipetting vigorously to create a bone marrow cell suspension. The cell suspension was centrifuged and the supernatant was discarded. The cell pellet was suspended in 1 ml of RBC lysing buffer (Sigma, St. Louis, MO) and incubated for 5 min. at room temperature. The cell solution was quenched by adding 10 ml T cell medium. After centrifugation at 500×g for 5 min, the supernatant was discarded. Washing was repeated one more time.

2.3.3 Bone Marrow Cell Culture with GM-CSF

At day 0, bone marrow leukocytes were cultured at a concentration of 1×10^6 cells/ml in a 25cm² culture flask (Corning Inc., Corning, NY). The cell culture medium contained GM-CSF (PeproTech, Inc., Rocky Hill, NJ) at 100 U/ml (10ng/ml) and IL-4 (PeproTech) at 10ng/ml. At day 3, 75% of the medium in the flask was aspirated without discarding any cells. Fresh T cell medium was added back into the flask with GM-CSF at 100 U/ml
(10ng/ml) and IL-4 at 10ng/ml. At day 4, large clusters of DCs developed and attached to the adherent cells (macrophages and fibroblasts) in the flask. After day 5 the clustering DCs were floating in cell culture medium. During day 6-10 the floating DCs were harvested by centrifugation at 1500 rpm for 5 min. After harvesting DCs, fresh medium was added to the flask with GM-CSF at 100 U/ml (10ng/ml) and IL-4 at 10ng/ml.

2.4 Spleen cell suspension

2.4.1 Spleen Removal

Standard fresh spleen cell culture medium was composed of T cell medium. A mouse was euthanized with CO₂ and cervical dislocation. Under a sterile workspace, the mouse spleen was taken from below the right side rib above the abdominal area using a sterile scissors and forceps. Any connective tissue around the spleen was cut away and only the spleen was placed in the fresh DMEM medium. In some cases, mesenteric, inguinal, brachial and axillary lymph nodes were also collected.

2.4.2 Preparation of Cell Suspension

In a tissue culture hood, the spleen in the fresh T cell medium was transferred into a sterile, loose fitting glass homogenizer. The rotator of the homogenizer disrupted the spleen tissue into a cell suspension. The suspension was centrifuged at 500 rpm for 30 sec to pull down the debris to the bottom of tube. The spleen cell suspension was separated
into a new tube and centrifuged to make the spleen cells pellet at 1500 rpm for 5 min. After discarding the supernatant, 1 ml of RBC lysing buffer (Sigma-Aldrich) was used to lyse red blood cells for 1 min at room temperature in the suspension and the cell solution was quenched with 10 ml of the fresh DMEM medium. The washing step to centrifuge, discard supernatant, suspend the cell pellet, and add fresh medium was repeated twice. About $10^8$ cells per spleen were prepared in a cell suspension for experiments.

### 2.5 Tumor cells

#### 2.5.1 GFP-P815

P815, a mouse mastocytoma-derived tumor cell line transfected with GFP plasmid (from Dr. Yan Cui, Louisiana State University Health Sciences Center), was used for the killing assay. Live GFP-P815 cells have green fluorescence since the GFP plasmid expresses green fluorescent protein. Dead GFP-P815 compared with live GFP-P815 lost the green fluorescence and by uptake of propidium iodide (Sigma-Aldrich) changed to red fluorescence. The color change was used as the marker of cytotoxic T lymphocyte (CTLs) activity against GFP-P815 tumor cells in vitro. The cell line was cultured in Dulbecco’s modified Eagle medium, DMEM with 10% fetal calf serum.

#### 2.5.2 EL-4
EL-4 is a mouse thymoma-derived tumor cell line. This tumor cell line was used for C57BL/6 since the vaccine showed more cytotoxicity in C57BL/6 against EL-4. The cell line was cultured in DMEM with 10% fetal calf serum.

### 2.6 Isolation of CD4$^+$ and CD8$^+$

Dynabeads® FlowComp™ Mouse CD8 (Invitrogen products, Life Technologies, Oslo) and Dynabeads® FlowComp™ Mouse CD4 kits (Invitrogen) were used for isolation of CD4$^+$ and CD8$^+$ cells from spleen cell suspensions. In the first step, a spleen cell pellet was suspended in cold isolation buffer and then incubated with biotinylated CD4 or CD8 T cell antibodies according to the target T cell type. The T cells which had bound the specific antibodies were captured by streptavidin-coated Dynabeads using the magnetic tube holder in the second step. Other cells were left over and discarded in the supernatant except for the target T cell with bound Dynabeads. In the last step, the attached Dynabeads were removed from the target T cells by adding FlowCompTMRelease Buffer. Continually during the separation process, isolation buffer was used, which was composed of Ca$^{2+}$ and Mg$^{2+}$ free phosphate buffered saline (PBS) with 0.1% BSA and 2mM EDTA. 0.1% BSA prevented T cells from attaching on the surface of the plastic tube.

### 2.7 Intracellular Cytokine Staining Assay

#### 2.7.1 Intracellular Cytokine, Interferon gamma (IFN-γ) Staining
After harvesting of DCs from cell culture, DCs were incubated with the vaccine peptide, Pam₃Cys MUC1 Tn (1µg/ml) for 2 hr at 37 degrees, 5% CO₂. CD8⁺ or CD4⁺ T cells derived from immunized mice were mixed with the DCs and incubated for 24 hr. at 4 degrees. Brefeldin A (eBioscience Inc., San Diego, CA) was added at 10µg/ml to the culture. BFA was used to cause accumulation of intracellular IFN-γ inside the cells. Antiviral and antibiotic BFA blocks intracellular protein, IFN-γ, transport after antigen stimulation from the endoplasmic reticulum (ER) to the Golgi complex.¹⁸ After 5 hr, cells were harvested and stained with the desired fluorescent antibodies. Positive control group DCs are pulsed by Concanavalin A (ConA) at 1µg/ml (Sigma) and negative control group DCs were not pulsed. Fix/Perm buffer (eBioscience Inc.) immobilizes intracellular IFN-γ and permeabilizes the cell to allow anti-IFN-γ antibodies to go through the cell membrane for binding with intracellular IFN-γ. The staining of the intracellular IFN-γ with anti-IFN-γ was done during overnight incubation at 4 degrees C. The level of intracellular IFN-γ was measured by the level of anti-IFN-γ (eBioscience Inc.) fluorescence using the Accuri C6 FACS machine (BD Bioscience).

2.7.2 Flow Cytometry
Flow cytometry describes the measurement (meter) of characteristics of single cells (cyto) suspended in a flowing saline stream. The flow cytometry measurement was by Accuri C6. The FACS machine allows collection of individual data from each cell. The

**Figure 5. T cell staining using antibody fluorescence**

The first row of two figures show CD8\(^{+}\) T cells which are positive for anti-CD3\(^{+}\) PE antibody, negative for anti-CD4\(^{+}\) FITC antibody and positive for anti-CD8\(^{+}\) APC. The second row of figures show CD4\(^{+}\) T cells which are positive for anti-CD3\(^{+}\) PE antibody, anti-CD4\(^{+}\) FITC antibody and negative for anti-CD8\(^{+}\) APC.
cell gating application was very useful to separate each CD4⁺, CD8⁺, CD4⁺IFN-γ⁺, and CD8⁺IFN-γ⁺ population from the large number of other cell populations using specific fluorescent antibodies. Anti-mouse CD4⁺ and CD8⁺ antibodies with FITC fluorescent labels were used to label target T cells. Iso-control and anti-IFN-γ antibodies had APC fluorescent labels for detecting only IFN-γ⁺ cells. Thus, CD4⁺ and CD4⁺IFN-γ⁺ T cells appeared in different areas of the flow cytometer figure. Likewise, CD8⁺ and CD8⁺IFN-γ⁺ were separated by different antibody fluorescence. All antibodies were from eBioscience. Figure 5 shows a representative staining.

2.8 Killing Assay

2.8.1 Killing assay using flow cytometry

Live GFP-EL4 expressed green fluorescence because they contained a transfected GFP gene, but dead cells lost the green fluorescence. Propidium iodide (PI, Sigma-Aldrich) entered the dead cell, therefore live cells were differentiated from dead GFP-EL4 cells by the presence of green fluorescence and lack of PI. In order to get 100% live cells, Ficoll-Hypaque (Sigma Histopaque 1083) was used at room temperature, which allows live cells to float on top of the dense layer. An equal volume of GFP-EL4 cell solution was underlayed under Ficoll-Hypaque in a tube. Centrifugation of the tube at room temperature at 3250 rpm for 15 min made the live cells float between the upper cell medium and lower Ficoll-Hypaque. Using a Pasteur pipette, only live GFP-EL4 was
removed. 100% dead cells were prepared by freezing-thawing with dry ice in acetone.

The samples were prepared as shown in Table 4.

Table 4. Live/Dead GFP-EL4 samples for a calibration curve

<table>
<thead>
<tr>
<th>Live/Dead Ratio</th>
<th>Calibration</th>
<th>Unstained</th>
<th>100:0</th>
<th>3:1</th>
<th>1:1</th>
<th>1:3</th>
<th>0:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live GFP-EL4 (μl)</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dead GFP-EL4 (μl)</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Staining Medium (μl)</td>
<td>349</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>DMEM Cell medium (μl)</td>
<td>116</td>
<td>300-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FACS Medium (μl)</td>
<td>535</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

Staining medium is PBS with 2% heat-inactivated fetal calf serum (FCS). The staining medium was used to dilute stock propidium iodide (1mg/ml) 1 to 2000. A calibration sample was prepared with 116μl of DMEM and 349μl of staining medium with propidium iodide. The cells were stained with the propidium iodide (0.5μg/ml) and analyzed on the Accuri C6 flow cytometer (Figure 6).
Figure 6. Killing Assay Data for Live/Dead Calibration Curve

Example of calibration curve samples, the gate P2 indicates live cells. FL1-A is for green fluorescent protein, FL3-A is for propidium iodide.

![Calibration Curve for Killing Assay of GFP-EL4](image)

Figure 7. Calibration Curve for Killing Assay

GFP-EL4 cells were stained as described in 2.8.1 and Table 4
A fixed volume of 50µl was collected per sample in an Accuri C6 FACS machine, allowing the number of live cells per ml to be calculated in event/µl. Event/µl × volume of sample (µl) = the number of cells in P2 in sample. % live cells = number of cells in sample (P2) / number of cells in 100% live sample. A calibration curve of GFP-EL4 fluorescence based on live cell number is displayed and allows us to determine the number of live cells by the number of live cells of the unknown sample in the Flow Cytometer (Figure 7).

2.8.2 JAM Assay Using ³H-thymidine Incorporation Assay

EL4 cells (nontransfected) in DMEM with 10% FCS were incubated with [³H] thymidine (5µCi/ml final concentration) at 37 degree, 5% CO₂ for 3 hr. Staurosporine (1µM in 200µl), an inhibitor of phospholipid/Ca²⁺ dependent protein kinase C that induces apoptosis in cells, was used as a positive control for cell death by apoptosis.¹⁹ The dilution series of staurosporine was prepared to determine the optimal concentration of staurosporine for killing EL4 cells. The drug was applied to the EL4 cells for different incubation times (6 and 21 hr). Staurosporine at 0.3µM killed EL4 effectively at 21 hr incubation at 37 degrees, 5% CO₂. Effector cells among activated CD8⁺ T cells killed the tumor cell EL4 labeled with [³H] thymidine. Dead cells produced fragmented DNA debris after killing by CD8⁺ T cells. The dead EL4 DNA fragments labeled with [³H] thymidine were separated from intact DNA during harvesting onto a Perkin Elmer Uni-Filter-96 GF/B filter. Radioactivity on the filter was determined by addition of scintillation fluid and counting with a Top Count scintillation counter. For the assay, EL-
4 cells in DMEM with 10% FCS were pulsed with MUC1Tn peptide and incubated at 37 degrees, 5% CO₂ overnight prior to labeling with [³H] thymidine for 3 hr, after washing, EL-4 cells (1×10⁴) were added per well to a round bottom 96-well plate, followed by addition of CD8⁺ T cells. The ratio of CD8⁺ to EL-4 was 10 to 1. The plate was centrifuged at 350×g for 5 min and then incubated for 6 hr at 37 degrees, 5% CO₂. The change of radioactivity was compared with positive control EL4 which was treated with Staurosporine. The decrease in retained [³H] thymidine showed the capability of CD8⁺ T cells to kill the tumor cell EL4. This assay is faster, more sensitive, easier to set up and safer than the standard ⁵¹Cr release assay which has been used to detect cell death using radioactive ⁵¹Cr. ²⁰ Percent of killing was calculated using percentage of live cells. The radioactivity (CPM) of sample was divided by the negative control sample’s radioactivity which had 100% live cells. The total number of percentage of live cells and killed cells in each sample should be 100%. Hence, the percentage of killed cells are calculated by the percentage of live cells and then the specific killing percentage was calculated by the difference of percent killing between samples with or without peptide.

2.9 ELISA

2.9.1 Anti-Rhamnose antibody ELISA

In the mouse model, mice were primed and boosted with Rha-Ficoll injection to produce anti-Rhamnose antibodies. To determine the level of anti-Rha antibody in mouse serum, an ELISA assay of anti-Rha antibody was prepared. Immulon ELISA assay plastic
96-well plates were coated with Rha-BSA (2µg/ml) or BSA (2µg/ml) in PBS and incubated overnight at 4 degrees. Then the plates were washed five times using washing buffer (PBS with 0.1 % Tween-20). Blocking buffer (1mg/ml BSA in washing buffer) was added to prevent other proteins from nonspecific binding to the plastic plate well surface. After 1 hr. incubation, unbound BSA was washed from the plate. Rha-Ficoll immunized mouse serum and non-immunized mouse serum were diluted in a five-fold series (1/5, 1/25, 1/125) with dilution buffer (1mg/ml BSA in PBS). 50μl of each sample was applied to the plate and the plate was incubated at r.t for 1 hr, followed by five times washing with washing buffer. After adding 100μl of HRP-goat anti-mouse IgG (Sigma), which was specific to Fab, diluted 1/5000 using dilution buffer, the plate was incubated at room temperature for 1 hr, followed by five times washing. In the last step, 100μl of one component HRP substrate (Bio, FX) was added to each well at room temperature. After allowing color to develop for around 10 to 30 min, the absorbance was measured in a SpectraMax ELISA reader machine at 620 nm wave length.

2.9.2 Interferon gamma (IFN-γ) ELISA

An ELISA assay using anti-IFN-γ was used to quantify the concentration of IFN-γ in the supernatant of stimulated T cells. The level of IFN-γ in the supernatant of T cells implied how effectively DCs with or without peptide presented the CD8⁺ epitope to CD8⁺ T cells and activated the CD8⁺ T cells. In order to accurately quantitate IFN, a recombinant murine IFN gamma ELISA kit (Peprotech) was used to generate a standard IFN-γ calibration curve and analyze samples. Anti-mouse IFN-γ monoclonal antibody
(capture antibody) was coated on a 96-well plate (100μl/well) at a final concentration of 1μg/ml and incubated at 4 degrees overnight. The plate was emptied and washed three times with washing buffer (PBS with 0.1% Tween 20) and blocked with 200μl of blocking buffer (1mg/ml BSA in washing buffer). After 1 hr incubation at room temperature, 100μl of standard IFN-γ samples diluted in a two-fold series (2, 1, 0.5, 0.25 ng/ml) and culture supernatant samples (1/1, 1/2 dilutions) were added to the coated plate and incubated at 4 degrees overnight in the sealed plate. After 5 times washes, 100μl of detection antibody (biotin-conjugated anti-mouse IFN-γ) was added to each well and incubated at r.t. for 1 hr. Wells were washed 5 times. 100μl of Avidin-HRP, which was diluted 1/2000 with buffer (1mg/ml BSA in PBS), was added to each well. After incubation at r.t. for 30 min, the plate was washed three times and 100μl of 1×TMB substrate solution at r.t. was added to each well. After 15 min, the plate was read in a plate reader at 620 nm wave length. Dilutions of standard IFN-γ samples and culture supernatant samples did not have the same background because culture samples were already in DMEM medium but the standard samples were in only dilution buffer. For selecting a reasonable solvent, an IFN-γ standard curve was measured using dilution buffer and DMEM medium as diluents. Detection of IFN-γ was more sensitive in dilution buffer, however DMEM T cell medium was used to dilute both standard IFN-γ and culture supernatant in the experiment (Figure 8).
For investigation of T cell proliferation, T cells (1×10^4) and irradiated (1500 Rads) DCs (1×10^3) were mixed to bring the total volume per well to 100μl in a flat-bottomed 96-well tissue culture plate. The 8 amino acid residue peptide of MUC1 Tn antigen peptide SAPDT-(Tn)RPA was used as the stimulating antigen. After incubation at 37 degrees and 5% CO₂ for 5 days, diluted [³H]-thymidine, 1μCi per well, was added to each well for incorporation into the DNA of CD8⁺ T cells. Harvest of radioactive DNA of CD8⁺ T cells was done by a cell harvester onto glass fiber filter paper (Perkin Elmer Uni-Filter-96 GF/B). After the plate had dried overnight at r.t., a white sticker was sealed on the bottom of the plate so that scintillation fluid did not leak. 35μl of scintillation fluid
(EcoLite) was added to each well of the 96-well filter plate. The plate was sealed over the top using a piece of transparent tape. The radioactivity of DNA of CD8+ T cells was measured by the TopCountNXT.
Chapter 3

Results and Discussion

3.1 Vaccine Test

The efficiency of the liposomal vaccine (MPLA/Rha-cholesterol/Pam$_3$Cys/MUC1-Tn), which had human MUC1 Tn antigen, 20 amino acid sequence PDTRPAPGST-(Tn)APPAHGVTSA, was tested using the BALB/c mouse model which was primed with Ficoll or Rha-Ficoll. After priming, injection of vaccine MPLA/Rha cholesterol/Pam$_3$Cys/MUC1-Tn was done every two weeks to each of five mice of group E* and F three times (Table 5).

Table 5. Mice model categories

<table>
<thead>
<tr>
<th>BALB/c group</th>
<th>E</th>
<th>E*</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primed by</td>
<td>Ficoll</td>
<td>Ficoll</td>
<td>Rha-Ficoll</td>
</tr>
<tr>
<td>Immunization with</td>
<td>No</td>
<td>MPLA/</td>
<td>MPLA/</td>
</tr>
<tr>
<td></td>
<td>Vaccine</td>
<td>Rha-cholesterol/Pam$_3$Cys/MUC1-Tn</td>
<td>Rha-cholesterol/Pam$_3$Cys/MUC1-Tn</td>
</tr>
</tbody>
</table>

After the second injection of the vaccine, an ELISA assay of anti-MUC1 Tn antibodies in immunized BALB/c serum was done to demonstrate that Rha-mediated uptake enhanced
the antibody response\textsuperscript{17}. Cells from these same mice were then tested for generation of CD8\textsuperscript{+} cytotoxic cells against the vaccine using the fluorescence assay. On day 6 after the third boost with the vaccines, two mice of each group E, E*, F were killed and the spleens were taken for separation of CD8\textsuperscript{+}T cells. Mouse tumor GFP-P815 (3×10\textsuperscript{4} in 100μl DMEM with 10% FCS) was pulsed with human MUC1 Tn pepteide (1μg/ml) for 2 hr and a Ficoll-Hypaque gradient was used to isolate all live cells. CD8\textsuperscript{+} T cells (1.5×10\textsuperscript{6} or 3×10\textsuperscript{6} in 100μl DMEM T cell medium) separated from each group of spleens were applied to the target tumor GFP-P815 (3×10\textsuperscript{4} in 100μl DMEM with 10% FCS) for 6 hr to allow killing. Propidium iodide was added to each sample and live GFP-P815 tumor cells were counted using the Accuri C6. The percentage of killing by cytotoxic T cells was analyzed (Table 6).

\textbf{Table 6. CTL cytotoxicity using MPLA/Rha-cholesterol/Pam\textsubscript{3}Cys/MUC1 Tn}

<table>
<thead>
<tr>
<th>BALB/c group</th>
<th>Primed by Ficoll</th>
<th>E</th>
<th>E*</th>
<th>F (Anti-Rha)</th>
<th>Rha-Ficoll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunization with</td>
<td>No Vaccine</td>
<td>MPLA/ Rha-cholesterol/Pam\textsubscript{3}Cys/ MUC1-Tn</td>
<td>MPLA/ Rha-cholesterol/Pam\textsubscript{3}Cys/ MUC1-Tn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLs of group</td>
<td>E</td>
<td>E*</td>
<td>E* &amp; ConA (1μg/ml)</td>
<td>F</td>
<td>No CTLs</td>
</tr>
<tr>
<td>GFP-P815:CTLs</td>
<td>1:100 1:300</td>
<td>1:100 1:300</td>
<td>1:300</td>
<td>1:100 1:300</td>
<td>Live GFP-P815</td>
</tr>
<tr>
<td>% of Lysis w/o peptide</td>
<td>12 30</td>
<td>69 71</td>
<td>71</td>
<td>62 66</td>
<td>0 100</td>
</tr>
<tr>
<td>% of Lysis with peptide (1 μg/ml)</td>
<td>22 28</td>
<td>44 61</td>
<td>73</td>
<td>62 54</td>
<td>0 100</td>
</tr>
</tbody>
</table>

Immunized mice were euthanized and CD8\textsuperscript{+} effector cells were separated from each mouse spleen. GFP-P815 was incubated with the vaccine peptide and mixed with CD8\textsuperscript{+} T cells based on each sample condition. CTL cytotoxicity was measured by the percentage of live GFP-P815.
CTLs of E* ConA (1 µg/ml) killed more GFP-P815 with Pam₃Cys MUC1-Tn (1 µg/ml).

CTLs of E or E* killed more GFP-P815 without Pam₃Cys MUC1-Tn (1 µg/ml). It was determined that the human MUC1 Tn antigen, 20 amino acid sequence PDTRPAPGST-(Tn)APPAHGVTSA did not generate significant CTL to kill tumor GFP-P815. The second assay data (Figure 9) showed more killing of GFP-P815 than the first data (Table 6) with vaccine peptide. Group F showed 30.6% specific killing compared to 11.8% specific killing for Group E*. However, the killing assay was not repeated therefore the vaccine efficiency to generate CD8⁺ that would kill tumor cells was not confirmed.

The generation of IFN-γ producing CD8⁺ cells was also tested. Separated CD8⁺ T cells (2×10⁶ in 500µl DMEM T cell medium) were applied to DCs (2×10⁵ in 500µl DMEM T cell medium) pulsed with Pam₃Cys MUC1 Tn (1 µg/ml) for 2 hr and positive control DCs were pulsed with ConA (1 µg/ml). After 20 hr incubation at 37 degrees, 5% CO₂, BFA at 10µg/ml was added to CD8⁺ T cells to fix intracellular IFN-γ and incubated for 5 hr at 37 degrees, 5% CO₂. Fc-block (6 µl) at 1/500 diluted in FACS medium was used to block anti-IFN-γ antibody nonspecific binding to Fc receptor on the cell surface and incubated for 10 min at 4 degrees. CD8⁺ T cells (1×10⁵ per tube) were aliquotted. The first antibody, anti-CD8⁺ FITC (100 µl at 1/100 diluted in FACS medium) was added to the CD8⁺ T cells (1×10⁵ per tube) and incubated for 30 min at 4 degrees. Fix-Perm buffer (200µl/tube) was added and incubated overnight at 4 degrees to allow the cell wall to be permeable. Perm buffer (2ml per tube) was used to wash the CD8⁺ T cells (1×10⁵ per tube). Iso-control APC antibody (40µl at 1/100 dilution in FACS medium) and anti-IFN-γ APC (40µl at 1/100 dilution in FACS medium) were added to stain the CD8⁺ T cells. After incubation at 4 degrees for 30 min, 100µl of cells per tube were applied to Accuri
C6 and analyzed for counting CD8+IFN-γ+ T cells. The % isotype+ cells was subtracted from the % IFN-γ+ cells. The results are shown in Table 7. It was concluded that the original vaccine was not effective to enhance the cytotoxic T cell response against tumor cells or to generate CD8+ effector cells.

![Percentage of Killing of GFP-P815](image)

**Figure 9. Percentage of Killing of GFP-P815**

The ratio of CD8+ to GFP-P815 was 50 to 1. Groups E and E* received Ficoll and Group F received Rha-Ficoll. Group E was not vaccinated and Groups E* and F received the MPLA/Rha-cholesterol/Pam3Cys/MUC1-Tn vaccine. E* with ConA was positive control sample.
Table 7. BALB/c mice CD8$^+$ IFN-γ$^+$ T cell response using MPLA/Rha-cholesterol/Pam$_3$Cys/MUC1 Tn

<table>
<thead>
<tr>
<th>BALB/c group</th>
<th>E</th>
<th>E*</th>
<th>E* &amp; ConA (1μg/ml)</th>
<th>F (Anti-Rha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primed by</td>
<td>Ficoll</td>
<td>Ficoll</td>
<td>Ficoll</td>
<td>Rha-Ficoll</td>
</tr>
<tr>
<td>Injection</td>
<td>No Vaccine</td>
<td>MPLA/ Rha-cholesterol/Pam$_3$Cys/ MUC1-Tn</td>
<td>MPLA/ Rha-cholesterol/Pam$_3$Cys/ MUC1-Tn</td>
<td>MPLA/ Rha-cholesterol/Pam$_3$Cys/ MUC1-Tn</td>
</tr>
<tr>
<td>Percentage of CD8$^+$ IFN-γ$^+$ cells with no stimulation</td>
<td>-0.60</td>
<td>0.30</td>
<td>1.15</td>
<td>0.65</td>
</tr>
<tr>
<td>Percentage of CD8$^+$ IFN-γ$^+$ cells with Pam$_3$Cys/ MUC1-Tn</td>
<td>-1.15</td>
<td>-0.60</td>
<td>-</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Examination of the vaccine sequence showed that the peptide might lack a good CD8$^+$ epitope. Hence, a new liposome vaccine, Rha-cholesterol/Pam$_3$Cys MUC1 Tn (human MUC 1 Tn antigen peptide sequence, HGVTSAHDT-(Tn)RPAPGSTAPPA, Figure 10) was designed to stimulate cytotoxicity of CD8$^+$ T cells and applied to C57BL/6 mice grouped as shown in Table 8. This peptide contains the 8 amino residue CD8$^+$ T cell epitope SAPDT(Tn)RPA$^{15}$. 

3.2 New Pam$_3$Cys MUC1-Tn Vaccine Test
3.2.1. Anti-Rha antibody production

Table 8. Mice model categories

<table>
<thead>
<tr>
<th>C57BL/6</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized with</td>
<td>None</td>
<td>Rha-Ficoll</td>
<td>None</td>
<td>Rha-Ficoll</td>
</tr>
<tr>
<td>Vaccinated with</td>
<td>Pam₃Cys/ MUC1-Tn</td>
<td>Pam₃Cys/ MUC1-Tn</td>
<td>Rha-cholesterol/Pam₃Cys/ MUC1-Tn</td>
<td>Rha-cholesterol/Pam₃Cys/ MUC1-Tn</td>
</tr>
</tbody>
</table>

In the C57BL/6 mouse model, mice were primed and boosted with Rha-Ficoll injection to produce anti-Rhamnose antibodies. To determine the level of anti-Rha antibody in mouse serum, an ELISA assay of anti-Rha antibody was prepared.
Rha-Ficoll immunized mice sera bound to Rha-BSA antigen with the highest absorbance (Figure 11). It was determined that Rha-Ficoll immunized mice had anti-Rha antibody in their serum. Hence, the C57BL/6 mice were ready to test the immune efficiency.

3.2.2 Anti-MUC1 antibody production

Using the same procedure, a new liposome vaccine (Figure 11. Rha-cholesterol/Pam₃Cys/MUC1-Tn or Pam₃Cys/MUC1-Tn), which had human MUC1 Tn
antigen, 20 amino acid sequence HGVTSAPDT-(Tn)RPAPGSTAPPA, was tested in C57BL/6 mice. After priming with Ficoll or Rha-Ficoll, groups of seven mice A, B, C, and D were injected every two weeks with liposome vaccine Rha-cholesterol/Pam3Cys/MUC1-Tn or Pam3Cys/MUC1-Tn. After a second injection of the vaccines, ELISA assay of anti-MUC1-Tn antibodies in immunized C57BL/6 serum was done to determine if the antibody response was enhanced by Rha-mediated uptake by APC (Figure 12).

**Figure 12.** ELISA assay of anti-MUC1 Tn antibody in C57BL/6 mice sera
ELISA plates were coated with MUC1-Tn (25μg/ml). Dilution of sera from groups A, B, C, and D were tested. Data analysis by Partha Karmakar.

Groups A and C mice were not primed therefore no anti-Rhamnose antibody was in C57BL/6 mice sera. Groups B and D mice were primed with Rha-Ficoll therefore anti-Rhamnose antibody was in C57BL/6 mice sera. Group D mice, which were primed with Rha-Ficoll and immunized with Rha-cholesterol/Pam3Cys/MUC1 Tn, showed the highest
absorbance with MUC1-Tn antigen therefore the largest amount of anti-MUC1-Tn antibody present in the sera.

### 3.2.3 CD8$^+ \text{ proliferation assay}$

CD8$^+$ T-cells were isolated from 2 mice spleens of each group A, B, C and D on day seven after the 3rd boost of rhamnose-liposome vaccine (Rha-cholesterol/Pam$_3$Cys MUC1 Tn) or normal liposome vaccine (Pam$_3$Cys-MUC1 Tn, Figure 10), using Dynabeads Flow Comp$^\text{TM}$ Mouse CD8 separation kit. CD8$^+$ T-cells ($2 \times 10^4$) and DCs ($2 \times 10^3$) were pulsed with rhamnose-lioposome (25$\mu$g/ml) or normal liposome vaccine (25$\mu$g/ml) and incubated for 6 days. On day 5, CD8$^+$ T cell DNA was labeled with radioactive $[^3\text{H}]\text{thymidine}$ at 37 degrees, 5% CO$_2$ overnight and at the end of day 6, the cells were harvested onto the glass fiber filter (Perkin Elmer Uni-Filter-96 G/B) using the Cell Harvester. After the plate had dried overnight, the plate was applied to Perkin-Elmer TopCount NXT. The radioactivity (CPM) showed how many proliferating CD8$^+$ T cells were in the culture (Figure 13).
Figure 13. C57BL/6 mice CD8$^+$ T cell Proliferation Assay

CD8$^+$ T cells were incubated with DCs and the indicated amount of antigen for 6 days, including an overnight pulse of $[^3]$H thymidine. Anti-Rhamnose antibody presence in sera was labeled by Rhamnose mice and absence as Non-Rhamnose. Normal liposome vaccine (25μg/ml) was indicated with Non-Rhamnose Liposome and Rhamnose-cholesterol liposome vaccine (25μg/ml) with Rhamnose Liposome. Data analysis by Partha Karmakar.

The level of $[^3]$Hthymidine (CPM) was similar in CD8$^+$ T-cells proliferation for the groups A, B and C whereas group D had much higher CD8$^+$ T-cell proliferation.
compared to the others. This demonstrated that effector CD8$^+$ T cells, which had responded to rhamnose liposome vaccine bearing human antigen MUC1 Tn in the presence of anti-Rha antibodies, were immunized better than effector CD8$^+$ T cells which had seem the normal liposomal vaccine in the absence of anti-Rha antibodies. Hence, it was concluded that the new vaccine was able to enhance the CD8$^+$ T cell response through Rha-mediated uptake by APC.

3.2.4. CD8$^+$ Cytokine production

For ICC assay, spleen cells (1×10$^6$ in 1ml of DMEM T cell medium per well) of each group A, B, C and D were incubated with DCs (1×10$^4$ in 100μl of DMEM T cell medium per well) and IL-2 (4μl at 20U/ml per well) at 37 degrees, 5% CO$_2$ for 14 days in order to culture CD8$^+$ T cells. The Pam$_3$Cys MUC1 Tn (5μg/ml) was a stimulator of CD8$^+$ T cell proliferation with IL-2. Every 3 days IL-2 (4μl at 20U/ml per well) was added the culture well. After 14 days, the intracellular cytokine staining assay attempted on the surviving cells, but most the CD8$^+$ T cells of A, B, C and D were dead. Thus, the CD8$^+$IFN-$\gamma^+$ T cell response with the new vaccine was not able to be analyzed in immunized C57BL/6 mice.

3.2.5. Small peptide (8 amino acids residue of new MUC1 Tn) test

We thought that detection of anti-MUC1 CD8$^+$ T cells in our assays would be low if the vaccine epitope was not efficiently cross-presented by MHCI. The new liposome
vaccine, Rha-cholesterol/Pam₃Cys MUC1 Tn (human MUC1 Tn antigen peptide sequence HGVT SAPDT-(Tn)RPAPGSTAPPA) was designed to stimulate the cytotoxicity of CD8⁺ T cells. A small peptide (8 amino acids sequence, SAPDT-(Tn)RPA) was selected as a CD8⁺ T cell epitope of the human MUC1 Tn antigen. If the 8 amino acid sequence had high affinity for murine MHC class I, H-2Kᵇ, it would bind to MHC-I without needing cross presentation by the DCs or tumor cells. The small peptide should be presented to CD8⁺ T cells and result in more CD8⁺ T cells stimulated by DCs’ or tumor cells’ MHC class I.

Group D mice CD8⁺ T cells (1×10⁴/well) and irradiated DCs (1×10³/well) were incubated with 8 amino acid sequence, SAPDT-(Tn)RPA at 37 degrees, 5% CO₂ for six days. The growth stimulant was the small peptide at a different concentration in each well (2.5, 5, 10, 20μg/ml). The DCs were irradiated with 1200 Rads to prevent proliferation of the antigen presenting cells.

On day 5, CD8⁺ T cells were incubated with radioactive [³H]thymidine at 37 degrees, 5% CO₂ overnight and at the end of day 6, the cells were harvested onto the glass fiber filter (Perkin Elmer Uni-Filter-96 G/B) using the Cell Harvester. After the plate had dried overnight, the plate was applied to a Perkin-Elmer TopCount NXT. The radioactivity (CPM) showed how many proliferating CD8⁺ T cells were in the culture. Lower CPM implied fewer dividing cells (Figure 14).
The data showed that the small peptide stimulated CD8+ T cells of group D, C57BL/6 mice. The high concentration peptide (25 μg/ml) increased proliferation of CD8+ T cells of group D, C57BL/6 mice. Hence, the new liposome vaccine, Rha-cholesterol/Pam3Cys MUC1 Tn (HGVTSAPDT-(Tn)RPAPGSTAPPA), is able to prime CD8+ T cells that can respond to the 8-residue peptide on DCs. Also, IFN-gamma production data supported the 8 amino acid residue test. C57BL/6 mice were vaccinated with Pam3Cys MUC1-Tn. APC were prepared using spleen cells (5×10^5) with and without the 8 residue peptide (10 microgram/ml). We expected antigen presenting cells among spleen cells might present the small peptide. Then the spleen cells were added to CD8+ T cells and incubated for 20 hrs. The cell culture supernatant (1 ml) was saved for IFN-γ ELISA assay. IFN-
gamma production would show that the small peptide presented on APC stimulated CD8\(^+\) T cells to produce the cytokine.

The difference of IFN-\(\gamma\) production was calculated by the equation derived in Figure 15. Specific stimulation (with 8 residue peptide-without peptide) of group D CD8\(^+\) T cells caused production of 103 pg/ml IFN-\(\gamma\).

3.2.6 JAM assay of C57/B6 with Pam\(_3\)Cys MUC1 Tn liposomal vaccine
A JAM assay of cytolytic activity was done using CD8\(^+\) CTL from Group B, C, and D C57BL/6 mice which were vaccinated with Pam\(_3\)Cys MUC1 Tn (HGVTSA\(\text{PDTR}\)PAPGSTAPP). Group A mice samples were not able to be analyzed because of losing the CD8\(^+\) T cells. The Pam\(_3\)Cys MUC1 Tn liposomal vaccine had either no Rhamnose or Rhamnose in it. The 8 amino acid small peptide was used to pulse EL-4 tumor cells because it was concluded that the small peptide (8 amino acids sequence, SAPDT-(Tn)RPAP) was a CD8\(^+\) T cell epitope of the human MUC1 Tn antigen. The small peptide should be presented to CD8\(^+\) T cells by MHC class I molecules of EL-4 tumor cells and result in stimulation of tumor antigen specific cytotoxic CD8\(^+\) T cells among the spleen cells. It was found that the tumor antigen specific cytotoxic CD8\(^+\) T cells of Group D killed more EL-4 cells than did those of Groups B and C with 8 amino acid peptide epitope (Figure 16). It showed the enhanced CD8\(^+\) T cell stimulation of the Rha-vaccine in immunized mice with Rhamnose antibodies in their sera.
3.3. Discussion

The CD8$^+$ T cell response is important to anti-tumor immune therapy. A more effective antigen specific CD8$^+$ T cell response is required to make enhanced anti-tumor activity in mouse models. In previous work by Sourav Sarkar, vaccination with a
rhamnose-liposomal vaccine showed enhanced antibody response and CD4⁺ T\textsubscript{helper} cell response against a tumor cell antigen but CTL cytotoxicity was not investigated. My project asked whether the liposomal vaccine peptide was taken up by APC and cross-presented to CD8⁺ T cells and if anti-rhamnose antibodies could enhance this cross presentation. In the beginning of this project, CD8⁺ T cell specific epitope sequence and the optimized data of vaccine peptide for the immune response were unknown. The previous 20 amino acid vaccine peptide was used to initiate a CD8⁺ T cell response. The cytotoxicity against tumor cells and production of IFN-γ were the biomarker to show an effective CD8⁺ T cell response. The previous 20 amino acid vaccine peptide was not effective to produce a strong CD8⁺ T cell response. So, a different vaccine peptide sequence was made to vaccinate mice, which contained an 8 amino acid sequence which was a known epitope for CD8⁺ T cells. Those data in this thesis were obtained using this peptide. The new liposomal rhamnose vaccine showed an enhanced CD8⁺ T cell immune response against the tumor antigen as measured by proliferation, IFN-γ production, and CTL killing. Also, the optimized amount of vaccine in mouse model was determined and the specific 8 amino acid sequence in new vaccine peptide was proved to be an epitope for CD8⁺ T cell. In the future, the immune efficiency of the new liposomal vaccine peptide will be tested for outcomes in tumor model.

In another way, the antigen specific CTL response may be induced by ex vivo targeting of the macrophage mannose receptor.\textsuperscript{22} In mice, oxidized mannan linked to MUC1 (M-FP), was injected to induce potent MHC-restricted CTL and tumor protection. Due to the resistance of cancer patients against immunization, ex vivo immunization of macrophage and DCs was conducted using oxidized mannan MUC1. The vaccine
targeted to the mannose receptor on APC and allowed the MHC I antigen restricted CTL activation. It resulted in high frequency of CTLs and protection against the tumor progression.\textsuperscript{22}
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