Developing a Novel Platform for Neoantigen Identification

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Developing a Novel Platform for Neoantigen Identification

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A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

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Abstract

Tumor infiltrating CD8+ cytotoxic T lymphocytes (CTLs) recognize tumor-derived peptide antigens, or neoantigens, presented on MHC Class I molecules to elicit an anti-tumor immune response. Neoantigens originate from the accumulation of tumor-specific somatic mutations in DNA sequences, such as point and frameshift mutations. These mutations generate modified peptides that can be found uniquely on the surface of a tumor cell, making them crucial target molecules in cancer immunotherapies for limiting off-target toxicity effects. The ability to identify neoantigens remains a great challenge in the field.

This thesis is focused on the development of a platform for neoantigen discovery and isolation employing a novel magnetic bead based system to enrich for cells expressing peptide MHC Class I complexes. Using a B16 F10 mouse melanoma cell line as a model system we present a unique approach for peptide presentation and peptide elution for mass spectrometry (MS) and high performance liquid chromatography (HPLC).

Utilizing this approach for detection of abundant peptides in an in vitro setting could then be translated to isolate neoantigens of low abundance in a clinical tumor model. This protein purification based method provides an alternative to current methods, such as high-throughput sequencing for neoantigen predictions, which may not be reliable. Improving upon methods to discover neoantigens is necessary for developing targeted, personalized treatment therapies that can boost anti-tumor immune responses.
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1. Chapter 1: Background

1.1 Background

Cancer is a major leading health concern in the world, affecting millions of individuals each year\(^1\). Its growing prevalence and complexity have created significant challenges in prevention, diagnoses, and treatment\(^2\). Traditional, non-specific cancer therapies including chemotherapy and radiation therapy pose the risk of secondary effects on normal cells causing patients to experience a variety of harmful side effects. For this reason, there has been a shift in focus to the field of cancer immunology to develop more targeted, cancer-specific therapies\(^3\).

Cancer immunology centers on exploring the interplay between the immune system and tumors\(^4\). By delving into such interactions, researchers have made significant advancements in learning how to manipulate one’s own immune response to eradicate cancers\(^5\). The immune system normally targets malignant tumors through the recognition of antigens, which are presented on Major Histocompatibility Complexes (MHC). The initial step involves the uptake of tumor antigens by antigen presentation cells (APCs), which then prime and activate CD8+ cytotoxic T lymphocytes (CTLs) in the lymph node. Once activated, CTLs infiltrate into the cancer site by responding to a variety of tumor-produced inflammatory chemokines, including CCL3, CCL5, and CX3CL1\(^6\). Within the tumor microenvironment, the receptors of CTLs engage tumor antigens presented on MHC I molecules on the surface of tumor cells to initiate an immune response\(^7\). This interaction between these tumor infiltrating lymphocytes (TILs) and corresponding tumor antigens has been a core focus of the field\(^8\).

One hallmark of malignant tumors is their ability to develop both direct and indirect mechanisms to evade the host’s normal immune response\(^5\). These evasion techniques include up-regulation of inhibitory receptors, down-regulation of MHC Class I molecules, release of
immunosuppressive factors, and recruitment of immunosuppressive cells. Such mechanisms prove challenging for the ability of immune cells to both recognize and destroy the tumor. The goal of immunotherapies is to overcome this inhibitory environment and clear out the tumor through stimulating robust anti-tumor immune responses. Therapies designed for this purpose have been developed in the form of antibodies, chimeric antigen receptor (CAR) T cells, and vaccines.

Antibodies designed for therapeutic purposes can act through a variety of mechanisms: coating cancer cells, triggering cell destruction, blocking cell growth, etc. Several therapies have been developed to inhibit growth and functional proteins expressed on cancer cells, including anti-HER2 receptor in breast cancer, anti-epidermal growth factor receptor (EGFR) in both colorectal and head and neck cancer, and anti-CD20 in B-cell lymphomas, all of which have shown improvements in clinical outcome. Other monoclonal therapies have been developed to block inhibitory protein receptors expressed on immune cells, such as cytotoxic T lymphocyte antigen-4 (CTLA-4, ipilimumab) and programmed death-1 (PD-1, pembrolizumab). These receptors are normally expressed on immune cells to limit autoimmune occurrences, though in the tumor microenvironment, malignant cells can upregulate corresponding inhibitory ligands to prevent immune cell activation and consequent effector functions. Also known as checkpoint blockade therapy, this method, although successful in various clinical applications to promote tumor cell death, comes with the undesired risk of immune-related adverse toxicity events, including diabetes, diarrhea, colitis and hepatitis, that may linked to high levels of inflammatory cytokine production. Current experimental trials are investigating how to manage such undesired effects while consequently improving the efficacy of the therapy.
Another common therapeutic strategy involves the use of CAR T cells. These cells consist of chimeric constructs that resemble the function of traditional T cell receptors by coupling a high-affinity antibody with a TCR’s natural co-stimulatory molecules to induce a more potent anti-tumor immune response\textsuperscript{16}. The receptors of these T cells are engineered to be directed at a tumor antigen of interest and the overall design of CARs has developed over time: first generation CARs consist of an activating Cd3ζ signaling molecule component, while second and third generation CARs, include various co-stimulatory molecules, such as CD28, 4-1BB, and OX40 linked to the CD3ζ signaling molecule,\textsuperscript{16} which provide secondary signals to enhance T lymphocyte activation. In current treatment models, T lymphocytes from the patient are transduced \textit{ex-vivo} environment with the CAR gene construct, expanded, and then transferred back into the patient. Once incorporated within the immune system, these cells are able to induce a strong, enhanced response after recognition of their target antigen\textsuperscript{5}.

Cancer treatment vaccines also serve as important immunotherapies which are administered using tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) combined with a chemical adjuvant for triggering strong anti-tumor immune responses\textsuperscript{17}. One such vaccine that has been approved by the FDA is Provenge (sipuleucel-T), administered for the treatment of prostate cancer, whereas a multitude of other promising therapeutic vaccines are undergoing clinical testing\textsuperscript{18,19}. Effective vaccines should be designed to enhance the inflammatory nature of APCs, improve activation of T cells, and promote lymphocytes infiltration to the target site as well as ability to respond to foreign antigens. Challenges have been encountered due to the difficulty in overcoming the immunosuppressive tumor environment, which can contribute to a lack of potency and efficacy\textsuperscript{20}. To create an additive, more potent anti-tumor response, combination therapies are currently being explored\textsuperscript{19}. 
One of the biggest challenges faced when developing immunotherapies is identifying TSAs, or neoantigens, which serve as crucial targets for immunotherapies. Neoantigens arise from tumor-specific somatic mutations in DNA, which alter the amino acid sequences to produce mutant proteins. These proteins can be degraded by the proteasome into small peptides consisting of 8-11 monomers and assembled onto an MHC Class I molecule in the Endoplasmic Reticulum. The peptide-MHC I complex is processed in the Golgi and transported to the surface of the tumor cell, to be recognized by CTLs. Unlike TAAs, which are found on both tumor and normal cells, neoantigens are expressed exclusively on tumor cells. Targeting tumor-specific antigens is especially important when dealing with therapy involving immune cells, due to their high sensitivity to antigens. This sensitivity creates a large risk of destroying normal healthy cells that may express a TAA at basal levels. Once successfully isolated, neoantigens can be incorporated into the development of therapeutic treatments that can provide a very robust, on-target, anti-tumor immune response to promote tumor regression with minimal side effects for patients, such as autoimmunity. Several personalized neoantigen-based immunotherapies have been or are being developed for various types of cancer, and show success in promoting strong T cell infiltration, activation and killing. Expanding the list of neoantigens that can be used for vaccine purposes remains a significant need in the field.

One of the first methods to identify neoantigens relied on using complementary DNA (cDNA) expression libraries. cDNA libraries are generated from tumor mRNA by a reverse transcriptase, incorporated into vectors in pools of 10 to 100, and transfected into an APC line expressing MHC I. The peptides expressed by the APCs are then screened by CTLs and evaluated based on their ability to trigger a cytokine-dependent T cell response. This method
was successful in isolation of various neoantigens, most abundantly in melanoma, including, but not limited to, mutated cyclin-dependent kinase 4 (CDK4)\textsuperscript{26}, mutated beta-catenin (CDC27)\textsuperscript{27} and melanoma ubiquitous mutated 1 (MUM-1)\textsuperscript{28}. The use of cDNA expression libraries to identify neoantigens is very limited in throughput and efficiency, mainly due to the necessity to assess the entire span of tumor-derived peptides, so alternate methods have since been explored.

![Figure 1](image.png)

**Figure 1.** The process of neoantigen-MHC I surface expression. (Image from Zolkind et al., Oral Oncology 2016).

Recent technological advances gave rise to high-throughput sequencing methods that have been applied to neoantigen identification. These *in silico* computational multi-step processes begin with whole-exome sequencing of both tumor and normal reference DNA. The sequences are then aligned to identify point mutations in the tumor DNA. The resulting mutated DNA sequences can then be converted to a list of possible mutated amino acid sequences. Next, peptide-processing algorithms are generated to develop a list of candidate peptide epitopes. The epitopes are analyzed using MHC I binding affinity algorithms to identify potential neoantigens, which can be evaluated against additional filtering algorithms, such as epitope abundance and
antigen processing\textsuperscript{29,30}. Though this method has led to the identification of potential neoantigens, including, mutant ERBB21P, MUM2 and spectrin B2, it has a major limitation: it relies solely on computational prediction algorithms, which can lead to the identification of candidates that are either not found in the tumor environment, or do not generate an anti-tumor immune response\textsuperscript{31}.

An alternate, more direct approach for neoantigen isolation is peptide purification, which focuses on capturing, separating, and isolating the protein present in the tumor environment\textsuperscript{32}. Examples of this technique include high-affinity chromatography and peptide elution. In the first, tumor tissue is extracted and put through an immunoaffinity purification column to extract MHC complexes. The complexes are then purified using High-Performance Liquid Chromatography (HPLC) and peptides are identified via Mass Spectrometry (MS). Candidate neoantigens are then validated based on their ability to induce an immune response after challenge with TILs or PBMCs. In the second approach, peptides are eluted off of MHC molecules on tumors using a mild acid solution, purified using HPLC, and sequenced via MS. The major limitation associated with such methods is sensitivity due to a low signal to noise ratio, derived from the large pool of isolated peptides\textsuperscript{33}. This poses a high risk of missing potential neoantigens.

To address the shortcomings of current peptide purification methods, we propose a method focused on the development of a novel platform for neoantigen discovery and isolation using a magnetic bead based system to enrich for cells expressing peptide MHC Class I complexes. Using a B16 F10 mouse melanoma cell line as a model system we present a unique approach to purify and elute peptides for downstream identification via a combination of mass spectrometry (MS) and high-performance liquid chromatography (HPLC). Optimization of this approach in an \textit{in vitro} setting can allow for eventual translation to isolate neoantigens of low abundance in a clinical tumor model (Figures 2, 3).
At the clinical level, our purification platform will be combined with dominant TIL clones isolated from the tumor to provide an even more effective method to identify neoantigens known for triggering an immune response. These dominant clones, which are identified via single cell sequencing, have T cell receptors (TCRs) specific to previously encountered tumor neoantigen. By providing an enriched environment of neoantigen-MHC I complexes and promoting a high avidity setting, the TCRs have a greater likelihood of recognizing and pulling down the specific neoantigen of interest to identify for patient-specific therapeutic purposes.
1.2 Schematic Figures

**Figure 2.** Model system to enrich for cells highly expressing ova-MHC I complexes.

**Figure 3.** Application of platform to enrich for tumor cells expressing neoantigen-MHC I complexes.
2. Chapter 2: Data and Methods

2.1 Short Introduction

To develop this platform for neoantigen discovery, we began our work in an established cell line for efficient MHC expression and peptide presentation. The cell line serves as an ideal cell model for purification of MHC I complexes as it provides two possible populations: a MHC I-deficient population and a MHC I-expressing population. The melanoma cell line B16 F10 does not express MHC I complexes due to the lack of expression of antigen presentation machinery (APM). However, stimulation with IFNγ induces the transcription of the APM, hence allowing MHC I to be expressed on the surface of the cell\textsuperscript{34}. This widely-used tumor model provides a well-defined cell line system for manipulating MHC I expression. As our target peptide to be presented, we used the MHC I restricted ova epitope, SIINFEKL, which induces a strong CTL response.

Two magnetic isolation methods were tested for enrichment of target MHC I expressing cells. The magnetic system integrated in our platform are MagCloudz\textsuperscript{TM} Streptavidin Beads from Quad Technologies, which consist of a hydrogel technology that allows for the release of highly viable, functional, magnetic free target cells available for downstream analysis (Figure 4, Quad Technologies). The conventional bead system used was the BD IMag\textsuperscript{TM} Streptavidin Particles Plus - DM, magnetic nanoparticles coated with streptavidin that can be used for positive and negative selection of target cell populations (BD Biosciences).
To validate the ability of our purification method to isolate the target ova-MHC$^+$ cell population, we used a 58C mouse cell T line transduced with a vector expressing a SIINFEKL-specific TCR for recognition of our target peptide, a CD8 co-receptor for activation, and GFP for easy detection.

**Figure 4.** MagCloudz$^\text{TM}$ Streptavidin Beads protocol for isolation of highly viable and functional target cells (Image from Quad Technologies)
2.2 Materials and Methods

2.2.1 Cell Culture

The B16 F10 (ATCC®) cells were grown in Dulbecco’s Modified Eagle Medium (Gibco™) with 10% Fetal Bovine Serum (Omega Scientific), 1% Penicillin Streptomycin (Gibco™) and 0.5% of a 1M HEPES buffer solution (Gibco™) in a 100 mm Standard Tissue Culture Dish (Corning®).

The EL4 (ATCC®) cells were grown in RPMI Medium (Gibco™) with 10% Fetal Bovine Serum (Omega Scientific), 1% Penicillin Streptomycin (Gibco™) and 1.1 µL/mL B-mercaptoethanol (Gibco™) in a 75cm² Rectangular Canted Neck Cell Culture Flask (Corning®).

Naïve 58C T cells and 58C OT-I T cells were grown in RPMI Medium (Gibco™) with 10% Fetal Bovine Serum (Omega Scientific) and 1% Penicillin Streptomycin (Gibco™) in a 75cm² Rectangular Canted Neck Cell Culture Flask (Corning®).

2.2.2 Expression of SIINFEKL-MHC I expression on B16 F10 cells

Treatment

The B16 F10 (ATCC®) cells were split into a 6-well Tissue Culture treated plate (Corning®) at low concentration in order to reach confluence in 3 days. Day 1 post seeding, cells were treated with 1µL of 20 µg/mL recombinant murine Interferon-Gamma (Peprotech®) per well. Two days following Interferon-Gamma (IFNγ) treatment, cells were pulsed with 2µL of 1 mg/ml SIINFEKL (Invivogen) for two hours before preparation for FACS (Flow Cytometry) analysis.
Preparation of Cell Sample

The cells were detached after incubation for 5 minutes using 0.25% trypsin-EDTA (Gibco™). The trypsin was inactivated using the B16 media and spun down at 230 g for 5 minutes at 4°C in a FACS Tube (Corning®).

Confirmation of SIINFEKL-MHC expression on B16 F10 cells

For FACS analysis, FITC anti-mouse H-2K^b Antibody (Biolegend®, 116505) was used to detect MHC Class I and APC anti-mouse H-2K^b bound to SIINFEKL Antibody (Biolegend®, 141605) was used to detect SIINFEKL bound to MHC Class I. Approximately 1 x 10^6 B16 F10 cells were stained in 100 μL of FACS Buffer (PBS + 2% FBS) with 1 μg of each antibody and incubated for 30 minutes on ice in the dark. The antibodies were diluted with 800 μL of FACS Buffer, centrifuged for 5 minutes at 230 g at 4°C and re-suspended in 300 μL of FACS Buffer. The cells were then kept on ice in the dark until analyzed. The cells were stained with both the antibodies at the same time after confirming that they did not interfere with one another (Appendix A). The samples were then analyzed by FACS.

2.2.3 MagCloudz™ Streptavidin Cell Separation Protocol (Modified from Quad Technologies MagCloudzTM Streptavidin Product Insert)

Preparation of Cell Sample & Labeling with Antibody

The starting cell sample was centrifuged (230 g for 5 minutes at 4°C) to pellet the cells. The supernatant was discarded and the cells were rinsed two times in 1 mL of 1x Cell Separation Buffer and spun down (230 g for 5 minutes at 4°C) to pellet the cells. The supernatant was
discarded after each wash. Approximately $1 \times 10^6$ cells were stained in 100 µL of 1x Cell Separation Buffer with 1 µg of anti-mouse MHC 1 (H-2K$^b$) biotin (Biolegend®, 116503) and incubated for 30 minutes on ice in the dark.

**Preparation of MagCloudz™**

MagCloudz were vortexed until evenly dispersed and transferred to a 1.7 Lo-Bind Eppendorf tube (Corning®) containing 850 µL of cold 1X Cell Separation Buffer. The tube was then placed on a magnetic stand (Dynamag™-2, Life Technologies) for 5 minutes, and the supernatant was discarded. The sample was then removed from the magnetic stand, resuspended in 1 mL of fresh 1x Cell Separation Buffer, and placed back on the magnetic stand for 5 minutes for a second wash step. MagCloudz™ were left in 1x Cell Separation buffer at 4°C until the cells were ready.

**Binding Target Cells to MagCloudz™**

After incubation with the biotinylated antibody, 850 µL of 1x Cell Separation Buffer was added to the sample tube containing cells and antibody, and centrifuged (230 g for 5 minutes at 4°C) to pellet the cells. The supernatant was carefully removed and discarded. The prepared MagCloudz™ were transferred to the cell pellet tube and pipetted gently up and down at least three times to re-suspend the cell pellet in the MagCloudz™ solution. They were then incubated with end-over-end rotation for 30 minutes at 4°C.

**Washing the MagCloudz™-Target Cell Complex**

After the incubation, the sample tube was placed on the magnetic stand for 5 minutes to separate the bound target cells. While on the magnetic stand, the supernatant containing unbound cells
was transferred to a clean FACS tube (Corning®) for further analysis. After removal from the magnetic stand, 1 mL of 1x Cell Separation Buffer was added to the sample, which was pipetted extremely gently to re-suspend the MagCloudz™ back into the solution. The tube was then placed on the magnetic stand for 5 minutes, and the supernatant was carefully removed and discarded. This step was repeated for a second wash.

Releasing Target Cells from MagCloudz

The sample was removed from the magnetic stand, and 1 mL of cold 1x Cell Release Buffer was added. It was then pipetted up and down vigorously at least five times to mix and was placed back on the magnetic stand for five minutes. While on the magnetic stand, the supernatant (Release 1) containing the target cells was transferred to a clean tube. The release step was repeated by removing the tube from the magnetic stand and adding an additional 1 mL of cold 1x Cell Release Buffer to the MagCloudz. The sample was then re-suspended vigorously at least five times to mix and then placed back on the magnetic stand for five minutes. The supernatant (Release 2) was combined with the Release 1 supernatant to pool the isolated target cells. The isolated target cells were then centrifuged (300 g for 5 minutes at 4°C) to pellet. The supernatant was carefully discarded, and the isolated target cells were re-suspended in FACS Buffer for further downstream analysis.

2.2.4 Confirmation of B16 F10 target cell binding to MagCloudz™ beads

1. Antibody Staining

The MagCloudz™ target cell complex was re-suspended in 100 uL of FACS Buffer. Fifty microliters of the sample was then transferred to a fresh FACS Tube (Corning®). An additional
50 µL of FACS Buffer was added to sample. The sample was then stained with 1 µg of APC anti-mouse H-2K<sup>b</sup> bound to SIINFEKL Antibody (Biolegend®) to detect the presence of the MagCloudz<sup>TM</sup> target cell complex. The supernatant from the wash step was centrifuged for 5 minutes at 230 g at 4°C and re-suspended in 100 µL of FACS Buffer, before staining with 0.5 µg of FITC Streptavidin (Biolegend®, 405201) to detect the presence of any unbound target cells. The tubes were incubated for 30 minutes on ice in the dark. After 30 minutes, the antibodies were diluted with 800 µL of FACS Buffer, centrifuged for 5 minutes at 230 g at 4°C and re-suspended in 300 µL of FACS Buffer. The cells were then kept on ice in the dark until analyzed.

2. **Cell Trace Dye Staining** (Modified from Invitrogen Cell Trace Cell Proliferation Kits Manual)

1 µL of CellTrace<sup>TM</sup> (Life Technologies) stock solution in DMSO was added to each mL of a 1 x 10<sup>6</sup> cells suspension in 1x PBS (Corning®) for a final working solution. The cells were then incubated at 37°C for 20 minutes, protected from light. 1 mL of culture medium was added to the cells, which where then incubated at 37°C for 5 minutes. The cells were then pelleted by centrifugation and resuspended for downstream analysis.
2.2.5 Streptavidin Particles Plus Binding Protocol (BD Biosciences) Modified from BD IMag™ Biosciences Streptavidin Particles Plus – DM Technical Data Sheet

Preparation of Cell Sample & Labeling with Antibody

A starting cell sample of $1 \times 10^6$ cells was stained in 100 µL of FACS Buffer with 1 µg of anti-mouse MHC 1 (H-2Kb) biotin (Biolegend®) and incubated for 30 minutes on ice in the dark. After incubation with the biotinylated antibody, 850 µL of FACS Buffer was added to the sample tube containing cells and antibody, and centrifuged (230 g for 5 minutes at 4°C) to pellet the cells. The supernatant was carefully removed and discarded without disrupting the cell pellet.

Preparation of BD IMag™ Streptavidin Particles Plus – DM and Binding of Target cells

The BD IMag™ Streptavidin Particles Plus – DM were vortexed thoroughly until evenly dispersed and 25 µL of particles were added to the FACS Tube containing $1 \times 10^6$ total cells. The contents were mixed thoroughly by pipetting up and down several times and were then incubated at 4°C for 30 minutes.

Washing the BD IMag™ Streptavidin Particles Plus – DM - Target Cell Complex

After incubation, 2 mL of 1x BD IMag™ buffer (1x PBS) was added to the sample, and the FACS Tube was immediately placed on a cell separation magnet for 6 to 8 minutes. With the tube still on the magnet, the supernatant containing the negative, unbound fraction of cells was transferred to a separate FACS tube for further analysis. The tube was then removed from the Cell Separation Magnet, and an additional 2 mL of 1x BD IMag™ buffer (1x PBS) was added, and the pellet was carefully resuspended by pipetting up and down several times. The tube was
then returned to the Cell Separation Magnet for another 2 to 4 minutes. With the tube still on the Cell Separation Magnet, the supernatant (wash fraction) was carefully removed. These steps were repeated for a second wash. After the final wash step, the tube was removed from the Cell Separation Magnet and the contents were resuspended in FACS Buffer for downstream analysis. The supernatant was stained with FITC Streptavidin (Biolegend®) to detect the presence of any unbound target cells.

2.2.6 T cell Stimulation with B16 F10 Protocol (Adapted from Steven Neier’s T Cell Stimulation protocol)

B16 F10 cells were plated in 2 6-well plates under the following conditions: Cells in the first plate were treated for optimal induction of ova-MHC I as previously detailed; cells in the second plate were only treated with 1µL of 20 µg/mL recombinant murine Interferon-Gamma (Peprotech®) per well on day 1 for induction of MHC I presenting endogenous peptide. Naïve 58C T cells were added to two wells from the first plate and two wells from the second plate in a 1:1 ratio. The same was repeated using 58C.CD8α.β.OT-1α.β cells. An additional plate for each T cell population was setup to include two-wells with only T cells and two wells with T cells treated with 3.08 µL Phorbol Myristate Acetate (PMA, Sigma Aldrich, 1mg/mL) and 2 µL Ionomycin (ION, Sigma Aldrich, 1 mg/mL) to serve as controls. T cells were stimulated for 4 to 6 hours. After stimulation, the supernatant containing the T cells was pipetted into FACS Tubes and centrifuged at 200 g for 3 minutes at 4°C. The supernatant was removed and the cells were re-suspended in FACS Buffer for an additional wash step. Cells were stained with CD69-Pacific Blue (Biolegend®) in 100 µL of FACS Buffer for 30 minutes on ice in the dark. 58C T cells
treated with PMA/ION and stained with CD69-Pacific Blue were used as a CD69 single color control and unstained 58C.CD8α.β.OT-1α.β T cells were used as a GFP positive single color control.
2.3 Results

2.3.1 Optimizing B16 F10 IFN treatment to maximize surface MHC I expression

To determine the optimal treatment method for efficient induction of MHC I surface expression, B16 F10 cells were tested under three different conditions: a single dose of IFNγ on day one (post-seeding), a single dose of IFNγ on day two, or one dose of IFNγ on day one and an additional dose on day two. Cells that did not receive IFNγ served as a negative control. Analysis by FACS on day three revealed that 97.1% of the B16 F10 cell population induced with one dose of IFNγ on day one expressed MHC I, the largest of the three conditions (Figure 5a).

To determine the best conditions for loading B16 F10 cells with ova peptide (SIINFEKL), B16 F10 cells were treated with IFNγ and pulsed with ova peptide for two hours or overnight. By FACS, 20.7% of the cells expressed ova peptide when pulsed overnight as opposed to 94.7% when pulsed for two hours (Figure 5b). Therefore, to ensure efficient ova-MHC expression, B16 F10 cells were treated with IFNγ for two days and subsequently loaded with ova for two hours (Figure 5c).

Figure 5. The optimal treatment strategy for high ova-MHC I expression on B16 F10 cells as determined by FACS: IFNγ treatment on day 1 (post-seeding) and an ova pulse for two hours. (a) Treatment conditions for MHC I induction. Untreated (blue), 1 dose IFNγ on day 3 (green), IFNγ on day 2 and 3 (red), IFNγ on day 2 (orange). (b) Treatment conditions for ova pulse. Untreated (blue), overnight pulse of ova (pink), 2 hour pulse of ova (orange). (c) Treatment conditions for ova-MHC expression. Untreated (blue), 1 dose IFNγ on day 3 and 2 hour pulse of ova (green), IFNγ on day 2 and 3 (red) and 2 hour pulse of ova, IFNγ on day 2 and 2 hour pulse of ova (orange).
2.3.2 Purification of MHC I\(^+\) B16 F10 using MagCloudz\textsuperscript{TM} Streptavidin Beads

To isolate and enrich for the target ova-MHC I\(^+\) B16 F10 cell population, we used a novel magnetic bead based purification system, the MagCloudz\textsuperscript{TM} Streptavidin beads (Figure 6).

![Figure 6. Microscopic images of B16 F10 cells and MagCloudz under both isolated and bound conditions. The number of bound beads varies from cell to cell. (a) MagCloudz\textsuperscript{TM} Streptavidin Beads. (b) B16 F10 cells. (c) B16 F10 cells bound to MagCloudz.](image)

The initial population consisted of a subset of B16 F10 cells expressing MHC I. After Magcloudz\textsuperscript{TM} for MHC I\(^+\) enrichment, the majority of our abundant target cell population remained in the unbound supernatant (Figure 7). Analysis of an EL4 positive control cell population with 100% cells expressing MHC I yielded similar results (Figure 8).
Figure 7. MagCloudz Streptavidin Beads are unable to bind target MHC I⁺ B16 F10 cells. FACS plots of B16 F10 cells before and after target cell purification. (a) Initial population of MHC I⁺ cells. (b) MagCloudz bound target cell population. (c) Unbound supernatant population. (d) Population of MHC I⁺ cells in unbound supernatant.

Figure 8. MagCloudz Streptavidin Beads are unable to bind MHC I⁺ EL4 cells. FACS plots of EL4 cells before and after target cell purification. (a) Initial population of MHC I⁺ cells. (b) Initial population of cells stained with Cell Trace Dye. (c) MagCloudz bound target cell population. (d) Unbound supernatant population. (e) Population of MHC I⁺ cells in unbound supernatant.
To determine the cause of low-efficiency binding, various aspects of the purification protocol were assessed. This troubleshooting consisted of testing the performance of the biotin H-2K\textsuperscript{b} antibody, varying the ratio of the target MHC I\textsuperscript{+} cell population and volume of MagCloudz\textsuperscript{TM} used, and using a larger surface area for the target cell bead binding incubation period (Appendix B). Based on these results, we believe there is some interference in the ability of the hydrogel on the MagCloudz\textsuperscript{TM} to bind and/or remain bound to the target cells.

2.3.3 Purification of MHC I complexes using BD Biosciences Streptavidin Particles Plus

Due to issues with binding of MHC I\textsuperscript{+} B16 F10 and EL4 cells to MagCloudz beads, we then tested with an alternative magnetic isolation system – the BD Biosciences Streptavidin Particles Plus – DM. This system, unlike the MagCloudz\textsuperscript{TM}, is composed of magnetic beads covalently conjugated with streptavidin directly on their surface. The experiments performed with these beads helped us to determine whether our cells possess some properties that render them unable to bind streptavidin beads.

Cells were treated to express ova-MHC I as previously described, and were bound to the BD Biosciences Streptavidin Particles using 1 \( \mu \)g biotin-H-2K\textsuperscript{b}, the optimal antibody concentration for binding the target cell population (Figure 9). This system was tested out on both B16 F10 and EL4 cell lines, with MHC I\textsuperscript{+} B16 F10 and EL4 cells stained with Pacific Blue for ease of detection. Analysis by FACS suggested that this magnetic purification system was able to successfully enrich for the target MHC I expressing B16 F10, along with an EL4 cell population with high purity, as a majority of the cells bound to the beads and only a small percentage of the population remained in the unbound supernatant (Figure 10, Figure 11).
Figure 9. BD Biosciences Streptavidin Particles bind EL4 cells most efficiently with 1µg of biotin H-2K\textsuperscript{b}. Microscopic images of EL4 cells after target cell purification. (a) EL4 cells + no biotin H-2K\textsuperscript{b} antibody (negative control). (b) EL4 cells + 0.5µg biotin H-2K\textsuperscript{b} antibody. (c) EL4 cells + 1 µg biotin H-2K\textsuperscript{b} antibody.

Figure 10. BD Biosciences Streptavidin Particles bind target B16 F10 cells with high efficiency. FACS plots of B16 F10 cells before and after target cell purification. (a) Initial population of MHC I\textsuperscript{+} cells. (b) BD Biosciences Streptavidin Particles bound target cell population. (c) Population of MHC I\textsuperscript{+} cells in unbound supernatant.

Figure 11. BD Biosciences Streptavidin Particles bind EL4 cells with high efficiency. FACS plots of EL4 cells before and after target cell purification. (a) Initial population of MHC I\textsuperscript{+} cells. (b) BD Biosciences Streptavidin Particles bound target cell population. (c) Population of MHC I\textsuperscript{+} cells in unbound supernatant.
An additional staining step was performed using APC anti-mouse H-2K\textsuperscript{b} bound to SIINFEKL Antibody to ensure that ova peptide remained bound to the MHC I\textsuperscript{+} target population after purification with the bead system (Figure 12).

![Graph showing FACS histograms of population of ova peptide positive B16 F10 cells before and after target cell purification using BD Biosciences Streptavidin Particles Plus – DM. Unstained negative control.]

**Figure 12.** Ova peptide remains bound to MHC I after purification with BD Biosciences Streptavidin Particles. FACS histograms of population of ova peptide positive B16 F10 cells before (blue) and after (green) target cell purification using BD Biosciences Streptavidin Particles Plus – DM. Unstained negative control (red).

### 2.3.4 Stimulation of 58C OT-I T cells with ova-MHC I

As a method to assess the ability of our purification approach to selectively isolate cells expressing the target peptide, ova, we challenged OT-I T cells with two populations: B16 F10 cells abundantly expressing ova and B16 F10 cells expressing endogenous peptide. In parallel, we challenged naive αβ-deficient 58C T cells with B16 F10 cells with the same two cell populations, to serve as additional controls. Naïve 58C T cells stimulated with PMA and ION, strong inducers of T cell activation, were used as a positive control\textsuperscript{35,36}. After a 4-6 hour stimulation period, we measured levels of a common T cell activation marker, CD69, for each condition (Figure 13).
CD69 up-regulation on 58C OT-I cells stimulated with ova expressing B16 F10 cells was slightly higher than on naïve 58C cells stimulated under the same conditions. Due to the weak signal, further testing is required to confirm activation was peptide-specific (Figure 13).

2.4 Brief Discussion

Using the B16 F10 cell melanoma cell line, we were able to successfully manipulate MHC I expression and purify the target ova-MHC I\(^+\) cell population with conventional magnetic streptavidin bead particles from BD Biosciences. This cell model provided a very useful basis for testing out the purification protocol using different starting populations of ova-MHC I\(^+\) cells. Though the MagCloudz\textsuperscript{TM} were unable to bind to the cells, the streptavidin bead particles were very effective in isolating our target cell population. Validation of the effectiveness of the purification strategy in isolating our target cell population with 58C CD8 OT-I cell stimulation was relatively weak, and further optimization of the system is required to obtain significant upregulation of activation after stimulating the OT-I cells with their receptor-specific peptide.
3. Chapter 3: Discussion and Perspective

The model platform developed for isolation of a cell population expressing target peptide in abundance provides the initial framework to build upon for ultimately isolating a cell population expressing rare neoantigens from cancer patients. At both levels, this enrichment process contributes to a greater likelihood of identifying the target peptide through MS. The use of dominant tumor-specific receptors to pull-down target neoantigens is a unique approach to address the limitations of current methods, specifically non-specific pull-down of large quantities of peptides.

Though advancements in technologies have created great promise for the field of immunotherapies, efficient identification of targets for such methods remains a significant challenge. Current methods being explored rely too heavily on identifying neoantigens through indirect, computer-based algorithms and hence should shift focus to improving upon more direct and reliable methods of isolation, such as peptide purification. The ability to extract neoantigens capable of inducing an anti-tumor response is pivotal for developing successful targeted therapies to treat cancer.

3.1 Limitations

One of the major limitations of the model system is the inability of the MagCloudz™ beads to efficiently bind to the target cell population. Unlike conventional bead systems, MagCloudz™ allows for the isolation of a magnetic-free, highly-viable, target cell population with maintained functionality and phenotype. This is crucial for downstream analyses for application to a clinical tumor model. Though the BD Biosciences Streptavidin Particles are able to successfully isolate our target cell population, they remain bound to the cells, which can disturb the functionality and viability of the cells. The inability of these particles to be easily
released from the target cell population is also problematic for experiments planned for clinical application. Further troubleshooting is required to optimize the MagCloudz™ system and ensure the ability to bind a biotinylated target cell population.

Another drawback of this system is the weak activation of 58C OT-I T cells when challenged with ova-MHC I expressing B16 F10 cells. With a large population of cells expressing ova peptide, the OT-I T cells were unable to produce a significant amount of signal. The weak signaling observed can be attributed to a variety of factors: sub-optimal transduction efficiency levels leading to weak expression of the OT-I αβ receptor, planar stimulation environment with adherent B16 F10 cells and suspension T cells, or inability of the 58C cell line to upregulate CD69 at high levels. Determining a better method for improving transduction efficiency, whether by altering the composition vector or using a higher concentration of polybrene reagent to enhance the process is crucial before moving forward.

### 3.2 Future Research

Following successful isolation and identification of abundant peptide, the sensitivity and specificity of the OT-I dependent system should be assessed. To test the range of detection, we will use ova at various concentrations and determine the consequent threshold required to maintain peptide-TCR interactions. By challenging OT-I T cells with a range of ova-derived peptides, we can determine the ability of a TCR to pull-down ova epitopes of lower affinity than SIINFEKL. These experiments are highly necessary and relevant since most neoantigens have a low affinity to TCR. To test specificity, we will use both a range of peptide epitopes derived from ova in addition to a mix of peptides from different protein sources. Testing the system with a mix of peptide epitopes more accurately models the clinical environment, where tumor cells abundantly expressing a diverse array of peptides.
In parallel, the system should be evaluated against a bead-bound cell population, to ensure beads used for purification do not interfere with optimal TCR-peptide binding interactions. Once confirming the ability of OT-I T cells in pulling down cells expressing ova, we can then test the effectiveness of single-chain OT-I CAR T cells. Presumably, the interaction between the CAR and ova should be stronger and more long lasting than that between the wild type TCR and ova. This strong interaction would greatly assist in pulling-down the target peptide for identification via MS.

Complete optimization and success of the *in-vitro* model platform for selectively isolating and identifying the target peptide should be achieved before translation into both a mouse and clinical human model. For *in-vivo* application, TILs isolated from the tumor environment will replace OT-I T cells. These TILs will have a receptor specific for the neoantigen previously recognized, thus possessing the ability to selectively pull-down and enrich for the same neoantigen when challenged with the MHC I⁺ tumor cell population. Being able to isolate neoantigens capable of triggering an immune response can help develop enhanced, on-target, patient-specific cancer immunotherapies.
4. Bibliography


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Appendices

Appendix A: Dual Staining with H-2Kb FITC and S-H-2Kb APC Antibodies

To ensure that both FITC anti-mouse H-2K\textsuperscript{b} Antibody and the APC anti-mouse H-2K\textsuperscript{b} bound to SIINFEKL Antibody did not interfere with each other, the amount of MHC I and ova expression under a dual staining condition was compared to single color control conditions. Analysis by FACS revealed that dual staining properly depicted ova-MHC I expression, at levels comparable to the single antibody controls. Dual Staining (blue), Single Staining (orange)
Appendix B: Troubleshooting the MagCloudz™ Streptavidin Bead System

H-2Kb Antibody Performance

To ensure that there was no fault in the ability of the H-2Kb antibody to bind to Streptavidin, the antibody was tested with different concentrations of a secondary Streptavidin FITC antibody. FACS analysis revealed that the FITC expression levels from biotin H-2Kb with Streptavidin FITC were compared to the H-2Kb FITC control.

B16 F10 cells retain MHC I expression over time

In order to assure that the cells maintained expression of ova-MHC I complexes throughout the course of the day, the cells were left at 4°C for several hours before being stained. Analysis by FACS revealed that expression remained at a comparable level to prior results. Unstained negative control (blue), B16 F10 + IFN + ova (orange)