DEVELOPMENT OF NOVEL CANCER IMMUNOTHERAPEUTICS UTILIZING CELL-TARGETING PEPTIDES

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DEDICATION

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DEVELOPMENT OF NOVEL CANCER IMMUNOTHERAPEUTICS UTILIZING CELL-TARGETING PEPTIDES

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Cancer immunotherapy is an emerging treatment option that offers high tumor specificity and efficacy. Immune therapies for cancer can be divided into two main types: active and passive. Active therapy strives to achieve a long term protective immunity against a tumor antigen while passive therapy supplies exogenous immunological reagents for anti-tumor effector functions. Both immunotherapies can be improved by utilizing cell targeting peptides.

Dendritic Cell Targeting Peptides: Cancer vaccines can elicit immune responses against tumor antigens. Antigen-pulsed *in vitro* matured dendritic cells (DCs) are used for higher efficacy. However, this method does not provide a significant therapeutic immune response. A more robust anti-tumor immune response could potentially be achieved through *in vivo* DC targeting of tumor antigens. Through phage-displayed peptide library panning protocol, four different DC-targeting phage clones were isolated. Of those, XS52.1 and XS52.3 bind specifically to the XS52 immature

dendritic cells. The XS52.3 phage clone also binds bone-marrow dendritic cells (BMDCs) from Balb/c and C57BL/J6 mice. Each phage clone elicited heightened anti-phage antibody production in both mouse strains. Potential future studies will determine if these peptides can be used to target antigen to DCs for *in vivo* cancer vaccines.

Peptide-Antibody Targeting: Monoclonal antibodies directed against tumor antigens have been successful in clinics, but problems remain with identifying and validating new targets. Modification of the antibody scaffold for distinct applications can also be problematic. Using our phage display panning protocol, we have identified ligands of high affinity and specificity against a panel of human non-small cell lung cancer (NSCLC) cell lines. Furthermore, these peptide-targeting ligands can be chemically synthesized and easily modified for different uses. In my studies, synthetic-peptide ligands have been used to redirect antibody targeting by using biotinylated-tetrameric peptides and anti-biotin antibodies. These results suggest that peptide-antibody conjugates utilizing isolated peptides can be used to redirect antibody targeting. This methodology would increase the antibody repertoire available for therapy.

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AAT - α1-antitrypsin

ADCC – antibody dependent cellular cytotoxicity

AF - alexafluor

AMP - ampicillin

APC – antigen presenting cell

BMDC – bone marrow derived dendritic cell

BSA – bovine serum albumin

CAR – chimeric antigen receptors

CD – Cluster of Differentiation

CMV – cytomegalovirus

COMP – rat cartilage oligomerization matrix protein

CQ – chloroquine

CTLA4 – Cytotoxic T-lymphocyte-associated antigen 4

DC– dendritic cell

DC-SIGN – DC specific intercellular adhesion molecule 3 grabbing non integrin

EDTA - Ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

EXO - exonuclease

FC – fragment crystallizable

Flt3L - Fms-related tyrosine kinase 3 ligand

GAM – Goat anti-mouse

GAR – Goat anti-rabbit

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

HPLC – high-performance liquid chromatography

HPV – human papilloma virus

IL – Interleukin

LAK – lymphocyte activated killer

LAL - Limulus Amebocyte Lysate

LOX1 - lectin-type oxidized low-density lipoprotein receptor 1

MAC1 - macrophage receptor 1

MALDI - Matrix-assisted laser desorption/ionization

MCIP – Myocyte-enriched calcineurin- interacting protein

mDC – myeloid DC

MHC – Major Histocompatibility Complex

NHS - N-hydroxysuccinimide

NIR – near infra-red

NSCLC – Non-Small Cell Lung Cancer

OS – overall survival

OVA – ovalbumin

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered solution

PCR – polymerase chain reaction

PD1 - programmed cell death protein 1

pDC – plasmacytoid DC

PE - phycoerythrin

PFA - paraformaldehyde

PFS – progression free survival

PI – protease inhibitor

RE – restriction enzyme

RT – room temperature

SA - streptavidin

SAP – shrimp alkaline phosphatase

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMCC - sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate

TAA – tumor associated antigen

TAM – tumor associated macrophages

TCR – T-cell receptor

TH – T helper cell

TIL – tumor infiltrating lymphocytes

TLR – Toll-like receptor

TMB - 3’3’5’5’tetramethylbenzidine

TNF – tumor necrosis factor

CHAPTER I

GENERAL INTRODUCTION

Cancer Immunotherapy

Traditional cancer therapy consists mainly of radiation, chemotherapy, and surgery. The advent of molecularly targeted therapies and improved surgical/radiation techniques have improved current available therapies, but all together these treatments are not very effective in patients with metastatic disease. The immune system has long protected humans against pathogenic intruders, but a spontaneous immunological response against a tumor antigen that leads to remission of established tumors is rare. Cancer immunotherapy started as early as 1774, where a Parisian doctor noticed a patient's breast cancer receding after infecting the patient with a pathogen. This practice continued to be utilized in 19th century Germany [1]. While this treatment sounds extreme, a modified version was used well into the 20th century by an American surgeon named Dr. William Coley. Dr. Coley used his concoction that consisted of toxins from *Streptococcus pyogenes* and *Serratia marcescens* to treat over 800 cancer patients. While his claim that half of his patients had a good clinical response has been deemed dubious, the American Medical Association did acknowledge that Coley's toxins may prevent metastasis and was occasionally curative for inoperable neoplasms [2, 3]. Since then, there have been many discoveries in the area of cancer immunology that have led to the development of improved immunotherapeutics to be used for cancer treatment. This is attributed to researchers discovering usable cancer antigens, understanding the

mechanisms that underlie tumor immunity and suppression, and learning how immunological tools can be used effectively for cancer treatment. However, there are still barriers that immunotherapy needs to overcome to be an effective treatment. A challenge that remains is the identification of appropriate TAAs for targeted treatment. Analysis of the tumor microenvironment has revealed the presence of immunological cells at the tumor site, but their role and how to stimulate these cells for antigen processing and tumor cell lysis is still poorly understood. The ultimate goal for tumor immunology is to harness the immune system to reject cancers, prevent metastasis, and provide a non-toxic alternative to current cancer therapies.

Two main immunotherapeutic approaches exist in today's cancer treatments: passive immunotherapy and active immunotherapy. Passive immunotherapy utilizes exogenous resources for immune function, while active immunotherapy directly activates the patient's immune system. These immunotherapeutic approaches can be further divided into those that induce an antigen specific response versus those that generally activate the immune system (Table 1). A general overview is given in this introduction of the advantages, barriers, and future potential of each type of immunotherapy. An entire section will each be assigned to *Cancer Vaccines* and *Antibody Therapies* to allow for a more in-depth look of these areas that are the focus of the dissertation research.

Table 1: Summary of Cancer Immunotherapeutics		
	Active	**Passive**
Specific	Cancer Vaccines	Monoclonal antibodies Engineered antibody derivatives Antigen-specific T-cells expanded *ex vivo*
Non-Specific	Coley's Toxins BCG Cytokines	Lymphokine-activated killer cells Tumor infiltrating lymphocytes

Table modified from review article [1]. Active and passive immunotherapies are further divided into therapies that induce a specific versus a non-specific response.

Passive Immunotherapy

A patient receiving an exogenous source of anti-tumor antibodies is the main form of passive cancer immunotherapy. Antibody treatment has been a time tested form of therapy with several FDA approved antibodies available for cancer treatment (Table 2). Before antibody therapy is to be further discussed in the *Antibody Therapy* subsection, an overview of other passive immunotherapy approaches is merited.

An early technique utilized for passive immunotherapy was allogeneic cell transfer [4]. Initially, general treatment with lymphokine activated killer (LAK) cell transfer produced seemingly positive results against metastatic melanoma and renal cell carcinoma [5-7]. Treatment with LAK cells consists of lymphocytes (NK, T-cell, and B-cell) cultured with IL-2 in order to induce anti-tumor cytolytic activity. This was further refined to expanding isolated tumor-infiltrating lymphocytes (TIL) *in vitro* that were later injected back into the patient [8, 9]. In cancer immunotherapy, an important step is migration of effector T-cells from the lymph nodes into tumor tissue for cytolytic activity. Effective migration requires coordination between surface markers of effector cells and vascular adhesion molecules. Manipulating T-cells *ex vivo* eliminates the dependency on *in vivo* T-cell activation. Infusion with donor lymphocytes and allogeneic bone marrow transplants is an effective treatment for some leukemias and lymphomas [10]. In some cases, isolated TILs were further modified with genetic transfer of the TNF cytokine gene [11, 12]. These therapies did not become popular because of high toxicity, but further T-cell strategies were developed, including using tetramer technology to isolate CD8+ T-cells with a specific antigen affinity [13, 14]. For example, Lee et al. isolated MART-1 melanoma antigen specific T-cells from patient PBMCs and measured

their ability to kill melanoma cells *in vitro*. In this study, antigen specific T-cells were found to be anergic and unable to lyse melanoma cells, even with stimulation of peptide antigen [14]. These cells were rendered useless against a TAA due to anergy, demonstrating that these cells are exposed to immunosuppressive factors *in vivo* that diminishes their ability to effectively recognize and attack tumor cells. A new direction that is being explored in T-cell therapy is the use of T-cell engineering with CARs (chimeric antigen receptors) for treatment of refractory chronic lymphocytic leukemia [15]. The major side effects of this treatment are lymphopenia and tumor lysis syndrome, which occurs because of the accumulation of by-products from apoptotic cells.

The main advantage of using passive therapy is the ability to induce an immediate effect on cancer cells without requiring an active immune response. Ultimately, passive therapy does not facilitate long term cancer immunity and therefore the patient is dependent on an exogenous source of active molecules/cells.

Table 2: FDA approved antibodies for cancer treatment				
mAb Name	**Trade Name**	**Target**	**Used to Treat**	**Approved**
Rituximab	Rituxan®	CD20	non-Hodkin lymphoma	1997
			chronic lymphocytic leukemia (CLL)	2010
Trastuzumab	Herceptin®	HER2	breast cancer	1998
			stomach cancer	2010
gemtuzumab ozogamicin*	Mylotarg®	CD33	acute myelogenous leukemia (AML	2000**
alemtuzumab	Campath®	CD52	chronic lymphocytic leukemia (CLL)	2011
ibritumomab tiuxetan*	Zevalin®	CD20	non-Hodgkin lymphoma	2002
tositumomab*	Bexxar®	CD20	non-Hodgkin lymphoma	2003
Cetuximab	Erbitux®	EGFR	colorectal cancer	2004
			head & neck cancer	2006
bevacizumab	Avastin®	VEGF-A	colorectal cancer	2004
			non-small cell lung cancer	2006
			breast cancer**	2008
			glioblastoma	2009
			kidney cancer	2009
panitumumab	Vectibix®	EGFR	colorectal cancer	2006
Ofatumumab	Arzerra®	CD20	chronic lymphocytic leukemia (CLL)	2009
Denosumab	Xgeva™	RANKL	cancer spread to bone	2010
Ipilimumab	Yervoy™	CTLA4	Melanoma	2011
brentuximab vedotin*	Adcetris™	CD30	Hodgkin lymphoma	2011

*conjugated antibody
**approval withdrawn

Modified from American Cancer Society table at cancer.org. Table consists of FDA approved antibodies used for cancer treatment. A Noteworthy item is the removal of approval for Mylotarg® and Avastin®. Mylotarg® was withdrawn in June 2010 when a clinical trial showed increased patient death and no benefit over conventional therapies. The approval of Avastin® for breast cancer was revoked November 2011 for not extending life in patients.

Antibody Therapy

Antibodies are used for the treatment of cancer, immune disorders, infections, and heart disease [16]. Initially, murine monoclonal antibodies were used because of their high affinities and specificities, but they were of limited use in humans due to immunogenicity, short serum half-life, and inefficient interaction with immune cells. Now chimeric, humanized, and fully human antibodies are used and produced through protein engineering, library technologies with phage (phage display), or by antibody production in transgenic mice [17, 18]. Antibodies, or immunoglobulins (Igs), exist in five separate forms distinguished by differences in their properties and functions determined by the constant region. The categories are IgA, IgD, IgE, IgG, and IgM. IgG is the isotype of most interest since it's the most common in cancer immunotherapy. This isotype is further divided into subtypes named IgG1, IgG2, IgG3, and IgG4 in humans. IgG1 has the highest abundance in serum and is also the isotype most used for cancer therapy. The IgG1 has the second-highest ability to induce complement fixation and has a high affinity for Fc receptors on immune cells. Antibodies consist of two antigen binding fragments (Fabs) where diversity of antigen specificity is determined and one constant fragment (Fc) that is important for ADCC and CDC. The Fc domain binds the IgG to immune effector mechanisms through the Fcγ receptors (FcγRs) on natural killer (NK) cells, neutrophils, monocytes, and DCs [19].

Antibodies are important in cancer therapy for their ability to initiate CDC and ADCC. Complement dependent cytotoxicity (CDC) is a mediator of anti-pathogenic immune response. CDC is a proteolytic cascade made up of over thirty proteins that lyses cells through assembly of the membrane attack complex (MAC) [20]. The classical

complement pathway is activated when two or more antibodies bind to a cell. It begins with binding of the C1 serine protease complex to the Fc region. Binding activates a proteolytic cascade that leads to the formation of MAC and subsequent cell lysis [21]. CDC is implicated in the anti-tumor activity of rituximab, an anti CD20 antibody approved for treatment of B-cell malignancies [22]. Genetic polymorphisms in patients' complement proteins are associated with response to rituximab therapy [23].

The Fc receptors on immune cells are the main mediators of ADCC. There are three activating FcγRs: FcγRI (CD64), FcγRIIA (CD32A), and FcγRIIIA (CD16A) and one inhibitory receptor, FcγRIIB (CD32B) [24, 25]. NK cells express FcγRIIIA and are the main effectors of ADCC. NK cells recognize antibody coated target cells through FcγRs and directly lyse cells through release of granzymes and perforin [26]. The importance of ADCC in cancer treatment is evidenced by the development of antibodies with enhanced Fc mediated function. AME-133 (Mentrik) and PRO131921 (Genentech) are antibodies that are engineered for increased ADCC activity [27, 28]. The AME-133 Phase I trial recruited patients with follicular lymphoma and a FcγRIIIa mutation (F-carriers) that results in decreased immune cell binding affinity to IgG's [29, 30]. These F-carriers tend to exhibit less rituximab efficacy [31-33]. The trial preliminarily showed efficacy in this patient population, thus illustrating the potential for engineered antibodies in special patient populations.

Currently there are more than 10 monoclonal antibodies FDA approved for cancer treatment (Table 2) [34]. Anti-Her2 antibody Herceptin® has been a major success story for treatment against breast cancer. Her2 is a receptor known to be up-regulated in 20-25% of breast cancer and is associated with poor clinical outcome [35,

36]. When combined with chemotherapy, treatment with Herceptin® increases disease-free survival in breast cancer patients [37, 38]. The efficacy of Herceptin® is attributed to its capacity to initiate ADCC and interfere with receptor dimerization required for signaling [39]. Evidence for the important role of ADCC in antibody efficacy includes murine studies done in mice lacking activating Fcγ receptors on immune cells that exhibited reduced or ablated anti-tumor function during antibody treatment [40]. In the same study Clynes et al. showed increased anti-tumor efficacy in mice lacking inhibitory receptor FcγRIIB. This receptor is not expressed in NK-cells; therefore the observed increase is attributed to monocytes and macrophages. There is also reduced lung metastasis in the FcγRIIB deficient mice. As a point of clarification, it is the IgG2a isotype that has heightened Fc function in murine models and is comparable to human IgG1. The importance of immunological cells for functionality of antibody treatment has also been observed in humans, where treatment of Trastuzumab with chemotherapy shows increased levels of NK cells in tumor sites which are not present in patients treated with chemotherapy alone [41]. In human studies, antibodies have shown ADCC function with immune cells isolated for *ex vivo* analysis.

There is ongoing evidence showing antibody therapies can also stimulate an adaptive immune response in patients treated with Trastuzumab and chemotherapy [42]. It is thought that opsonized tumor cells enhance DC internalization and processing of tumor antigens which leads to an immune-complex mediated induction of tumor immunity, though this has mainly been detailed in murine studies [43]. This immunity has been attributed to cross presentation; a DC antigen processing mechanism which is enhanced by antibody treatment and increases immunity towards melanoma in human

cells *in vitro* [44]. Studies in patients demonstrated that not only does induced humoral immunity from Trastuzumab/chemotherapy show favorable clinical response, but it also increases HER2 specific CD4+ T-cells [42]. Antibodies have also been used for their adjuvant effect in whole-cell vaccines, where opsonized tumor cells can lead to increased tumor free survival in transgenic mice [45].

An advance in antibody therapy that is of particular interest to this project is the production of peptide-antigen conjugates. Amgen has produced a peptide antibody conjugate with a thrombopoietin mimetic peptide isolated from phage display[46]. Nplate® is a recombinant fusion protein that contains human immunoglobulin IgG1 Fc domain covalently linked at the C terminus to a peptide containing two TPO receptor-binding domains. Nplate® has no amino-acid-sequence homology to endogenous TPO. It increases platelet production through binding and activation of the TPO receptor. While these fusions are commonly produced through genetic engineering, Dr. Barbas and colleagues have isolated a catalytic antibody capable of chemical conjugation to peptides for production of peptibodies. This work with peptide antibody conjugates will be further discussed in Chapter's III introduction under *Previous development of peptide-antibody conjugates.*

Active Immunotherapy

Active immunotherapy utilizes the patient's immune system to produce an immune response against tumor associated antigens (TAAs) without depending on an outside source of antibodies. Current forms of active immunotherapy include the use of cytokines, pathogenic components, or vaccines in order to activate the host immune

response towards cancer. While cancer vaccines are explored as a method to direct an antigen specific immune response against a tumor, cytokines and other immunological agents are used to initiate a non-specific response.

A historical example of pathological components used for cancer treatment is the Coley's toxin that was discussed earlier in the introduction, where a cocktail of immunogenic toxins was used to generally activate the immune system in hopes that the tumor would be recognized in the immunologically heightened state. Bacillus Calmette-Guérin (BCG) is another example of a pathogenic cocktail that is used to activate the immune system against cancer. BCG was originally developed as a tuberculosis vaccine produced from *Mycobacterium bovis*, but studies have shown that administration of BCG can be used as treatment for superficial early stage bladder cancer and to prevent recurrence [47, 48]. The exact mechanism of action is unknown, but it is hypothesized that BCG initiates immunological responses that lead to tumor cell death. Studies in rodent bladder cancer models showed increased presence of T-cells in BCG treated bladders, while studies in athymic rodent models showed that BCG had no anti-tumor effect until T-cells were transferred [49, 50]. Most recently, *in vitro* studies using human cancer cell lines and PBMC from donor patients show that BCG can increase tumor MHC class I presentation and Th1 cytokine production, resulting in higher T-cell cytolytic ability [51]. Other studies have shown that BCG treatment was ineffective in mice that were NK-cell deficient or that underwent antibody depletion of NK-cells, leading to a proposed model in which BCG treatment supported by T-cells initiates Th1 cytokine secretion that in turn activates NK-cells [52]. BCG is the only agent FDA approved as primary therapy of bladder cancer, but this efficacy, along with the

intravesicular route of administration, is not translatable to other tumor types.

Cytokines have also been used on their own as a treatment for cancer. The main cytokines utilized are IFNα, IL-2, GM-CSF, and IL-12. Cytokines are employed in cancer therapy to modulate immune responses. Host immune responses are complex and require several different cell types; therefore administration of cytokines can enhance anti-tumor responses through modulation of different immune cells [53]. IFNα has been used in clinical trials for treatment of melanoma, leukemia, and advanced kidney cancer [54, 55]. GM-CSF has been explored for efficacy against melanoma and IL-12 was examined for efficacy against advanced malignancies [56, 57]. Most of these cytokines showed initial positive results but were unable to show long term and consistent efficacy. The most striking example of cytokine treatments' ineffectiveness can be observed in melanoma clinical trials. Several melanoma clinical trials that were performed using IL2 alone or with IFNα showed no significant increase in response rate or survival [58]. Biochemotherapy, a combination of IL-2 and/or IFN-α with various chemotherapeutic agents such as dacarbazine and cisplatin, has also failed to affect survival. Biochemotherapy improves response rates but not overall survival, and therefore has been largely abandoned [59-61]. The haphazard nature of the treatment as well as side effects that include fever, chills, flu-like symptoms, and overall feeling of illness makes it difficult to harness the response in a consistent fashion useful for broad cancer treatment.

Treatments that have received FDA approval in the area of active immunotherapy are Proleukin® and Provenge®. Proleukin® is a recombinant IL-2 cytokine that was approved in 1998 for metastatic melanoma [62]. Since then, vemurafenib (B-Raf inhibitor) and ipilimumab (anti-CTLA4 antibody) received FDA approval for melanoma

treatment and have replaced Proleukin® for first line therapy. Proleukin® is now being studied for treatment of HIV and as a vaccine adjuvant [63, 64]. Cytokines by themselves do not direct an immune response toward a specific TAA, but rather modulate a heightened immunological state where cancer immunity may occur if the appropriate cells are activated. In contrast, vaccination treatments such as Provenge® allow the host to elicit an antigen specific immune response. Provenge® is a recent breakthrough in the field of cancer immunotherapy as the first cancer vaccine to be approved by the FDA. Even this breakthrough is starting to see detractors, most notably Marie L. Huber who wrote a scathing article published in JNCI claiming that Provenge® lacks demonstrable tumor responses and therefore calls into scrutiny the original trial design [65]. She claims that previously unpublished data show worse overall survival in older versus younger patients in the placebo groups. Also, placebo patients had cells harvested but not reinfused, adding to the detrimental effect of repeated cell loss that explains the survival "benefit" of Provenge®. Regardless of the outcome of this controversy, a long list of other cancer vaccines has been deemed unsuccessful, therefore necessitating further investigations into ways to improve therapeutic responses. A more detailed description of cancer vaccines will be discussed in the *Cancer Vaccine* section of this introduction.

Combination and Alternative Approaches to Immunotherapy

Years of failure in using single approaches have yielded attempts to combine several immunotherapies for cancer treatment. The most logical transition is the use of cytokines that had shown low efficacy on their own in combination with vaccination strategies [66]. An example of this is Provenge®, the first FDA approved cancer vaccine. Like its

predecessors it is a DC based vaccine, but what makes it unique is the recombinant protein that conjugates the antigen with GM-CSF. Other combination strategies plan on using targeted molecular therapies in conjugation with immunotherapy. An example is using vemurafenib, a B-RAF inhibitor for melanoma patients carrying the V600E activating mutation, in combination with ipilimumab to potentially enhance immunity against endogenous tumor antigens that are released during vemurafenib-induced death [34]. Ipilimumab is an anti-CTLA4 antibody that sustains an active response by blocking inhibitory signals. CTLA4, also known as CD125, is a receptor expressed on helper T cells that downregulates the immune system by acting as an "off" switch for antigen-mediated T cell attack. CTLA4 is similar to CD28 on T cells that binds to B7 and molecules on APCs during antigen stimulation. Without this signal T cells are in a perpetual "on" state and can continue with tumor cell lysis without disruption. There is also evidence that immunogenic cell death induced by chemotherapeutics can lead to enhancement of an anti-tumor immune response [67]. Cytotoxic agents capable of exposing the immune system to potential tumor antigens should be considered as candidates for combination treatment with immunotherapy, though great care should be taken to not initiate tumor suppression.

One factor that needs to be taken into account during development of cancer immunotherapy is the role of immunosuppression in tumor development. Studies show that tumors can hide from the immune system by eliciting tumor suppressive factors to inhibit activation of surrounding immune cells. Adenosine, which is released by tumor cells under hypoxia, can suppress T-cell activation while enhancing tumor growth and angiogenesis [68]. In addition, tumors can downregulate MHC class I antigen expression

to evade immunological mechanisms based on antigen expression [69, 70]. Tumors can also upregulate inhibitory surface ligands such as PD-L1 to mediate T-cell anergy [71]. While these are only a few examples, the extent that tumors are able to undergo mechanisms for evasion of immunosurveillance can be appreciated.

In addition to changes in the tumors themselves, several studies have looked at how the immune cells interact with the tumors. The role of these cells within the tumor is still not well understood, but the most recent studies point to mechanisms the immune system undergoes that aids in tumor immunosuppression. Accumulation of CD4+/CD25+/FOXP3+ T cells (T regulatory cells, or T-regs), whose main function is immunosuppression to prevent autoimmunity, predicts poor survival in ovarian cancer patients [72]. T-regs are especially cumbersome and are thought to dampen T-cell immunity to TAAs and be the main obstacle to successful vaccination [73]. Other suppressive lymphocyte subsets that are present in the tumor include: IL-10 producing B-cells and myeloid-derived suppressor cells [74, 75]. In addition, non-immunological cells such as cancer-associated fibroblasts can aid tumor growth through secretion of chemokines that recruit immunosuppressive immune cells [34, 76]. The tumor microenvironment has factors from both the cancer cells and surrounding cells that are actively hiding the tumor from recognition

The most recent developed immunotherapeutic that addresses the role of immunosuppression during tumor development is ipilimumab, an anti-CTLA4 antibody that down-regulates inhibitory signals for T-cell activation. Ipilimumab has shown potential in clinical studies involving metastatic melanoma, showing increased survival even when used without a melanoma TAA vaccine [77]. However, there are side effects

associated with the treatment including hepatitis, inflammation of the eyes and pituitary gland, and nephritis. These side effects are the direct result of down-regulating the same mechanisms that are necessary to prevent autoimmunity. Ipilimumab is the first immunomodulatory cancer treatment approved by the FDA, paving the path for other similar molecules such as anti-PD1 antibodies to block immune checkpoints during cancer immunotherapy [78]. PD-1 limits T-cell activity in peripheral tissues during inflammation and prevents autoimmunity [79-81]. Through upregulation of PDL-1 in tumors, which binds to PD-1 on T-cells, this mechanism is utilized for immune resistance during cancer [82]. A Phase I study with anti-PD-1 antibody BMS-936558 showed patient safety and durable responses in NSCLC, melanoma, and renal cell carcinoma and that PD-L1 expression on tumor cells can serve as a potential predictive biomarker [83]. In addition, another phase I trial with anti-PD-L1 antibody showed durable tumor regression and prolonged stabilization of disease in the same advanced cancers [84]. Interest in these clinical trials points to the potential for immunomodulation as a form of cancer treatment.

The most effective treatment against a poorly immunogenic tumor will likely be a combination of therapies that will induce an anti-cancer immune response and utilize immunomodulation to counteract immunosuppressive signals. Progress towards this goal necessitates a better understanding and improvement of cancer vaccines.

Cancer Vaccines

Cancer vaccination is a heavily researched immunotherapeutic approach that offers the promise of initiating an immune response to TAAs that will result in tumor elimination with reduced toxicity to surrounding normal tissue [85]. Cancer vaccines can be used for a wide array of cancer types; currently cancer vaccines are being tested in clinical trials for breast, cervical, prostate, lung, and many other types of cancer [86]. Breast cancer has been shown to be an excellent candidate for cancer vaccine therapy because of known breast cancer antigens, demonstrating the therapeutic capability of vaccines for other cancers given further identification of usable TAAs [87]. A successful vaccine has the capability of activating CD4+ and CD8+ T-cells for a specific immune response [88]. However, the field of cancer vaccine development has been viewed with skepticism due to past failures, especially during phase 3 clinical trials [89]. In *Types of Cancer Vaccines*, an overview will be given on the different approaches to cancer vaccines as well as their failures and future potential.

Types of Cancer Vaccines

Cancer vaccines have included anything from whole-cell vaccination, full-length proteins, or antigenic peptides. Early vaccines used whole-cell or tumor lysates to elicit a polyvalent immune response, since single tumor antigens had not been identified [90, 91]. This vaccination with thousands of antigens did not elicit a tumor specific response [92]. Efforts were taken to use genetically modified vaccines to replace these inconsistent mixtures [93]. This includes using tumor cells that were infected with the influenza virus

or transduced with viral genes to increase immunogenicity in mouse models [94, 95]. Animals that rejected the transfected tumor cells lead to rejection of non-transfected tumor cells. In a similar effort to make tumor cells visible to the immune system, tumor cells have also been transfected with genes for cytokines and costimulatory molecules [96]. Phase I clinical trials in patients with advanced renal cancer preliminarily showed increased immunogenicity of autologous cells transduced with GM-CSF compared to non-transduced cells [97].

The simplest formulation is vaccination with small peptide/epitope antigens that were identified as TAAs (ex. MAGE-1) [98, 99]. Small peptides identified for vaccination are usually those presented on MHC. The first example of utilizing MHC restricted peptides is during vaccination against different viruses [100, 101]. Vaccination with cytotoxic T-cell epitope peptides protect against tumors induced by HPV in murine studies [102]. It was later found that non-targeted vaccinations that utilized high dose injection of antigenic peptides alone induced tolerance instead of protective immunity in lymphocytic choriomeningitis virus murine models [103]. Peptide cancer vaccines tested in clinical trials with melanoma patients used MAGE-3 or gp100 antigens in combination with Freud's incomplete adjuvant or IL-2 in order to prevent tolerance [104-106]. Rosenberg et al. used peptides from the gp100 melanoma-associated antigen and a synthetic peptide, designed to increase binding to HLA-A2 molecules, as cancer vaccines to treat patients with metastatic melanoma. In total 91% of patients were successfully immunized with the synthetic peptide, and 13 out of 31 patients (42%) receiving the peptide vaccine plus IL-2 had objective cancer responses. Parkhurs et al. vaccinated with three common gpl00 epitopes and saw melanoma-reactive CTL could be induced *in vitro*. Restimulations were

required and specific reactivity could not be generated in many patients. To enhance immunogenicity of gpl00 peptides amino acid substitutions were introduced which enabled the induction of melanoma-reactive CTL from all seven patients. These studies illustrate the delicate balance of tolerance and protective immunity that must be addressed in cancer vaccines.

Another engineering approach to cancer vaccines takes advantage of the immunogenicity of viruses to create recombinant viral vaccines. Rodent studies with recombinant Poxvirus vector vaccines that encode tumor antigens show tumor rejection and protective humoral and CD8+ immune responses [107-109]. Recombinant viral vaccines against cancer have been tested in clinical trials, but only recently was there success [110]. A phase II clinical trial in 2010 showed an improvement in overall survival in prostate cancer with a recombinant viral vaccine that encoded PSA and three immune costimulatory molecules [111]. Certain strains of recombinant bacteria have also been utilized because of their potential to be taken orally and because of their ability to infect monocytes and macrophages [112-114]. In particular, *Listeria monocytogenes* has been used in murine tumor models and it enables antigen processing through MHC class I and class II because of its two-phased intracellular life cycle in infected macrophages [115]. These recombinant vaccines are an interesting approach to enhancing antigenicity of TAAs through delivery with a highly immunogenic vehicle.

Naked DNA vaccinations have also been explored as an alternative to protein based formulations. Naked nucleic acids have less potential than recombinant viruses for vaccine usage. This is because DNA encoding TAAs are not highly immunogenic and there is no amplification of genetic material through viral reproduction. However, naked

plasmid DNA vaccines provide practical benefits for large-scale production that are not available for recombinant protein or whole tumor cells [116, 117]. The ability to design expression constructs makes DNA vaccines valuable for cancer immunotherapy. DNA vectors have been explored in mouse tumor models as a means of cancer vaccination, where inoculation with different viral and bacterial vectors encoding a model antigen resulted in some protection against tumors [118]. Follow up experiments showed that APCs are able to present the antigens encoded by the naked DNA, thereby verifying the ability of DNA vaccines to mediate an antigen specific response [119]. The major immunogenic component that activates APCs in DNA vaccines is the unmethylated CpG motifs that exist in bacterial vectors [120, 121].

If a cancer vaccine is unable to mediate a response on its own, the next step is to utilize adjuvants or other means of increasing immunogenicity of cancer antigens. The FDA approved Provenge® vaccine utilizes a recombinant protein that is a fused antigen-cytokine molecule. This allows for DCs to receive both antigen and an activating cytokine at the same time during maturation. Adopting a similar approach, a recent clinical trial showed improved progression-free survival and overall survival in melanoma patients by administering a gp100 antigenic peptide with systemic IL-2 [122]. Other forms of cancer therapy have the potential to increase immunogenicity without direct administration of cytokines. It is thought that chemotherapy can enhance tumor immunogenicity by decreasing regulatory T-cell function and increasing antigen load through tumor cell apoptosis [123, 124]. This would serve useful in future combination studies utilizing chemotherapy and immunotherapy.

Ultimately, all forms of cancer vaccines suffer from the same problem; the

inability to activate a therapeutically relevant immune response against a non-immunogenic cancer antigen that hides within a network of immunosuppressive factors (discussed in *Combination and Alternative Approaches to Immunotherapy*). Therapeutic cancer vaccines must break tolerance in order to be effective, a key point that has not been understood until recently. One way to break tolerance is to ensure delivery of a high load of antigen to professional APCs in order to activate a heightened immune response. Therefore, the potential to create an anti-tumor immune response with dendritic cell (DC) based vaccines has been explored. There is currently a need for vaccination formats that can target DCs directly *in vivo* in order to induce an optimal immune response against a desired antigen. Before this concept is further discussed, an overview of DCs will be presented in the next section.

Dendritic Cells

The major definition of efficacy for an APC is its ability to activate both naïve B-cells and T-cells in an MHC restricted fashion. The reason for targeting DCs is that they are a more potent APC than both B-cells and macrophages. In particular, DCs are the only APCs able to activate naïve T-cells *in vivo* [125, 126]. The reasons for their unique potency in activating T-cells include: higher expression of MHC molecules necessary for T-cell receptor engagement and an extremely high expression level of costimulatory molecules [93]. The signals required for activation of naïve T-cells are i) the presentation of the antigenic peptide fragment on the MHC scaffold for recognition by TCRs and ii) costimulatory molecules from the APC that are recognized by the T-cell and act as a secondary activation signal. The MHC class I scaffold supports an 8-10 amino acid long

sequence from an antigen for presentation to the immune system [127]. Without the appropriate co-stimulatory molecules present, the T-cells are induced into anergy which leads to tolerance instead of activation towards an antigen. These co-stimulatory molecules are present on APCs during certain levels of activation. Important co-stimulatory molecules include the B7 family (CD80, CD86) and CD40. Non-APC cells lack these stimulatory molecules and therefore are unable to activate an appropriate T-cell response. The traditional DC antigen processing model is that A) endogenous proteins (ex. viruses or tumor antigens) are processed and presented through the MHC class I pathway for activation of CD8+ cytotoxic T-cells for lysis of virally infected cells [128] and B) exogenous proteins internalized by the APC are processed and presented via MHC class II pathway for activation of CD4+ helper T-cells for B-cell and CD8+ T-cell activation. There is an exception to this model where antigens can be cross-presented, meaning that exogenous proteins can be processed and displayed on the MHC class I scaffold [125]. This mechanism only occurs in DCs and could potentially be utilized for MHC class I and II antigen presentation for activation of CD4+ and CD8+ T-cells.

DCs are derived from hematopoietic stem-cells and are located in lymphoid and non-lymphoid tissues where they coordinate the innate and adaptive immune response [129, 130]. While identification of DCs in lymphoid tissues first occurred by Steinman, DCs were originally identified in the skin by Paul Langerhans, hence the name Langerhans for epidermal DCs [131]. Based on location, DCs can be divided into lymphoid-organ resident and migratory. Lymphoid DCs reside in bone marrow, spleen, thymus, and lymph nodes [132-135]. During development DCs migrate to reside in different tissues including the skin, intestine, kidney, and liver [136-138]. Another

component to the mechanism set in place for DCs to resist infection and tolerance is the development of functional plasticity and subsets [139]. The two major subsets are the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). This diversity allows for the adaptive immune response to elicit different types of responses [140]. pDCs are front-line in anti-viral immunity because of their ability to secrete IFNα in response to viral infection [141]. IFNα secretion may also promote immunogenic maturation of other DC subsets of DCs. Furthermore, activated pDCs can induce B-cell maturation through cytokine production and surface signaling [142, 143]. Human blood mDCs share have the high capacity to capture exogenous antigens for cross presentation. They also are equipped for activation of CD8+ T cell-mediated immune responses because of expression of receptors against chemokines expressed by activated CD8+ T cells [144, 145]. Other human mDCs such as Langerhans cells are also able to cross-present antigens [146]. Langerhans are more efficient in cross presentation of antigens to CD8+ T cells and can differentiate CD8+ T cells into effector cytotoxic T-cells. Therefore, the Langerhans DC subset would be an optimal target for vaccination strategies.

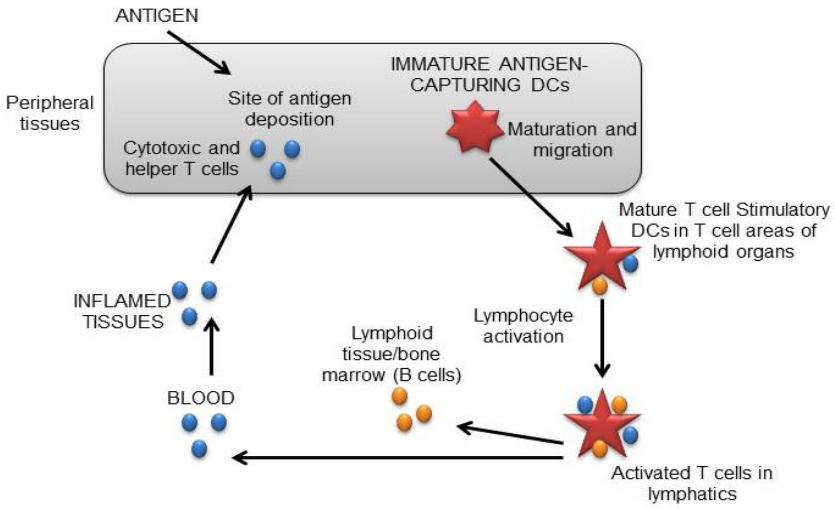


FIGURE 1 – IMMUNE SYSTEM ACTIVATION PROCESS BY IMMATURE DCS DURING ANTIGEN EXPOSURE

Figure modified from Banchereau et al. review [126]. Antigens are captured by DCs in peripheral tissues and processed for MHC presentation. Immature DCs begin to mature and express molecules for binding and stimulation of T cells in lymphoid tissues. If the antigen has also been captured by B cells, then both B and T cells can cluster with DCs. After activation, T-cells and B-cells migrate. B-cells secrete antibodies and T-cells respond to cells that are presenting antigen. This limits the T-cell response to the site of initial antigen exposure during pathogenic infection.

Dendritic Cell Vaccines

In general, vaccines using peptides, proteins, and DNA elicit a weak antigen specific immune response. These vaccines usually require an adjuvant in order to elicit a response against a TAA. DC's are targeted for cancer vaccines because, as explained in the previous section, they are potent antigen presenting cells (APCs) capable of activating naïve T-cells and eliciting a T-cell mediated immune response. Prior studies have pulsed antigenic peptides into isolated DC precursors *in vitro* and reintroduced these cells into the host [147]. Human DCs can be isolated from PBMCs and cultured in the presence of cytokine combinations (TNF-α, GM-CSF, Flt3 ligand, or CD40 ligand) and antigen to induce an antigen specific mature DC [148]. Several methods have been explored for introduction of antigen into DCs *in vitro.* The most common method is pulsing with peptide or protein, but other methods include adeno and lenti viruses, mRNA penetration, tumor-DC fusions, tumor lysates, and heat-shock protein/antigen complexes [149]. The benefits of these procedures are i) direct exposure of the antigen to DCs, increasing antigen uptake and processing and ii) immunosuppressive factors which downregulate DC activation are not present during *ex vivo* maturation. DCs can also be stimulated in a controlled environment where maturation status can be checked before administration [150]. However, there are caveats to this DC vaccination method. First, there is increased cost and time needed in the isolation and maturation of DC precursors *in vitro.* Cells need to be in culture for approximately a week to differentiate into immature DCs before exposure to antigen(s). In addition, adequate storage and production facilities are required to efficiently produce DCs for each individual patient. These costs are evident in the high price of Provenge®, which is an estimated $100k for a 4 month increased survival.

Another problem with this methodology is that the cells that have been matured and pulsed with antigen(s) *in vitro* are unable to migrate and activate naïve T-cells in lymph nodes [151]. Therefore, a novel vaccination approach needs to be developed that will ameliorate these problems that exist in current DC vaccination schemes.

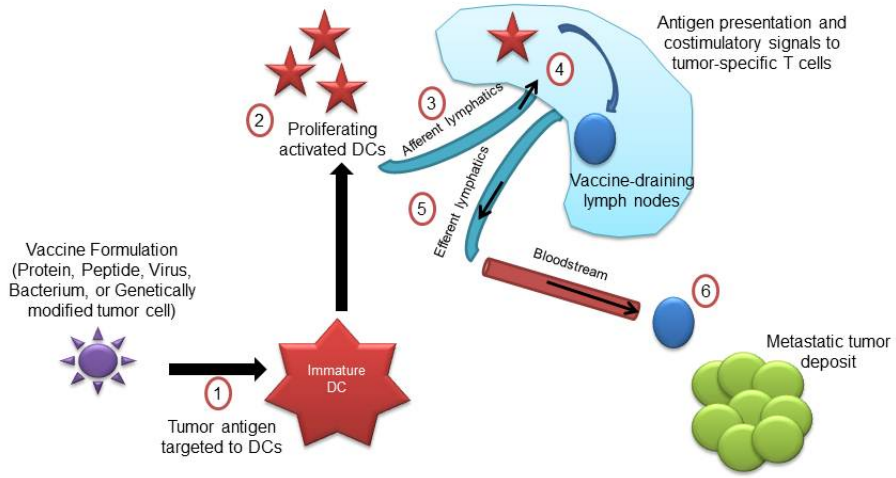


FIGURE 2 – MECHANISM OF ACTION FOR DC BASED CANCER VACCINES: Figure modified from Pardoll et al. review [92]. The figure shows the role of DCs in generating vaccine induced anti-tumor immune responses. Cancer vaccines consist of many formulations where the antigen is a protein, peptide, recombinant virus or bacterium, or whole tumor cell. The first step is for antigen(s) to be targeted to DCs (1). An ideal vaccine will activate DCs into proliferation (2). Steps 1 and 2 can be accomplished ex vivo with DC vaccines (ex. Provenge®). Activated DCs loaded with tumor antigen migrate to draining lymph node (3). In the lymph node, they present processed antigen(s) to T-cells and activate tumor-specific T-cells (4) that are otherwise antigen tolerant. Activated T-cells leave draining lymph nodes (5) and enter the bloodstream. They exit bloodstream in peripheral tissues, where they seek out and recognize tumor cells and kill them (6).

DC targeted antigen delivery is used to increase the efficacy of DC based vaccines. Previous studies have utilized a variety of targeting moieties including antibodies, TLR ligands, and carbohydrates. Antibody targeting to CD11c for antigen delivery allows for a murine immune response and rapid antibody production [152, 153]. Another study took advantage of mannose receptors that are highly expressed on DCs and created mannosylpolyethylenimine conjugates for DNA vaccination [154]. It was demonstrated that this vaccination produced activated antigen-specific T-cells. Ant-tumor activity was not tested in this publication. Other carbohydrate vaccinations also showed therapeutic value. Synthetic high-mannose oligosaccharide-antigen constructs were developed to target DC-expressed lectins [155]. TLR-2 ligands Pam2Cys, Pam3CysK4, and CpG target DCs and other immunological cells *in vivo* [156, 157]. These vaccines were also capable of inducing either CD8+ T-cell or antibody-mediated immune responses. These synthetically made TLR targeting vaccines also served as their own adjuvant. However, other non-TLR-2 expressing cells were also being targeting, bringing into question the ability of TLR ligands to act as specific targeting agents. In addition to utilizing targeting moieties, production of nanoparticle antigen carriers that are targeted to DC C-type lectin receptor DC-SIGN have been explored [158]. Cruz et al. utilized both carbohydrates and antibodies to target their nanoparticle to DCs for vaccination. In their comparison, they found that even with a higher number of surface molecules in the carbohydrate coated nanoparticle, the antibody nanoparticle had greater binding and internalization by human DCs *in vitro.* The author's conclusion from this study is that antibodies are more effective than carbohydrates for DC targeted vaccines. Another component to the targeting moiety is ligand density. Bandyopadhyay et al. produced nanoparticles with different densities of

anti-DEC-205 antibodies[159]. Unexpectedly, the nanoparticles induced a different cytokine response dependent on ligand density. Surface density increased the amount of anti-inflammatory IL-10 produced by DCs and T-cells. This is an interesting result that adds another component to creating DC targeted vaccines.

The development of targeting ligands begins with identification of a potential receptor for targeted delivery. Many receptors have been utilized for antigen delivery. The main type of receptor targeted is the C-type lectin receptor family, which includes the mannose receptor, CD205, and DC-SIGN (Table 3). Other receptor families include Fc Receptors and integrin. Prior *in vivo* dendritic-cell targeted vaccines have used antibodies against DC markers such as CD11c and DEC-205 to deliver antigens for vaccination [160, 161]. Other receptors that are targeted are expressed on other immune cells other than DCs, which may decrease efficacy of these *in vivo* targeting reagents. What is evident in Table 3 is that the receptors that are currently used for *in vivo* targeting strategies are not exclusively expressed on DCs. Many of these receptors are not only expressed on other immune cells, but on other non-immunological cells as well [162]. An example of this lack of selectivity with current targets is the widely used anti-DEC205 antibody. The DEC-205 target is expressed in subsets of peripheral DCs, including Langerhans, in murine models [163]. Initial studies showed that targeting DEC-205 with antibody-antigen fusions led to antigen specific immune responses in mice [164, 165]. In addition, DEC-205 targeted delivery of gp100, a melanoma tumor antigen, cured 70% of tumor bearing mice [166]. While targeting DEC-205 as a DC marker worked well in mice, this marker is expressed in various immune cells in humans [167]. Many other promiscuous receptors have been successfully targeted for eliciting immune responses,

despite their expression on non-DCs [168]. While targeting these promiscuous receptors do not seem to effect induction of immunity, it appears that it is still primarily the DCs that are responsible for T-cell priming and activation [169-171]. DCs dominate the immunological outcome of vaccination strategies, and therefore targeting antigens to promiscuous receptors may dilute vaccination efficacy or potentially induce tolerance. Therefore, there needs to be a targeting ligand that is able to deliver antigens specifically to DCs for future *in vivo* vaccination strategies.

Table 3: DC receptors targeted for *in vivo* vaccination strategies		
Receptor Family	**Targeted Receptor**	**Expression by human cells**
C-type Lectin	Mannose Receptor	Immature DCs, monocytes, macrophages
	CD205	Mature DCs, monocytes, B cells, NK cells, T cells
	DC-SIGN	Immature DCs, macrophages
	LOX1	Immature DCs, macrophages
	Dectin-1	Immature DCs, macrophages, neutrophils, B cells
FcR	FcγRI	DCs, macrophages, monocytes, neutrophils
	FcγRIIa	DCs, macrophages, monocytes, neutrophils, eosinophils
	FcγRIII	DCs, NK cells, macrophages, neutrophils, eosinophils
	FcγR	Mature DCs, monocytes, macrophages, neutrophils
Integrin	CD11c-CD18	DCs, monocytes, macrophages, NK cells, T and B cells
	MACI	DCs, monocytes, macrophages, NK cells, T and B cells
TNF-receptor superfamily	CD40	DCs, B cells, macrophages, keratinocytes, hematopoietic cell progenitors

Summary of dendritic cell (DC) surface receptors that have been used for the targeting of antigens to DCs (Modified from Tacken et al). The table shows which cells express the targeted receptor in human cells.

Hypothesis and Specific Aims

The necessity for improved cancer therapies has led to efforts in utilizing the immune system to recognize and destroy tumors [172]. Two main immunotherapeutic approaches, DC vaccination and antibody therapy, will be the focus of the project. The primary approach proposed is to use DC targeting peptides to deliver antigens directly to DCs for vaccination, thus eliminating the need for ex vivo manipulation of DCs. Most DC markers are shared with other immune cells, so it is difficult to directly target a specific subset of immature dendritic cells. Highly specific DC ligands are needed for the development of *in vivo* DC cancer vaccines. The Brown Lab has developed a peptide presenting - phage library panning method through which cell-specific peptides can be isolated without prior knowledge of the cell surface [173]. Peptides that can directly target immature dendritic cells and deliver antigens *in vivo* have been isolated with this technique [174]. The main goal of the project is to use the isolated DC peptides as delivery reagents for generating an immune response.

An additional component to improving cancer immunotherapeutics would be the development of a novel antibody therapeutic. A peptide-antibody conjugate would combine the immunogenic capabilities of an antibody with the peptides' ability for easy modification and interchangeability. Several lung cancer targeting peptides have been isolated and tested for binding in the Brown Lab. These peptides will be utilized to create a peptide-antibody format that will allow for facile and fast production of lung cancer targeting reagents for treatment.

The specific aims will test the ability of peptides to improve on cancer

immunotherapeutics, mainly the ability to deliver antigens to DCs for increase immunogenicity and to deliver immune reagents to cancer cells.

- **Specific Aim 1: Test the ability of DC peptides to elicit an antigen specific immune response.** The primary goal of this aim is to test whether the DC targeting-peptides can elicit a heightened immune response against a model antigen.
    - **Subaim 1a: Isolate and test DC peptide specificity *in vitro*.** DC-targeting peptides can be utilized for antigen delivery to this important subclass of APCs. Using a phage-displayed peptide library, DC targeting peptides will be isolated. The specificity of the isolated targeting peptides for DCs compared to other immune cells will be determined.
    - **Subaim 1b: Further test peptide DC binding on *ex vivo* cells.** The experiments performed in subaim 1a utilize cell lines. Subaim 1b will test the binding capabilities of the DC peptides on *ex vivo* immune cells. The transition to *ex vivo* cells will test the peptides' binding ability on BMDCs. Results will serve as an indication to peptides' ability to bind DCs not derived from a cell line and are more representative of *in vivo* DC subsets.
- **Subaim 1c: Test DC peptides in an *in vivo* murine vaccination model.** The DC peptides will be tested for ability to elicit a heightened immune response. The model antigen will be the phage antigens present on the targeting phage. These experiments aim will give an indication of whether these peptides merit further development and testing in tumor models.

- **Specific Aim 2: Test the ability of lung cancer peptides to target cells on an antibody scaffold.** The main goal of this aim is to preliminarily develop and test a peptide-antibody conjugate utilizing previously isolated lung cancer targeting peptides. There is a need for antibody treatment for lung cancer, and this need can be met by combining already isolated lung cancer peptides with an easily modifiable antibody scaffold. Specifically, this conjugation would combine the benefits of antibodies (immune function) with the benefits of peptides (easy production and isolation). This aim would develop a foundation for further work into developing a lung cancer antibody therapy.
    - **Subaim 2a: Develop a peptide-antibody conjugate and test binding abilities.** A lung cancer targeting-peptide will be conjugated to an antibody scaffold and tested for cell-specific binding on lung cancer cell lines.
    - **Subaim 2b: Determine targeting ability of conjugate in murine tumor model.** The peptide-antibody conjugates will be tested for their ability to target lung cancer tumors in an *in vivo* model. This will be the ultimate determination for the further utility of this conjugate for immunotherapeutics.

CHAPTER II

DC TARGETING PEPTIDES FOR VACCINATION

INTRODUCTION:

The Brown Laboratory specializes in the isolation of cell targeting ligands through biopanning live cells with a phage display library [175-179]. While the majority of the work in the Brown Lab has focused on isolating targeting agents to lung cancer cells, there have been many peptides isolated that target immune cells [179, 180]. This includes targeting peptides for macrophages, B-cells, and dendritic cells. The hypothesis of this project is that the DC targeting peptides can be utilized to elicit a heightened immune response by delivering an antigen to the DCs.

Phage Library and Previously Isolated DC peptides

A challenge that needs to be overcome is identification of new ligands that can bind to immature DCs with high affinity and specificity. In addition, multiple cell-binding ligands need to be isolated for a higher probability of acquiring the desired immune function. Biopanning of phage displayed peptide libraries on intact cells results in isolation of cell-specific ligands (Figure 3). In particular, this method can be used to isolate peptides that distinguish between different DC activation states. While antibodies have been the most common targeting ligand used for in vivo targeting strategies, peptides offer a less expensive and cumbersome approach to isolation of targeting ligands that are easily modified for down-stream applications. The library was constructed to express peptides at the N terminus of pIII fd phage coat protein. In addition, the vector

has a tetracycline resistance that allowed cloning with randomly synthesized oligonucleotides [181]. This synthetic phage library is not derived from a biological source which increases the potential to find high affinity ligands that do not exists in nature.

There have been other groups who have isolated DC targeting peptides. Curiel et al. also utilized phage display to isolate DC targeting peptides[182]. They used a 12-mer library and found 20 candidates that bound monocyte-derived human DCs. They were able to narrow down the candidates to 3 peptides through flow cytometric analysis of binding. The peptides did not bind to monocytes, T-cells, or B-cells. The peptides did bind DCs from chimpanzees, and only one peptide bound to murine DCs. These peptides were not specific for immature DCs and still bound to DCs that had been activated with LPS. The DC peptide fused to a hepatitis C viral protein was incubated with DCs *in vitro,* and the authors found that cytokine production by T-cells was higher when incubated with DCs pulsed with the targeted fusion protein. This heightened T-cell activation was also seen in a humanized murine model. The main problem with this study is that DCs were incubated with the targeted protein fusion, but there was no direct experiment to test the ability of the peptide to target DCs *in vivo.* The results indicate that the peptide does allow for higher loading of antigen, but whether this peptide can target an antigen to DCs *in vivo* was not adequately tested. One of the authors of this study, Mansour Mohamadzadeh, has done further work with the peptides by using a probiotic vector to make an oral vaccine that would deliver antigens to mucosal DCs for vaccination against pathogenic microbes [183]. Other peptides that have been used for DC targeting are derived from ICAM-4 that binds to CD11c/CD18 [184]. In addition to these peptides that

were derived from known ICAM-4 binding sequences, another peptide was identified through phage display [185]. This peptide was isolated by panning random peptide phage libraries on purified CD11c/CD18. They identified a phage expressing the circular peptide that bound specifically to CD11c/CD18 expressing monocytes but not CD11c/CD18 negative lymphocytes. The peptide sequence revealed a similar sequence in ICAM-1, and a soluble fusion protein containing the extracellular domain of ICAM-1 eliminated phage binding to CD11c/CD18. The synthetic circular peptide also inhibited ICAM-1 binding. The authors concluded that multimeric display of the selected peptide is essential for high affinity binding, and that the DC targeted phage binds specifically to CD11c/CD18 by structurally mimicking the interaction site of ICAM-1. Faham et al. used 2 peptides with sequence homology to human ICAM-4 to target CD11c/CD18 heterodimeric integrin in addition to the previously isolated phage display peptide [186]. Liposomes conjugated to the 3 peptides showed binding to CD11c+ cells *in vitro* and *in vivo*. Vaccination of mice with the antigen bearing liposomes induced antigen specific T-cell activation and antibody productions. Of the three peptides used, the one isolated through phage display was among the 2 that showed greatest efficacy. It also showed antitumor efficacy when vaccinated in a tumor mouse model. Unlike the Curiel study, this group administered the vaccine through i.v. injection, which is a more accurate portrayal of DC *in vivo* targeting. However, the targeted receptor (CD11c) is not exclusively expressed on immature DCs. These DCs are the subset most receptive for antigen processing and presentation. The proposed study will find DC targeting peptides with specificity to immature DCs and the ability to enhance immune response.

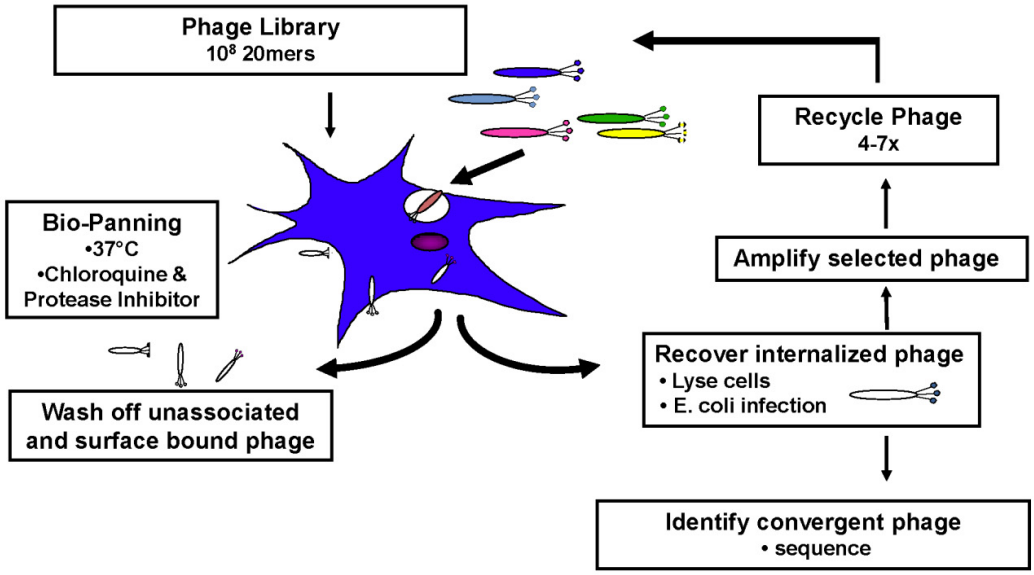


FIGURE 3 – SCHEMATIC OF PHAGE LIBRARY PANNING ON LIVE CELLS FOR ISOLATION OF TARGETING PEPTIDES:
Live cells were incubated with phage library (10^9) in culture for 1 hour. Phage solution contained phage in PBS+0.1%BSA with protease inhibitor and chloroquine to stop degradation of internalized phage. After incubation, the cells of interest were washed 4 times with PBS+0.1%BSA and 2 times with a low pH acid wash to remove unassociated and surface bound phage. Cells were incubated in 30mM Tris-HCl pH 8.0 to lyse cells for phage recovery. Recovered phage was incubated with K91 E. coli bacteria for infection and then plated on YT tetracycline plates and grown overnight. Agar plates were soaked in LB and scraped to acquire all phage clones from previous round of panning. The phage was purified and titered for use in the next round of panning.

Preliminary data with XS52 targeting peptides

In order to accomplish *in vitro* antigen targeting of immature DCs, the Brown Lab isolated targeting peptides to this cell population by biopanning a peptide presenting - phage library on a DC line in culture (Table 4). This panning protocol has two main advantages when selecting specific peptides to cell types: 1) The protocol allows for isolation of cell-specific peptides that undergo internalization by the cell. This is important when considering that DC's induce an immune response through internalization and presentation of antigens on the MHC class II scaffold. 2) Little to no knowledge of cell surface receptor expression is required for isolation of targeting peptides. This allows isolation of a cell specific targeting ligand without knowing the cell surface profile. It also keeps receptors in a relevant cellular context. The XS52 immature DC cell line was employed for biopanning a phage-displayed peptide library comprised of 10^8 different 20 amino acid peptides. A single phage clone displaying the peptide sequence GPEDTSRAPENQQKTFHRRW was isolated as the predominant species. This phage clone, named XS52.1, preferentially associates to XS52 cells, as witnessed by fluorescence microscopy using anti-phage antibodies (Figure 5). The functionality of the XS52.1 peptide outside of the phage scaffold was tested by utilizing peptides for Q-dot delivery to cells for imaging (Figure 5). This peptide is capable of targeting and delivering to only XS52 cells and not other immune cell types such as T-cell and B-cells (Figure 4). In addition, this peptide has reduced binding to XS106, which is a mature DC cell line. The peptide has specificity to the immature DC subset which is most capable of acquiring, processing, and presenting a tumor antigen to the immune system.

To further expand the number of DC-targeting peptides, the XS52 cells were panned using the original library but under more stringent conditions. Biopanning was also performed using a different de novo synthesized phage library containing 4 x 10^7 diverse sequences. From these pannings, another 3 DC-binding phage clones were isolated. To further analyze the functionality of these peptides during *in vivo* immunization, phage was administered by intradermal injection to mice with phage presenting peptide XS52.1, XS52.2.1, XS52.3, XS52.4, or control phage. Preliminary experiments showed XS52.1 and XS52.3 induced the highest anti-phage immune response of the different peptides at 35 days post-immunization (Figure 6). The antibodies produced in the mice were specific for the phage coat-proteins and not for the targeting peptide. These functional studies demonstrated that the isolated peptides target DCs *in vivo*. Further analysis visualized liposomal gene product delivery to DCs in migratory lymph nodes. These results support continuing this project with the DC peptides. Further experiments need to address the ability of the peptides to bind DCs *ex vivo* and to elicit a heightened antigen-specific immune response.

Table 4: Isolated DC targeting phage clones		
Phage Clone Name	**Peptide Ligand Sequence**	**Selectivity**
XS52.1	GPEDTSRAPENQQKTFHRRW	360
XS52.2	GLERGSGRPTSGGVPSALFG	80
XS52.3*	SGETGSNLVGHELDFRPGSPSP	70
XS52.4*	SGGGATKGPDGLRSAGTSSARG	60

XS52 immature DC cell line is an untransformed cell culture derived from the epidermis of a BALB/C mouse. Peptides were selected from two different phage-peptide libraries panned on the XS52 cell line. Phage clone names marked with an asterisk were selected from a second 22mer phage library that had a constant SG amino sequence at the N-terminus. This motif was added for facilitated bio-conjugation for downstream applications and is not required for specificity. Selectivity was calculated as the output phage to input phage ratio and was normalized to a non-specific control phage.

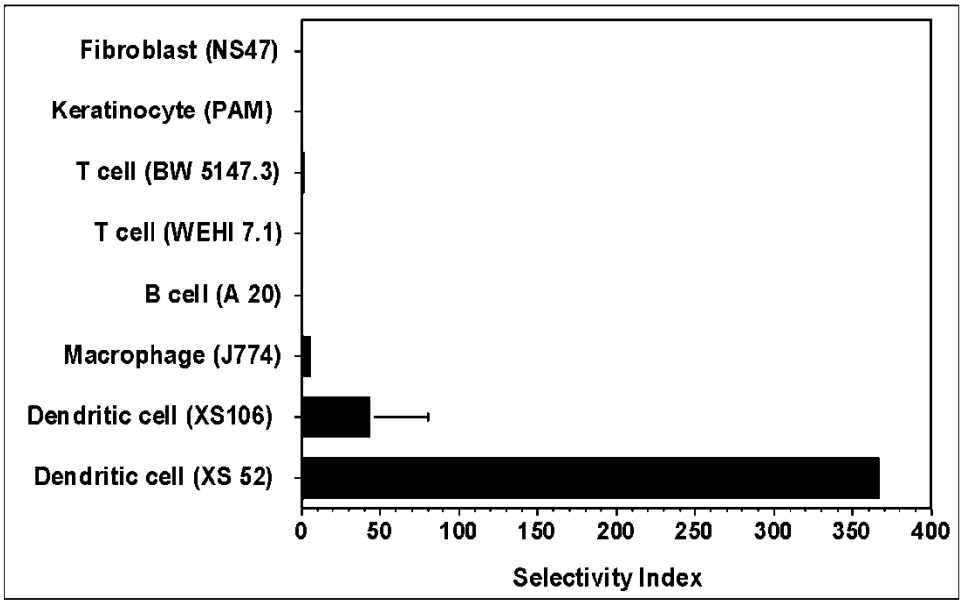


FIGURE 4 - XS52.1 PHAGE CLONE SELECTIVITY COMPARING XS42 CELLS AGAINST DIFFERENT IMMUNOLOGICAL CELL LINES:
Isolated XS52.1 DC phage clone was tested through comparative binding against different cell lines for selectivity based on phage output/input normalized to a non-binding control phage (selectivity index). Cells are incubated with either control (NGRGTELRSPSVDLNKPGRH) or XS52.1 phage. After incubation, the cells are washed. Cells are lysed for phage recovery and phage is incubated with K91 E. coli bacteria and plated. Colonies are counted for calculation of phage input and output. Name and mouse strain: NS47 (Balb/c), PAM 212 (Balb/c), BW5147.3 (AKR/J), WEHI 7.1(Balb/c), A20 (BALB/cAnN), J774A.1 (Balb/cN), XS106 (A/J), and XS52 (Balb/c).

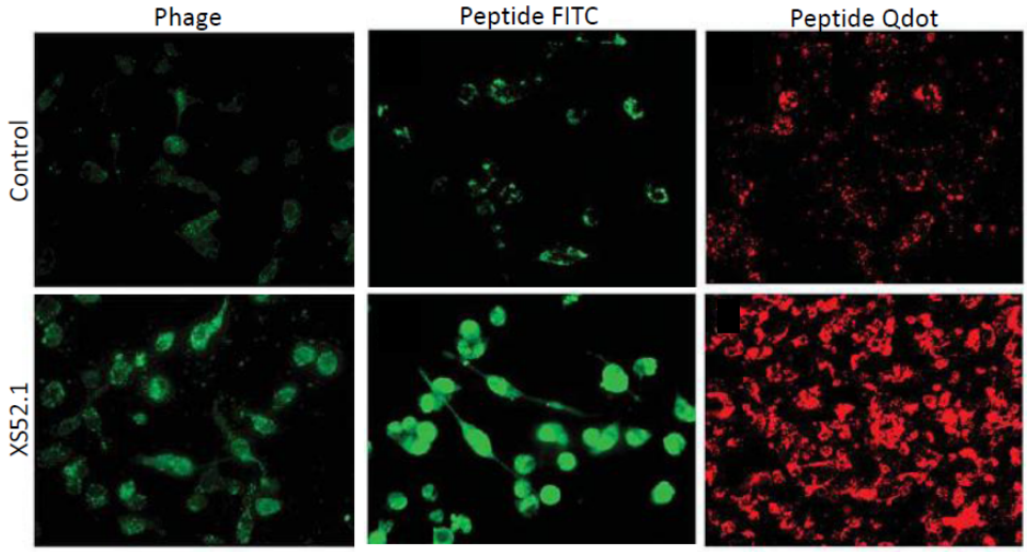


FIGURE 5 - XS52.1 PHAGE/PEPTIDE TARGETING OF XS52 CELLS:
Phage was detected by using an anti-phage murine antibody made in house from sera of vaccinated mice. XS52 cells were grown to 70% confluency in an eight-well chamber slide. Cells were incubated with 10^8 phage and then fixed with 4% paraformaldehyde. Cells were permeabilized and blocked with 2% normal goat serum. Cells were incubated with mouse antiphage antibody and then incubated with FITC-conjugated goat antimouse IgG antibody. Nuclei were stained with Hoechst dye. For peptide imaging, tetrameric peptides were synthesized with a biotin moiety to allow binding to NeutraAvidin-FITC and Strepavidin Qdot605. For peptide-NeutraAvidin FITC, cells were first incubated with peptide and then permeabilized and incubated with FITC-conjugated NeutraAvidin. For Qdot imaging, the biotinylated peptide (200 nM) was incubated with Streptavidin-Qdot 605. Excess SAQdot605 was blocked with free d-biotin. The peptide-SAQdot605 solutions were diluted with serum free RPMI to a final concentration of 20 nM. Cells were incubated with peptide-Qdot605 and then fixed and visualized. Cells were visualized by fluorescent microscopy on a Nikon Eclipse TE2000 fluorescent microscope.

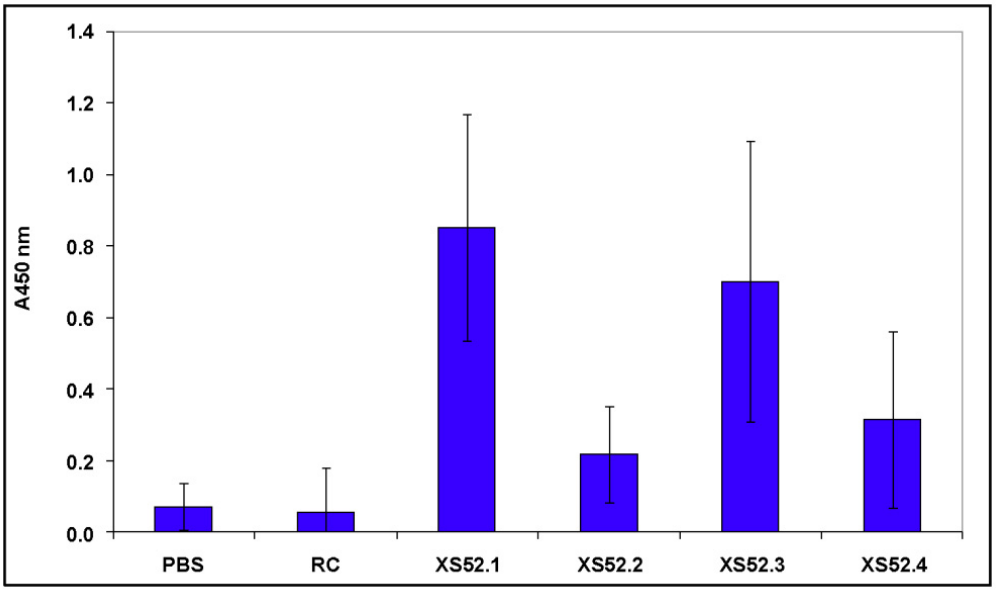


FIGURE 6 - PRELIMINARY BALB/C DC PHAGE CLONES VACCINATION: Groups of five BALB/c mice were injected intradermally with 10 ng of the phage. Mice were boosted on day 21 and blood was collected on day 35. Sera were analyzed for anti-phage antibodies through ELISA plates coated with 10 µg/ml whole control phage (peptide sequence LFMGAGMEVGLGGAPLKSQT). Sera were diluted 1:250 and incubated for 2 hours. The secondary anti-mouse horseradish-peroxidase conjugated antibody was used for visualization with TMB substrate. Reaction was stopped at 10 minutes with 1 M sulfuric acid and absorbances were read at 450 nm.

RESULTS:

In Vitro Phage Binding Results

Based on the previous published results, it was known that the XS52.1 phage clone binds to XS52 cells in culture. Both the XS52.1 and XS52.3 phage clones were isolated from panning of the phage display library on XS52 Balb/c Langerhans cell line. However, only the XS52.1 had been analyzed for binding to other immune cells. The comparative binding assay, which measures binding of a phage clone to its original cell type versus other cell lines, was performed with the XS52.3 phage clone to determine its specificity for XS52 cells. XS52.3 phage bound selectively to XS52 cells in culture based on the selectivity indexes calculated from the comparative binding assay (Figure 7). Numerical values for the selectivity index of XS52.3 phage clone on XS52 cells have previously been determined in the laboratory. The XS52.1 peptide binds to XS52 cells 360 times better than control phage, and the XS52.3 binds 70 times better. According to one-way ANOVA analysis, the difference between the selectivity index of XS52.3 binding on XS52 cells compared to NS47, S49.1, A20, and XS106 cell lines is statistically significant. XS106 is a mature DC cell line that was developed by Akira Takashima, the same laboratory that developed the XS52 cell line. This indicates that XS52.3 does not associate with other immune cell types and can differentiate between mature and immature DCs. There was no statistical difference in XS52.3 selectivity index between J774A.1 and XS52 cells. However, the selectivity index for J774A.1 was under 20, which has traditionally been used as the cut-off point for what is considered binding. A potential explanation for XS52.3 phage's small affinity to macrophages is that this

phage clone binds to a common myeloid/ monocyte marker. In comparison, the XS52.1 phage clone binds to XS106 cells (FIGURE 4) which would indicate binding to a common Langerhans marker. For future analysis, it is desirable to determine whether the DC phage clones are capable of binding to DCs that have not been immortalized for cell culture.

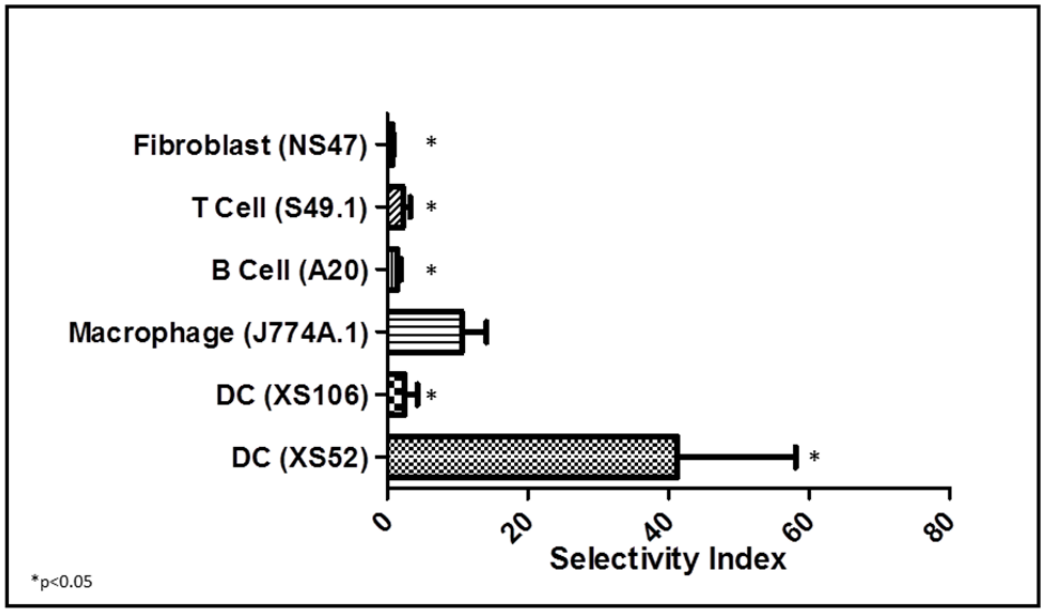


FIGURE 7 - XS52.3 PHAGE CLONE COMPARATIVE BINDING WITH XS52 CELLS VERSUS OTHER IMMUNOLOGICAL CELL LINES:
Selectivity values of XS52.3 phage on other immune cells were measured through comparative binding assays (same protocol as figure 2). Selectivity index was a measure of targeted phage clone binding to cells compared to a control phage clone. Cells analyzed were NS47 (BALB/c Fibroblasts), A20 (BALB/cAnN B cells), J774A.1 (BALB/cN Macrophages), XS106 (A/J mature DCs), and S49.1 (BALB/c T cells). All floating cell lines (A20, XS106, and S49.1) did not undergo acid wash. S49.1 cells did not undergo pre-clear step (2 hour incubation in RPMI) due to the delicate nature of cells.

Ex Vivo BMDC Phage Clone Binding Results

Bone marrow from mice were isolated and cultured for characterization of DC phage binding on *ex vivo* DCs. Flow cytometric analysis of DC markers MHCII and CD11c was performed on BMDCs as an indication of the population of immature DCs that were derived after culture. Defining immature DCs as cells that express both MHCII and CD11c markers, BMDC cultures from both Balb/c and C57BL/6J mouse strains resulted in an estimated 30% immature DC population (Figure 8). Culturing of bone marrow cells in GM-CSF is the simplest and most common means of differentiating monocytes to DCs, and this is the preferred methodology to obtain a large amount of immature DCs ex vivo. After verification of immature DC status, immunofluorescence imaging of phage was performed to visualize phage clone binding onto BMDCs (Figure 9). BMDC's were incubated with Control, XS52.1, or XS52.3 phage clone in culture for 10 minutes. XS52.3 phage clone preferentially associates with BMDC's from both Balb/c and C57BL/6J strains when compared to Control and XS52.1 phage clones. This result correlates with the comparative binding assay results and indicates that XS52.3 targets a general DC marker while XS52.1 targets a marker more exclusively expressed on Langerhans cells. This also justifies further analysis of the DC targeting phage clones' ability to elicit a heightened immune response in murine vaccination models. Dual staining with DC markers was not performed due to technical difficulties with visibility of staining antibodies during microscopy. Mainly, the CD11c marker was not visible during microscopy and therefore the double population could not be determined. Initial imaging showed that there were cells that expressed MHC II without phage, but any cells with phage had MHC II expression. However, MHC class II not exclusively expressed on

DCs and therefore was not used as an indication of phage binding to immature DCs. Mature DCs, monocytes, and macrophages all express MHC class II markers.

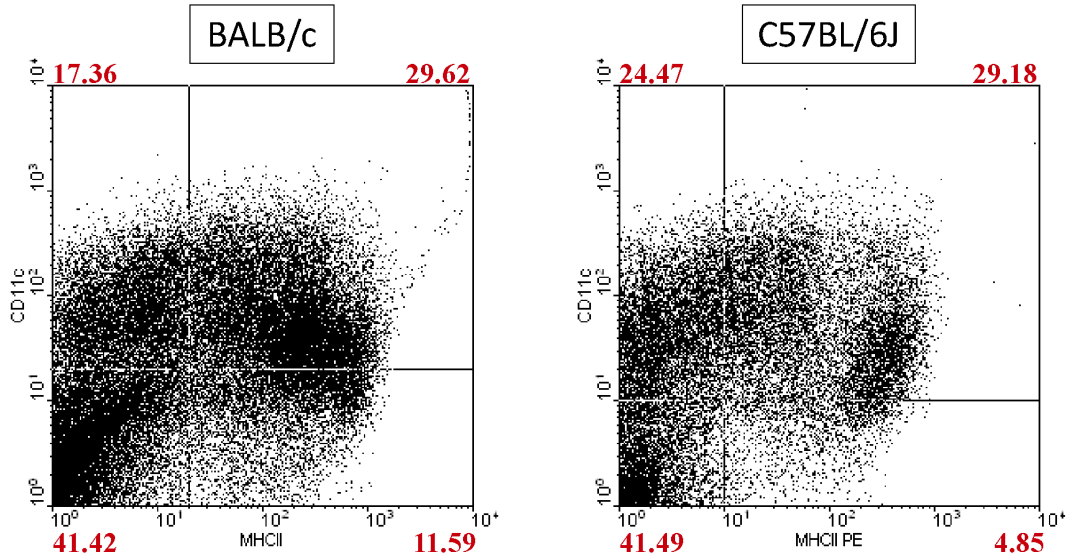


FIGURE 8 - FLOW CYTOMETRIC CHARACTERIZATION OF BMDCS FOR PURITY ANALYSIS:
Bone marrow from BALB/c and C57BL/6J mice was flushed out of the femur and tibia and treated with Red Blood Cell Lysis Buffer for 3 minutes. Cells were reconstituted in RPMI with 10% FBS, 10mM HEPES, and 50 μM 2-ME with 1:100 dilution of mouse recombinant GM-CSF (Gift from Dr. Pasare). Cells were plated on a 24-well dish with1mL per well at a cell concentration of 700,000 cells/ml. Fresh media with GM-CSF was added to cells on day 2 and day 4 of culture. Non-adherent cells were collected on day 5 of culture. Cells were resuspended in blocking buffer (PBS w/ 2%BSA) with 1:50 FcBlock for 30 minutes at 4°C. Cells were spun and resuspended in blocking buffer with antibodies (1:200 of CD11c PE/Cy5, 1:700 of MHCII PE) for 30 minutes in 4°C. Cells were then washed 3 times in PBS+0.1%BSA and resuspended in 1 mL solution for flow cytometry analysis on FACScan. Quadrants are drawn based on non-stained cells (less than 5% outside of lower left quadrant).

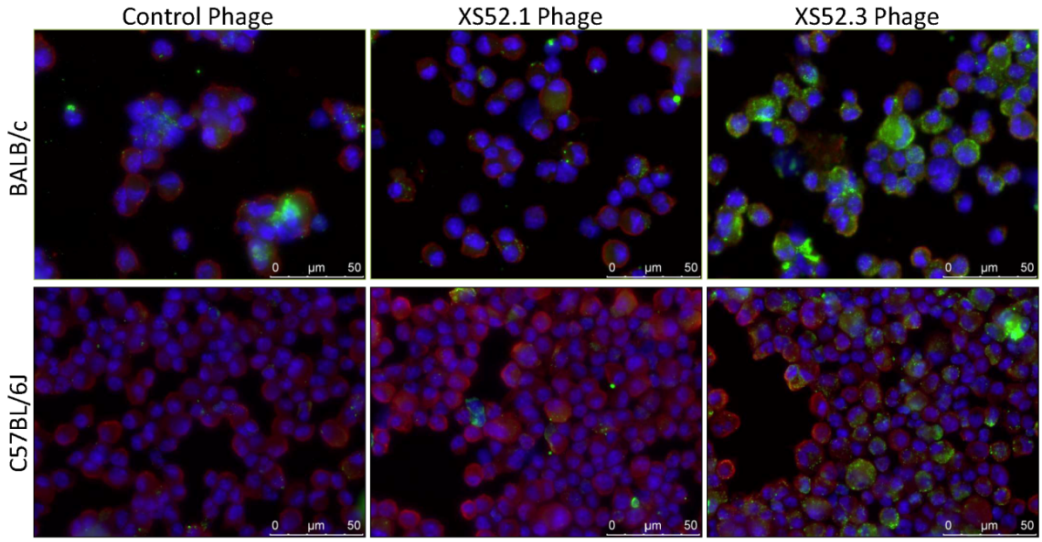


FIGURE 9 - DC PHAGE CLONES BINDING TO EX VIVO BMDCS:
BMDCs were generated *ex vivo* from bone marrow of BALB/c and C57BL/6J mice. On the 5th day, floating cells were harvested and incubated with phage solution for 10 minutes at 37°C. Cells were washed and spun onto poly-d-lysine slides. Cells were fixed to the slides and prepared for imaging. GAM AF488 antibody was used to visualize phage. Cells were stained with WGA TxRED to visualize cell membranes. XS52.3 showed binding to BMDCs from BALB/c and C57BL/6J mice, but not XS52.1. This suggests that XS52.3 phage is capable of binding several different DC subsets while XS52.1 is specific to Langerhans.

In Vivo Phage Vaccination Results

Balb/c and C57BL/6J mice were injected intradermally with XS52.1, XS52.3, or Control to analyze the capability of the DC targeting phage clones in eliciting an anti-phage humoral response (Figure 10). Intradermal injections were done to ensure Langerhans antigen exposure; intradermal injection increases the local concentration of antigen in the region of the cellular subset being targeted. The XS52 targeting peptides were isolated from a Langerhans cell line, and intradermal injection increases the local concentration of antigen in the region of the cellular subset being targeted. Mice were injected with 50 ng of phage from phage stocks that were endotoxin purified to reduce any interfering adjuvant effect not associated with the phage. 50 ng of targeting-phage was determined to be the minimum amount against which an immune response was seen in mice, with control phage not eliciting a response until injection of 500-1000 ng phage (Figure 11-1). ELISA plates were coated with empty phage that did not contain the peptide to measure antibody response against phage proteins. XS52.1 and XS52.3 DC targeting phage clones elicited a heightened anti-phage humoral response compared to Control phage as measured through ELISA. This difference is statistically significant according to one-way ANOVA statistical analysis of day 35 ELISA results. The timeline graph shows that antibodies were produced as early as day 14 with an increase after the booster shot administered on day 28. The sera from mice with an elicited immune response were further diluted to determine whether there was a difference between the two DC targeting phage clones. A difference was seen between Balb/c XS52.1 vs all other groups at 1:500 and 1:1000 sera dilution, but this difference was not statistically significant. Western blots using combined sera as the probing antibody against empty

phage showed that all groups responded strongly to the PIII phage protein except for XS52.1 in Balb/c. This group produced an antibody response to various different phage proteins other than the main PIII protein. The western blot also showed a small response to a minor phage protein in Balb/c mice injected with Control phage. This was not seen through ELISA and was only detected with the western blot. To obtain an indication of whether a TH1 or a TH2 response is being elicited, antibodies from the sera were isotyped and IgG2a/IgG1 ratios calculated. Only mice that responded in the initial ELISA were isotyped; Mice whose sera did not show an absorbance through ELISA were excluded from the ratio calculation. The results indicate that XS52.1 and XS52.3 targeting phage do not bias the TH response, except for Balb/c mice injected with XS52.1 phage. There seemed to be a TH1 bias in this vaccination group. The potential benefit fo a TH1 bias is the production of antigen specific cytotoxic T cells which are important for tumor elimination. The overall results indicated that DC targeting phage clones elicited a humoral response, with XS52.1 phage eliciting a different humoral and TH response only in Balb/c mice. The bias that this phage had did not seem to be universal and was limited by strain genotype. This could be due to genetic variances that affect immunological response. This phenomenon has been seen in other vaccination studies where both BALB/c and C57BL mice respond with a different TH response to the same antigen administered through the same route.

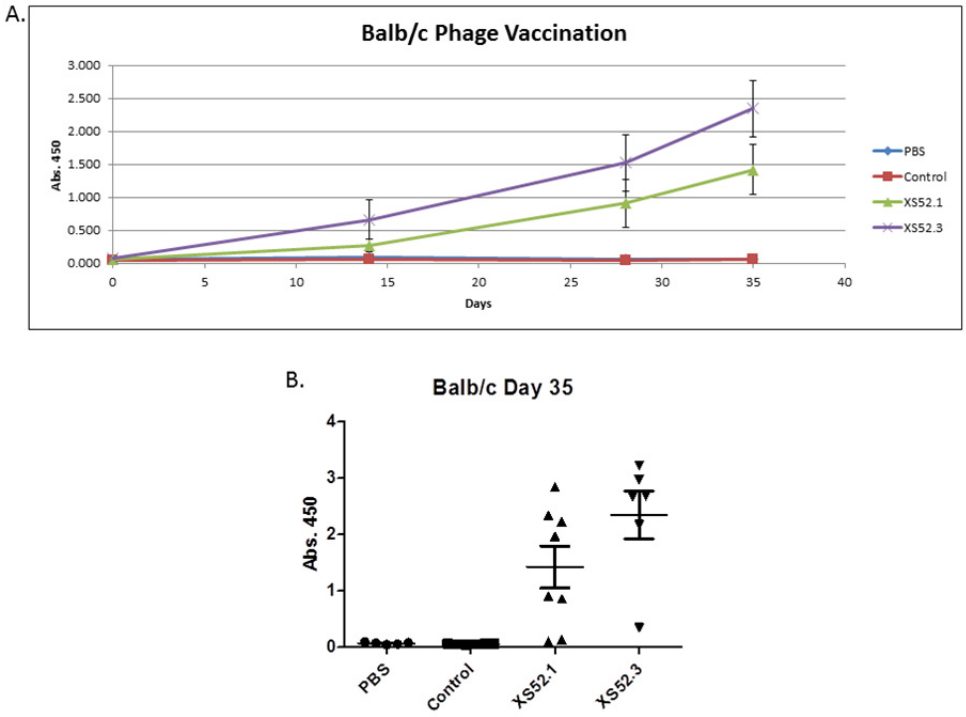
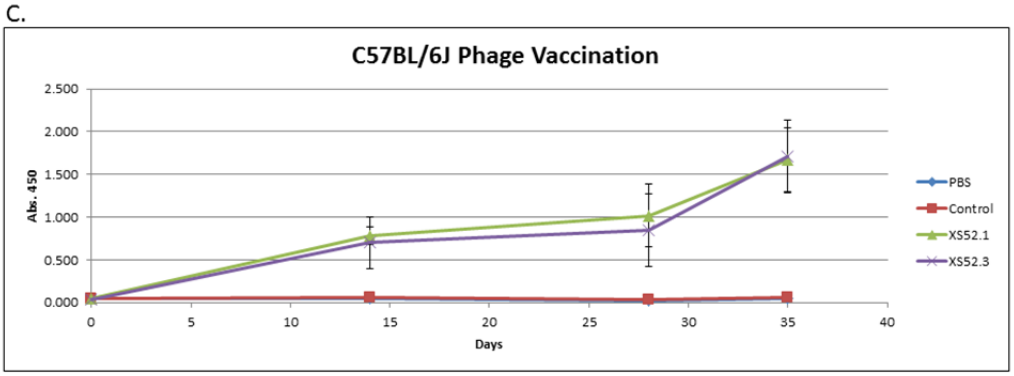


FIGURE 10-1 - DC TARGETED PHAGE CLONES ELICIT HEIGHTENED ANTI-PHAGE HUMORAL RESPONSE IN BALB/C MICE:
BALB/c (A and B) mice were injected intradermally with either PBS (endotoxin free), or 50ng of either Control phage (133.1 Eu/mL), XS52.1 phage (272.8 Eu/mL), or XS52.3 phage (384.8 Eu/mL). Initial injection occurred on day 0 and a booster shot was given on day 28. Stock phage solutions were endotoxin purified using a polymixin B gel resin column. Sera from these mice were tested in an ELISA on empty phage (no peptide presented) coated plates. The development of anti-phage antibodies with XS52.1/XS52.3 is shown over time (A) and the final titer on day 35 (B). Error bars show SEM.

C.



D.

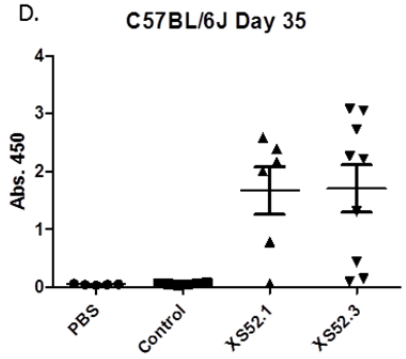
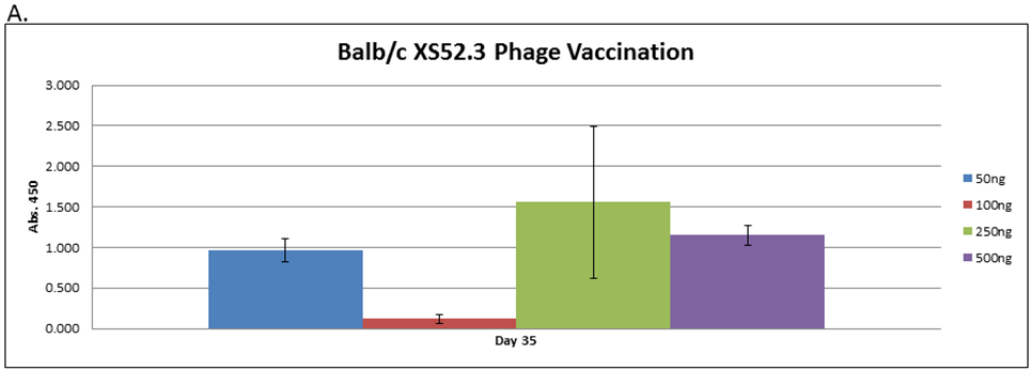


FIGURE 10-2 - DC TARGETED PHAGE CLONES ELICIT HEIGHTENED ANTI-PHAGE HUMORAL RESPONSE IN C57BL MICE:
C57BL/6J (C and D) mice were injected intradermally with either PBS (endotoxin free), or 50ng of either Control phage (133.1 Eu/mL), XS52.1 phage (272.8 Eu/mL), or XS52.3 phage (384.8 Eu/mL). Initial injection occurred on day 0 and a booster shot was given on day 28. Stock phage solutions were endotoxin purified using a polymixin B gel resin column. C showed the presence of anti-phage antibodies over time. D showed different responses of individual mice on day 35. Error bars show SEM.

A.



B.

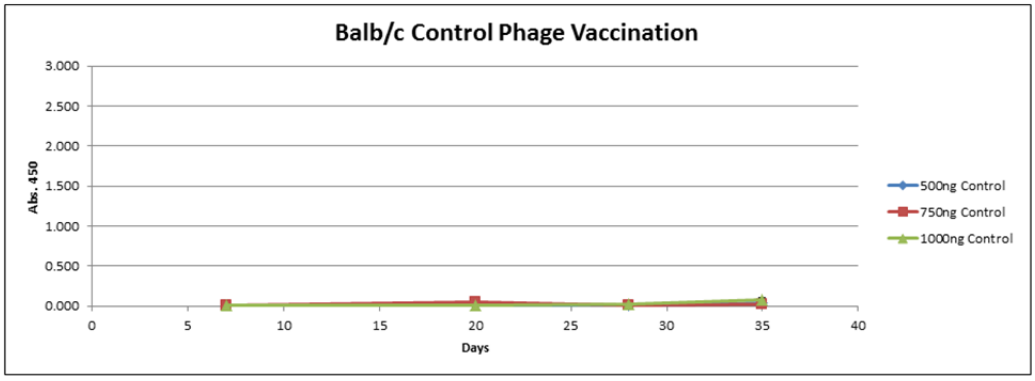


FIGURE 11-1 – PHAGE VACCINATION EXPERIMENTS TO DETERMINE ANTIGENICITY IN BALB/C MICE

BALB/c (A and B) mice (2 per group) were injected intradermally with different phage concentrations. A shows measurement at day 35 while B shows time-course measurements. Initial injection occurred on day 0 and a booster shot was given on day 28. Sera from these mice were tested in an ELISA. A shows immune response against 50 ng XS52.3 phage, while B shows no response. Error bars show standard deviation.

C.

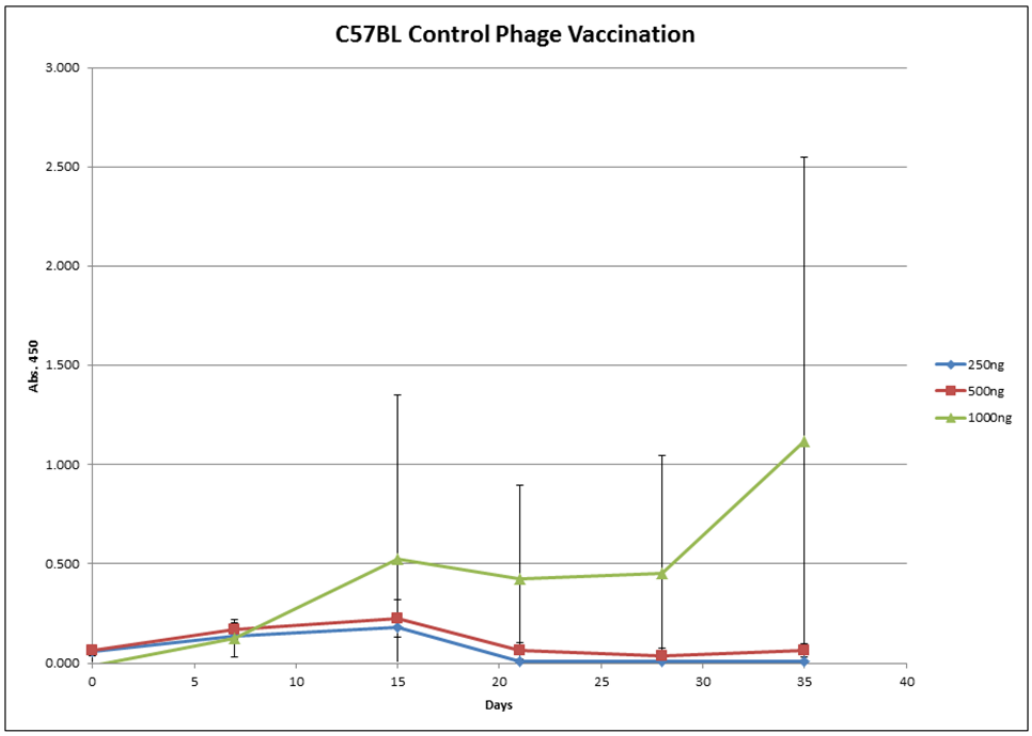


FIGURE 11-2 – PHAGE VACCINATION EXPERIMENTS TO DETERMINE ANTIGENICITY IN C57BL MICE

C57BL/6J (C) mice (2 per group) were injected intradermally with different phage concentrations (time-course measurements). Sera from these mice were tested in an ELISA on empty phage (no peptide presented) coated plates. No response until 1000ng is seen. Error bars show standard deviation.

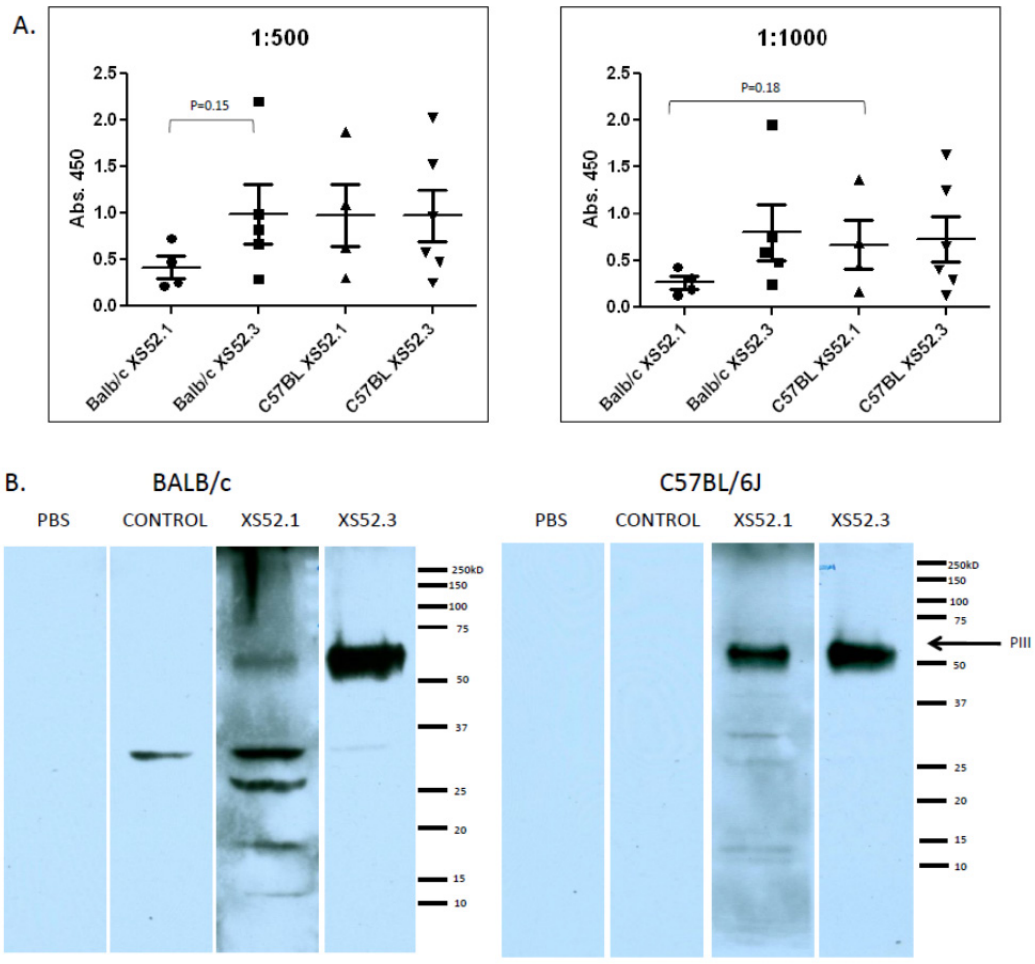
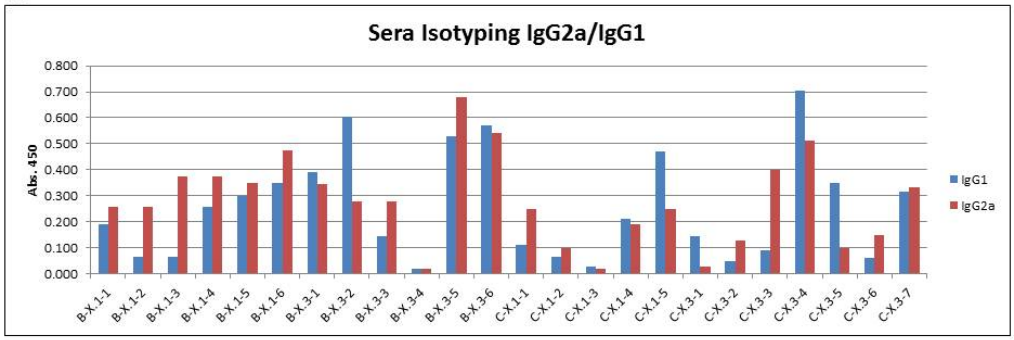


FIGURE 12 - CHARACTERIZATION OF DC-TARGETED PHAGE ELICITED HUMORAL RESPONSE:

A. ELISAs were done on highest responders from Figure 9 at either a 1:500 or 1:1000 sera dilution. Though SEM bars showed Balb/c XS52.1 to have a lower response than other groups, statistical analysis (Student paired T-test) showed this difference is not significant. B. Empty phage (1ug per well) was run on an SDS-page gel and transferred to a nitrocellulose membrane. Western blots were performed by using pooled sera from vaccinated mice as primary antibody and using a goat anti-mouse HRP secondary and SuperSignal® Chemiluminescent substrate. A strong response for PIII phage protein was seen in all groups except for Balb/c XS52.1 mice.

A.



B.

		MOUSE						
STRAIN	PHAGE	1	2	3	4	5	6	7
BALB/c	XS52.1	1.36	3.99	5.82	1.45	1.16	1.35	
	XS52.3	0.89	0.46	1.92		1.29	0.94	
C57BL/6J	XS52.1	2.24	1.55		0.90	0.53		
	XS52.3	0.18	2.74	4.38	0.73	0.29	2.38	1.05

TH1 TH2

FIGURE 13 – ANTBODY ISOTYPING OF ANTIBODIES IN SERA OF VACCINATED MICE FOR TH RESPONSE ANALYSIS:
IgG2a/IgG1 isotyping was performed on mouse sera from targeted phage vaccinated groups. ELISA plates were coated with empty phage Isotype secondary was added at 1:1000 dilution and tertiary HRP conjugated antibody was used for detection with TMB. Original ELISA results (A) and calculated ratios of IgG2a/IgG1 (B) are shown. Preliminary data showedTh1 response in BALB/c injected with XS52.1 phage, but all other groups showed no bias in Th1 versus Th2. Only mice that showed immune response in primary ELISA experiment were isotyped. Black boxes represent mice that were tested but had low signal in the isotyping ELISA.

OTHER EXPERIMENTS WITH DC PEPTIDES:

Attempts to utilize the DC targeting peptides outside the context of the phage were undertaken in order to test the utility of the peptide in eliciting an antigen specific immune response. The vaccination formats that were created include a DNA vaccine and a peptide-antigen direct conjugate; both utilizing the DC targeting peptides outside the context of the phage. These formats should give an indication of whether these peptides are useful in the *in vivo* targeting of antigen to DCs. This would also lay claim to the versatility of these peptides for being used in multiple formats for immunotherapeutic approaches.

DNA Vaccination

The initial experiments used a ballistics gene gun for DNA vaccination. The gene gun is a biolistic particle delivery system that introduces genetic material into the dermal layer of the skin using pressurized inert gas. This methodology had previously been utilized in the laboratory of Stephen Johnston with a humoral immune response elicited to antigens encoded in a genetic plasmid under the CMV promoter [187]. To test the ability of the DC targeting peptide to deliver antigen during DNA vaccination, a genetic construct containing a leader secretion sequence, Kozak sequence, and peptide was used. The proposed mechanism of action is that the gene gun will introduce the genetic construct into the dermal layer of the ear of vaccinated mice. Most cells receiving the construct will be the abundant keratinocytes. These cells will produce and secrete the antigen-peptide conjugate which will targeted DCs and hopefully initiate antigen specific

immunity. Some constructs will contain a COMP domain that will allow for multimerization of the antigen-peptide conjugate, mimicking peptide presentation that exists on phage. Multivalent binding can improve binding affinity for weak binders [188]. Antigen targeted to DCs through this mechanism will be internalized and presented through the MHCII pathway that activates CD4+ T cells. A small percentage of Langerhans cells will also receive the construct. When they produce the peptide antigen it is anticipated to be processed and presented through the MHCI pathway. This pathway will directly activate CD8+ cytotoxic T-cells, the cell type most capable of directly lysing tumor cells in a cancer model. Therefore, the benefit of using this vaccination strategy is the ability to elicit immune responses mediated by MHCI and MHCII antigen presentation [125].

The initial idea was to create a construct to encode the antigen-peptide conjugate. The model antigen of choice is ovalbumin chicken egg protein (OVA), since it is not a mouse self-antigen and is highly immunogenic. This model antigen has been well studied and characterized for vaccination models. The full length mRNA sequence of OVA was inserted into the genetic construct to demonstrate that the delivery system could elicit a response to a full length protein without biasing the response to a single antigen sequence. This would be especially useful in downstream applications where the antigenic peptide is unknown for a TAA. In addition, the genetic construct was created to have interchangeable components to facilitate downstream modifications. This includes an interchangeable peptide sequence, antigen domain, and the ability to remove the COMP sequence to easily create the monomeric mimetic antigen-peptide conjugate.

While the construct had been designed for easy modifications, difficulties arose that included accurate insertion of the OVA sequence and low success rate of integration of a new peptide sequence. For these reasons, only the targeting constructs were created. An initial experiment to test the efficacy of the construct was conducted by vaccinating Balb/c mice with the genetic construct using the ballistics gene gun. The construct was coated onto gold beads to make the DNA bullets, using a protocol previously optimized in the laboratory. Five groups had gold bullets that also contained genetic constructs that encoded the GM-CSF and Flt3L adjuvants. This combination of adjuvants in the genetic format had also been previously utilized to enhance humoral immune response against the antigen encoded by a genetic construct. Unfortunately, no humoral immune response was observed on day 35 in all groups as determined by ELISA. The lack of an immune response could be from a faulty vaccination construct or from the gene gun vaccination.

To further analyze the complications that occurred with the initial experiment, mice were vaccinated with the constructs encoding the DC targeting peptides with no COMP domain compared to a group vaccinated with a positive control construct encoding the human AAT protein. This construct contained the same CMV promoter as the DC targeting construct, the only difference being that the AAT construct does not encode for any peptide, Control or target. Previous experiments with this construct showed a strong humoral immune response against this immunogenic antigen, therefore it should serve as a strong positive control for the DNA vaccination. No antibody production was observed by ELISA with any of the constructs, even the AAT positive control. Upon further analysis of the DNA content of the DNA bullets, it was found that only 10-15% of the 1ug of DNA originally added was attached to the bullets. This low

DNA loading efficiency, along with the failure of the positive control to elicit a humoral immune response, indicates that the most likely explanation for the lack of immune response is the low amount of DNA introduced during vaccination. For these reasons, no clear conclusion can be drawn about the efficacy of the DC targeting DNA constructs until loading efficiency of the bullets is improved.

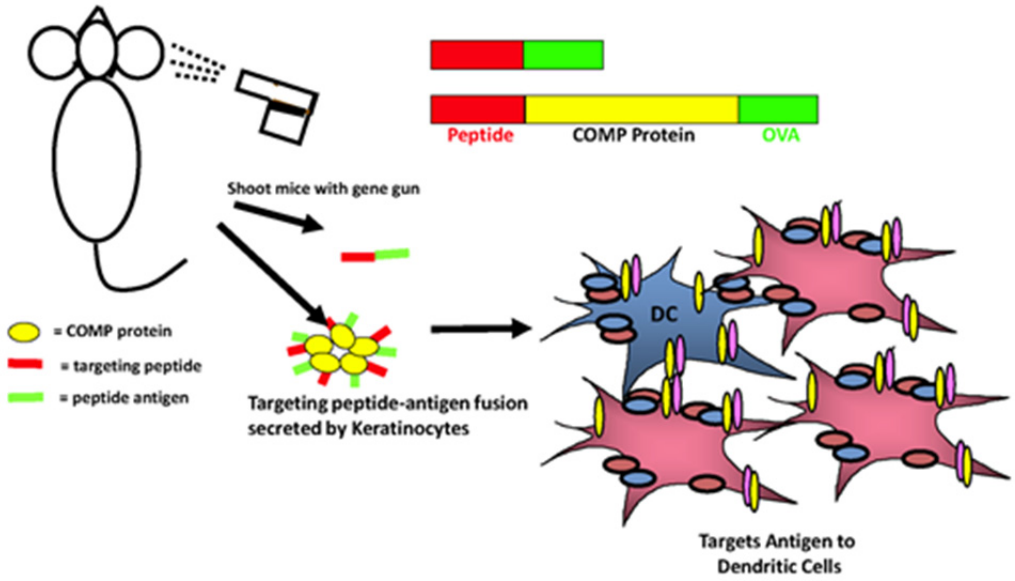


FIGURE 14 - DNA GENETIC VACCINATION SCHEMATIC USING GENE GUN:
The schematic demonstrates how the DNA vaccination will theoretically work. Constructs in an expression plasmid (CMV promoter) will contain a leader sequence for secretion, a DC targeting peptide sequence for delivery, a rat cartilage oligamerization matrix protein (COMP) sequence for multimerization, and the full length sequence of the model antigen, ovalbumin (OVA). The construct is ballistically shot into the ear of a mouse with a gene gun. The DNA will be delivered indiscriminately to all cell types of the skin, including immature DCs and keratinocytes. The protein fusion is expressed, secreted, and the peptide antigen conjugate will be targeted to the immature DCs in the skin for vaccination against OVA.

A.

B.

Peptide	COMP	Antigen	Obtained
Control	-	OVA	No
Control	+	OVA	No
XS52.1	-	OVA	YES
XS52.1	+	OVA	YES
XS52.3	-	OVA	YES
XS52.3	+	OVA	YES

FIGURE 15-1- DNA VACCINATION CONSTRUCTS AND RESULTS ON SUCCESSFULLY MADE CONSTRUCTS:

A. The genetic construct was engineered through cloning techniques to contain restriction enzyme sites for interchangeable components. This was done to facilitate insertion of different peptide sequences, addition/removal of COMP domain, and switching of antigen system. B. Chart indicates constructs that were made successfully. Constructs containing the control peptide sequence were never obtained.

C.

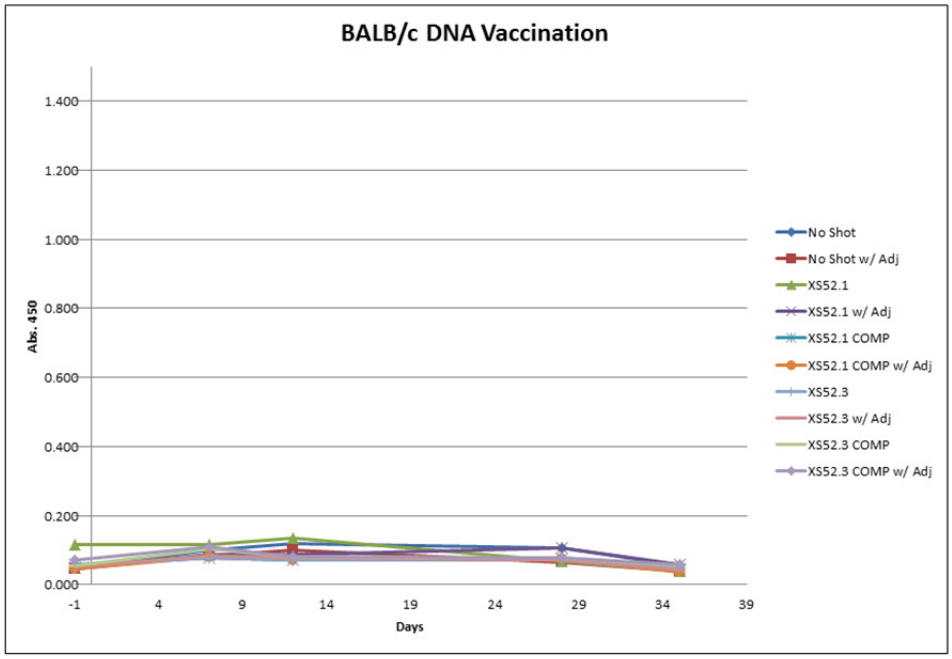
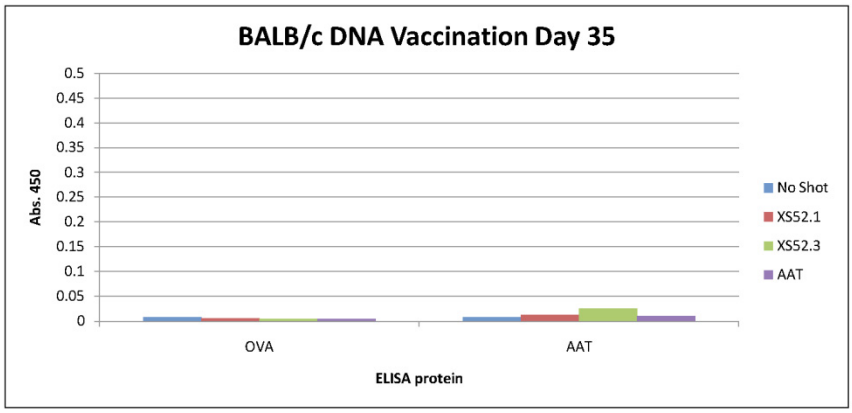


FIGURE 15-2- DNA VACCINATION CONSTRUCTS AND ELISA RESULTS:
C. Mice were vaccinated using a gene gun with DNA constructs on day 0 and day 14. Some groups received 2 additional constructs containing GM-CSF and Flt3L (w/ Adj) as adjuvants. DNA bullets were loaded with 3ug DNA. DNA vaccination (4 mice per group) sera were analyzed through ELISA using 100ng/mL OVA protein to coat plates. No anti-OVA response was seen with tested sera. Anti-OVA commercial antibody was used as a positive control to test ELISA plates.

A.



B.

GROUP	XS52.1	XS52.3	AAT
DNA (ng)	120	148	80

FIGURE 16 - INVESTIGATIVE DNA VACCINATION TO DETERMINE POTENTIAL PROBLEM:

A. Vaccinated 4 mice per group with either XS52.1 no COMP construct, XS52.3 no COMP construct, or human alpha-1 antitrypsin (hAAT) construct under a CMV promoter. This construct has previously produced an immune response using gene gun DNA vaccination. ELISA plates were coated with either OVA protein or AAT protein at 100ng/mL. Mice received initial vaccination on day 0, and a booster on day 14. Sera from day 35 was measured. No immune response was seen with control hAAT construct. B. DNA bullets were measured for DNA content. Mice were vaccinated with 1 bullet per ear, with 0.5mg of gold and 1ug of DNA loaded onto each bullet. DNA was loaded onto gold through incubation with 50mM spermidine and 1M CaCl2. After bullet production, water was added to 2 bullets and DNA content was measured using a nanodrop spectrophotometer. The table shows the average of the 2 bullets for each vaccination group. Measurements showed an 8-15% efficacy in DNA loading.

Peptide-Antigen Conjugate

A second vaccination format that may have promise is a peptide-antigen conjugate created through bioconjugate chemistry. This would provide a fast and easy way to modify any antigen of interest and increase its immunogenicity by adding the DC targeting peptide. This would especially be useful for non-immunogenic TAAs that are difficult to elicit an immune response with high efficacy. For an initial test, the XS52.1 monomeric peptide that contained a reactive free thiol group was used for conjugation to the OVA protein. For bioconjugation of the peptide-antigen conjugate, the OVA protein was treated with sulfo-SMCC, which contains a reactive NHS ester to modify primary amines on OVA for maleimide activation. The maleimide activated OVA reacts with the free thiol group on the peptide for conjugation. The products of this reaction were analyzed by SDS-PAGE using Coomassie stain to visualize the protein species. Up to 2 peptides were conjugated per OVA molecule. The reaction contained a majority of peptide conjugated OVA with extreme molar excess of sulfo-SMCC. While initial results showed successful conjugation of peptide to the OVA antigen, subsequent immunofluorescence assays did not detect binding or uptake of the conjugate to DCs. These experiments were performed by incubating XS52 cells with the peptide-antigen conjugate, and then probing for OVA with an anti-OVA antibody for immunofluorescence. No fluorescent signal was seen above background for cells incubated with the conjugate compared to OVA alone. Unfortunately, the experiment was unable to detect OVA in the positive control (EG7-OVA ovalbumin expressing cells). Therefore, conclusions about the targeting potential of this peptide-OVA conjugate cannot be drawn. Potential modifications to the protocol include using a different primary

antibody for OVA detection and development of a tetrameric peptide-antigen conjugate. Further analysis of the tetrameric format using other means of peptide-antigen conjugation would be optimal to accurately analyze binding of the recombinant protein.

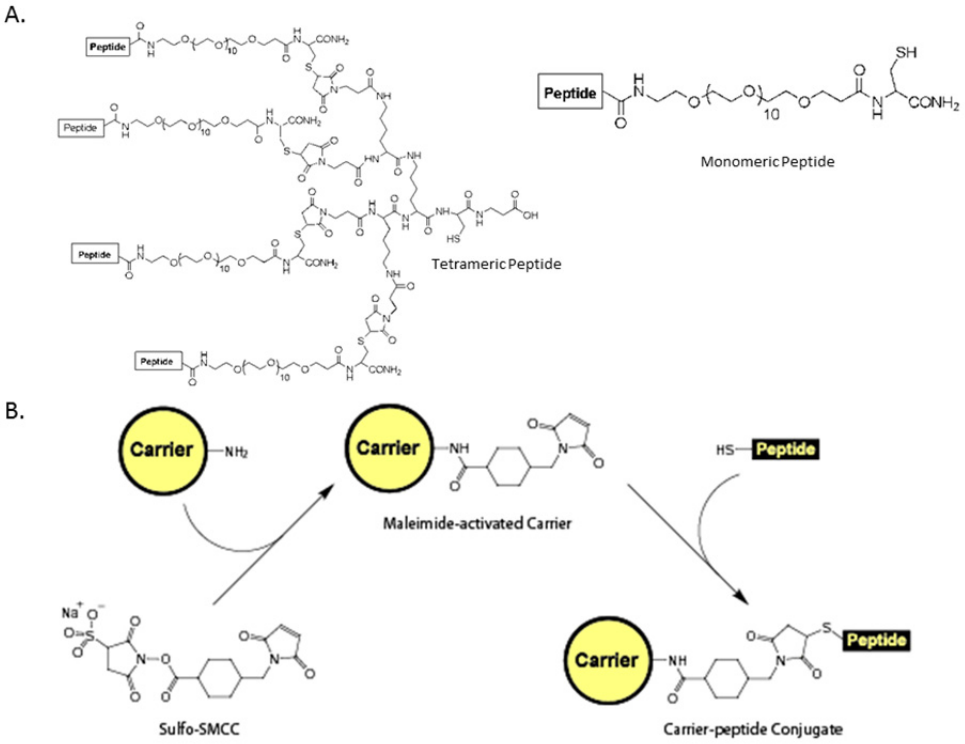


FIGURE 17-1 - PEPTIDE-OVA CONJUGATE SYNTHESIS SCHEMATIC WITH PEPTIDE STRUCTURES:

A. Chemical structure of monomeric and tetrameric peptide with free thiol group used for conjugation to OVA protein. Only XS52.1 monomeric peptide (3.1 KDa) was tested for conjugation. B. Bioconjugation schematic of peptide conjugation to OVA protein. OVA was first treated with sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) with a reactive N-hydroxysuccinimide (NHS ester) to modify primary amines on OVA and maleimide activate OVA protein. The maleimide activated OVA reacted with the free thiol group on the peptide for conjugation

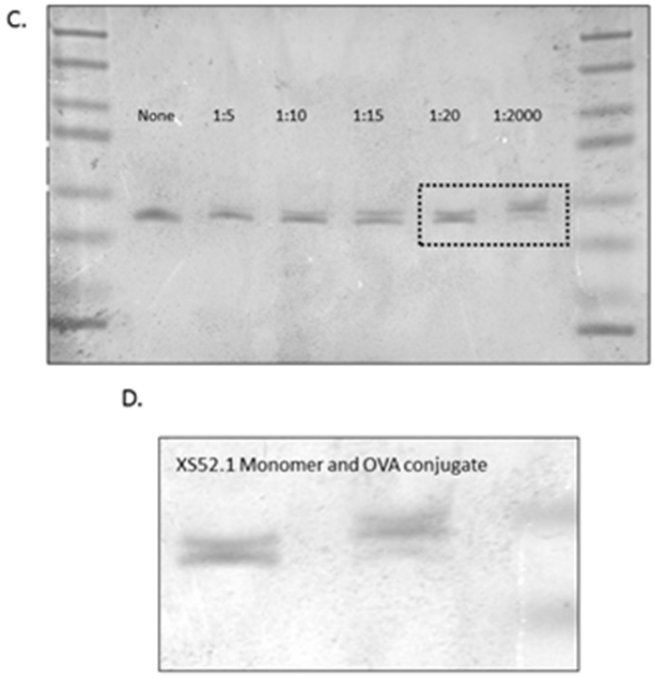


FIGURE 17-2 - PEPTIDE-OVA CONJUGATE SYNTHESIS CONFIRMATION:
C. SDS page protein gel was ran and stained with Coomassie Brilliant Blue R-250 to characterize products. Reaction at all molar ratios had 1nMol of OVA reacted. "None" showed 47ug of unreacted OVA. Double bands were seen at 1:10 molar ratio OVA:SMCC reaction, and the majority of OVA protein was reacted with peptide at the 1:2000 molar ratio. D. A magnified view of the same gel on C, shows that potentially up to 2 peptides were conjugated to OVA at the 1:2000 reaction ratio.

DISCUSSION:

The goal of this project is to develop DC-targeting peptides that can deliver antigen to this important class of APC *in vivo*. The results show that the targeted phage clones are capable of binding specifically to immature DC cell lines, to BMDCs, and are capable of eliciting a heightened humoral immune response. While further work is needed to test the vaccines in other murine strains, this preliminarily shows that these peptides have great potential as novel DC targeting reagents.

The DC-targeting phage clones were isolated on XS52 cells in culture. While preliminary data showed improved binding of these targeting phage clones above control, there was no guarantee that these phage clones wouldn't bind any other immune cells. There was also no guarantee that these DC-targeted phage clones would bind BMDCs. The peptide in the context of the phage is capable of binding to DCs *ex vivo* as shown through imaging on BMDCs. This had not previously been characterized. The XS52.3 phage clone's ability to bind BMDCs makes immunological sense when the comparative binding assay is taken into account. This assay shows some XS52.3 phage clone binding to a murine macrophage cell line. BMDCs consist of dendritic cells that are derived from monocytes in culture. The data suggests that the XS52.3 phage clone binds to a general DC receptor that is shared between Langerhans cells, macrophages, and BMDCs. This is consistent with a marker that is expressed in monocytes or in an early shared macrophage/DC precursor. In contrast, the XS52.1 phage clone did not bind BMDCs and was shown to be exclusively binding to immature DCs with reduced binding to mature DCs and no binding to macrophages. XS106, the mature DC cell line used for analysis,

was derived by Takashima in the same manner as the XS52 cells except they are Langerhans cells that exhibited markers of maturation (increased MHC class I, CD40, CD80, CD86, CD54, and CD205) [189]. Therefore, the XS52.1 is potentially binding to a marker that is not expressed until DCs migrate to the dermis to become Langerhans cells.

Speculation over the receptors targeted by the DC-peptides leads into the question of receptor identification, a topic that has been explored in the lab. One current method to potential receptor identification would be BLAST sequencing for homology to known ligands. This method was utilized in the initial identification of αvβ6 as the target receptor for H2009.1 NSCLC targeting peptide. However, in all other cases no sequence homology was found for receptor identification. BLAST does not always yield adequate homology because the peptides presented in the phage library are not from a biological source. While the synthetic nature of the peptide sequences allows for isolation of novel ligands, it is not guaranteed that an isolated peptide will target a known receptor.

It is not known whether DC-targeted phage clone binding is restricted to a DC subset. DC subsets include plasmacytoid DCs, myeloid DCs, and Langerhans. Langerhans cells are only one of several tissue resident DC subsets that exist. Other subsets are based on cytokine secretion (IL-10, IL-4, TNF, and IL-15) [190]. This raises the question of whether it is beneficial to target a single subset of DCs or a general DC receptor. Targeting the Langerhans would be useful for intradermal vaccination methods, but systemic administration of vaccine would require the capability to target many DC types. Further analysis into receptor identification and peptide usage would potentially answer these questions, and whether in fact it would be beneficial to use both peptides at the same time for enhanced antigen delivery. Further consideration for future experiments

should include receptor identification as well as an experiment to directly trace peptide delivery of antigen to DCs *in vivo*.

The functional test of the DC-targeting phage clones is their ability to produce an anti-phage immune response that is greater than non-targeted phage. Mice injected with endotoxin purified XS52.1 or XS52.3 phage produced a humoral response above that of control phage. The control phage did not elicit an immune response, as indicated when compared to mice injected with PBS alone. In addition, higher doses of endotoxin purified phage maintained this increased efficacy of the targeted phage. Even at doses of up to 1000 ng phage, no response is observed for the control phage in Balb/c. In these experiments no adjuvant was utilized, demonstrating the potential of these DC-targeted phage clones to elicit a heightened immune response without additional necessary reagents. An immune response was seen as early as 14 days, and the booster shot at day 28 induced beneficial increase of antibody production a week later. These experiments demonstrate that the DC peptide has the potential to increase the immunogenicity of a model antigen. While the hypothesis is that the peptide is delivering the antigen to DCs and therefore eliciting a heightened immune response, this has not completely been proven with the peptides. It has not been ruled out that the phage clone is binding and/or being processed by other immunological cell types. In particular, other APCs such as macrophages and B cells could be responsible for the increased immune response. While previous comparative binding assays were performed on murine cell lines as confirmation of specificity, further analysis needs to occur with isolated *ex vivo* macrophages and B cells. In addition, further *ex vivo* culture conditions with different cytokines can be utilized to determine DC subset binding. These types of experiments,

using the liposomal format or a directly labeled phage, would have to be performed to solidify the argument that the heightened immune response is a direct result of antigen delivery to DCs mediated by the peptide.

While imaging of the phage on BMDCs was successful, other means of visualization with the peptide on a fluorophore was not. No increased fluorescence was seen above background when comparing control versus targeted peptides. These studies were done with peptide-biotin with SA dye and a direct conjugate. Further analysis should be done to confirm specificity. One possible explanation for these results is that the receptors are being presented differently on the XS52 cell line than on *in vivo* cells. Another possible explanation is the spacing of peptides on the phage scaffold is necessary for peptide binding. The spacing of the peptide on the tetrameric scaffold may not be appropriate for binding. A peptide-PIII fusion may be needed to represent adequate spacing of peptides needed for DC binding. This would facilitate the transition from peptide presentation on the phage to another non-phage scaffold. Previously published data utilized liposomes, which presents the peptide in repetition over a large surface area. This closely mimics how the peptide is presented on the phage. The peptide on the tetrameric scaffold would not be presented in a large area as would occur on the liposome or on the phage. Therefore, further development of peptide-presenting scaffolds should be developed and tested in a vaccination strategy. This would best indicate optimal use of the peptides for DC targeting.

T-cell activation analysis during vaccination is another important direction that should be followed. Isotype analysis gave a preliminary indication that the peptides do not give a biased response during T-cell activation. The exception is the XS52.1 phage

clone in Balb/c. Further vaccinations in other mouse haplotypes would be required before any conclusion can be reached as to whether this difference is based on a biological component of the DCs or if this is exclusive to Balb/c mice. If seen only in Balb/c, this would indicate a genetic variance in this strain that affects peptide activation of T-cells. Initial studies to elucidate TH response were performed to determine if there is increased T-cell activation after 10 days post vaccination. The cytokines analyzed were IFNγ, TNFα, IL-10, and IL-4. TH1 (IFNγ and TNFα) or TH2 (IL-10, IL-4) response was determined by increased cytokine production. No increase was seen in these experiments. However, the results of these experiments are unclear. A positive control (IFNγ and TNFα secreting cells) for appropriate intracellular cytokine staining for flow cytometry showed adequate staining. The percentages of secreting cells correlated with manufacturer standards (BD Pharmingen). Positive controls that consisted of cell cultures activated with PMA/Ionomycin showed low and variable secretion of TH1 cytokines. Therefore, while the protocol for measuring cytokine secretion through flow cytometry functioned properly, it was questionable whether the PMA/Ionomycin utilized for T cell activation could function consistently. In addition, further positive controls for the vaccination were not used due to unavailability of appropriate adjuvants. In the future a positive control (Phage plus Freund's Complete Adjuvant) would be compared to the control and targeted phage vaccinations. If no immune response is seen with the positive control, this may point to a T-cell independent immune response. If this is true, the targeted phage clones are directly activating B-cells, which is problematic since this indicates that the phage is not being processed by DCs as previously thought. If the targeted phage is directly activating B cells for antibody production, this would indicate

that the B cells are activated independent of T cell response. This would further indicate that the B cells are not activated through MHC class II antigen presentation from DCs. If this is the case, then the DC-targeted phage are in fact targeting B cells and not inducing a response through DC internalization. This is unlikely since all prior evidence does not point to this direction. To clarify the antigen processing that is mediated by the DC-targeted peptides, the internalization mechanism in DCs should be further elucidated. This would be performed through imaging experiments to see co-localization of phage and lysosome/endosome markers. Knowledge of mechanisms of DC internalization will aid future experiments in determining the best vaccination strategy for tumor mouse models.

Before determining the utility of these peptides for a vaccination scheme, further analysis should be performed to measure the ability of the DC-targeting peptides to bind human DCs. This could be performed through imaging experiments with cultured PBMCs from human samples. Imaging would have to be performed with the phage for initial tests of binding. It would be of interest to test the peptide on these cells to determine if the peptide on the tetrameric scaffold is capable of binding human DCs. If binding to human DCs is seen, then the targeted peptides are binding to a receptor that is evolutionarily conserved in DCs across different species. Further *in vitro* analysis would determine if human DCs incubated with a peptide-antigen conjugate are capable of activating T and B cells. Further vaccination studies could be performed in humanized mice that contain humanized DCs and other immune cells. These studies would determine the utility of the DC-targeting peptides outside of the mouse model. If there is no binding seen on human DCs, the next step would be to redo the panning procedure on

human DCs in order to isolate human DC-targeting phage clones. This illustrates the benefit of utilizing the phage library for isolating targeting ligands where peptides can easily be isolated with any cell type of interest. The experiments performed in the vaccination project lays down the foundation for future studies by showing the possibility of isolating immune-cell targeted ligands for induction of an antigen specific immune response.

MATERIALS AND METHODS:

Mouse Strains and Cell Lines. BALB/c and C57BL/6J mice were received from UT Southwestern breeding core. XS52 and XS106 cell lines were a kind donation from Dr. Akira Takashima at the University of Toledo.

Library Phage Selection of XS52 Phage Clones. XS52 cells were grown in complete RPMI supplemented with granulocyte macrophage-colony stimulating factor (GM-CSF, kind gift from Dr. Chandrashekhar Pasare) and NS supernatant [191], then panned for phage clone isolation. Cells were plated in a 12 well culture dish and grown to 90% confluency. Before panning on XS52 cells [175, 192] surface receptors were cleared in serum free RPMI at 37°C for 2hrs. Phage solution consisted of 10^{11} phage in PBS+0.1%BSA with protease inhibitor 25X without EDTA (Boehringer Mannheim, Indianapolis, IN) and 100X 10mM chloroquine to block acidification of endosome and enhance phage recovery. 50μL of the phage solution was saved for input measurements. Phage solution was incubated on cells for 1 hour at 37°C, after which the cells were washed 4 times at room temperature with PBS+0.1%BSA. Cells were then washed 2 times with an acid wash (0.1M HCl-glycine, pH 2.2 + 0.9% NaCl) to remove most surface-bound phage and enrich for phage clones that mediate internalization. Cells were lysed by incubation on ice in 30 mM Tris pH 8.0 for 30min., then placed at -20°C from 1 hour to overnight. Internalized phage clones were recovered and amplified by incubating cell lysates with Escherichia coli (K91) and harvesting phage infected colonies. Bacterial tittering was used to calculate the number of phage particles in the output fraction. Further rounds of panning were performed by incubating XS52 cells in culture with the

phage clones recovered from the previous round. The ratio of output phage to input library phage was calculated at each round to monitor progress of the panning process. Isolation of XS52.1 phage clone has previously been characterized and published [3]. XS52.2 phage clone was isolated with the same phage library from which XS52.1 was derived, and XS52.3 and XS52.4 were isolated from another synthetic phage library. The new synthetic library is comprised of 22 amino acid peptides in which a constant serine-glycine (SG) amino sequence was added at the N-terminus. The SG cap facilitates bio-conjugation for downstream applications and is not required for specificity.

Phage Clone Selectivity Assays. Assays were prepared similar to the library selection protocol. Cells (10^6) were overlaid with a 1-mL solution containing 10^8 phage clones of either the selected DC-targeting phage clone or the control phage clone. The non-binding control phage clone displayed the peptide NQRGTELRSPSVDLNKPGRH, which has previously been validated for non-binding in previous publications [177]. The cells and phage were incubated at 37°C for 10 minutes and washed similar to the library selection protocol. For non-adherent cells (A20, XS106), the media and washes were removed by centrifugation. Cells were osmotically lysed and used to infect E. coli. Selectivity index was defined as the output/input ratio of the targeted phage divided by the output/input ratio of the control phage.

Fluorescence Imaging of Phage in XS52 cells. XS52 cells were cultured overnight in 8-well poly-d-lysine chamber slides. Cells were then incubated for 10 minutes at 37°C with Control, XS52.1, or XS52.3 phage in solution (10^8 phage per mL in PBS+0.1%BSA with PI and CQ). Cells were washed 4 times with PBS+0.1% BSA and 2 times with an acid wash. Cells were then fixed for 10 minutes in 3.7% PFA in PBS, then washed in

PBS for 10 minutes, and lastly blocked/permeabilized for 30 minutes. Block solution contained PBS with 2% BSA and 2% goat serum (Sigma, St. Louis, MO). For the blocking/permeabilization step, 1:500 dilution of Fc block (Rat anti-mouse CD16/CD32 antibody, BD Pharmingen San Diego, CA) and 0.1% Triton-X was added to block solution. Block solution containing 1:250 dilution of primary anti-phage antibody (from mouse sera) was incubated onto cells for 30 minutes. Cells were washed 3 times with PBS+0.1%BSA and then incubated with 1:250 goat anti-mouse AF488 in block solution for 30 minutes. Cells were washed 3 times in PBS+0.1% BSA and prepared for imaging with DAPI fluoromount G (Southern Biotech, Birmingham, AL). Phage was detected by using an anti-phage primary antibody (from mouse sera of phage vaccinated mice created in house by Dr. Michael McGuire) and a secondary Goat anti-mouse AF488 conjugated antibody (Invitrogen, Eugene, OR).

Generation of Bone Marrow Derived DCs. Bone marrow from Balb/c mice was flushed out of the femur and tibia with a syringe and treated with Red Blood Cell Lysis Buffer (Sigma, St. Louis, MO) for 3 minutes. Cells were resuspended in RPMI with 10% FBS, 10mM HEPES, and 50 μM 2-ME with 50ng/ml of mouse recombinant GM-CSF (Gift from Dr. Pasare). Cells were plated on a 24-well dish (Becton Dickinson, Franklin Lakes, NJ) with 1mL per well at a cell concentration of 700,000 cells/mL. Fresh media with 50ng/ml GM-CSF was added to cells on day 2 and day 4 of culture. Non-adherent cells were collected on day 5 of culture.

Fluorescence Imaging of Phage in BMDCs. BMDCs were cultured as previously described. The cells were exposed to 10^8 phage particles of an isolated phage and incubated for 10 minutes at 37°C. Cells were washed and fixed with 4% PFA,

permeabilized with 0.1% Triton-X in PBS/BSA, and blocked with 2% normal goat serum in PBS with 2% BSA for 20 minutes at RT. Mouse anti-phage antibody (1:250 dilution) was overlaid on the cells and incubated for 30 minutes at RT. The cells were washed in PBS/2%BSA 3 times, and then incubated with AF488-conjugated goat anti-mouse IgG antibody (1:250 dilution) (Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 30 minutes at RT. Excess antibody was removed by 3 washes in PBS and the nuclei were stained with DAPI. Cells were visualized by fluorescent microscopy on a Leica DM5500 B fluorescent microscope.

Phage-Mediated Humoral Response. Groups of Balb/c or C57BL/6J mice were injected intradermally with either PBS or 50ng phage in 20 µL of endotoxin-free PBS. Phage solutions were endotoxin purified using Detoxi-Gel™ endotoxin removing columns (Thermo Scientific, Rockford, IL) twice and then eluted with endotoxin-free PBS. Samples tested low for endotoxin when tested with the Limulus Amebocyte Lysate (LAL) test kits (Sigma-Aldrich, St. Louis, MO). Mice were boosted with the same phage solution on day 28. ELISA assays for phage-specific antibodies were conducted. Nunc maxisorp microtiter plates (Nunc, Rochester NY) were coated at 4°C overnight with 100ng/mL of empty phage (no peptide sequence expressed) in 50mM Tris-HCl pH 8.0 as the target antigen and blocked with PBS+0.1%BSA overnight. Protein concentration was measured by Bradford protein assay (Bio-Rad). Sera were diluted 1:250 into block solution and then incubated on ELISA plates for 30 minutes. The secondary antibody horseradish-peroxidase conjugated, anti-mouse, IgG (Pierce, Rockford, IL) was used for visualization with 3’3’5’5’tetramethylbenzidine (TMB) substrate (Calbiochem, La Jolla, CA). Reactions were stopped at 10 minutes with 1 M hydrochloric acid and the

absorbance was read at 450 nm. Statistical analysis for efficacy includes a one-way ANOVA with Tukey post hoc test. Final p values were calculated with a student t-test.

Creation of Genetic Constructs for Vaccination. DNA constructs were created to contain peptide sequence and full sequence of the Ovalbumin model antigen (from the pCB7OVA plasmid, a kind gift from Dr. Cathryn Nagler at the University of Chicago). The pcDNA3.1+ construct (Invitrogen, Grand Island, NY) was previously modified to contain the MCIP antigenic peptide and targeting peptides through insertion of multiple oligos through RE digest and ligation. Interchangeable components were designed to contain RE sequences by inserting oligos (Operon, Huntsville, AL) that reverted the original RE site to the new sites according to construct design. Construct components included: a secretion sequence from human gene encoding AAT (Leader Sequence: MPSSVSWGILLLAGLCCLVPVSLAEDL), the peptide sequence, a COMP domain for oligomerization (GDLAPQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMECDACGDDP) [193, 194], and a CMV promoter. Correct modification of constructs was verified through agarose gel electrophoresis and Big Dye sequencing after colony PCR purification with Exo1 and SAP (UT Southwestern Sequencing Core). Amplification of genetic constructs required transformation through heat shock of competent XL1 Blue E. coli cells (Stratagene, Santa Clara, CA). Transformed cells were selected with LB AMP plates. GenElute™ HP Plasmid Maxi prep kits (Sigma) were used for construct amplification and purification. DNA construct was precipitated after Maxi prep using EtOH and NaOAc. DNA was quantified with a NanoDrop 1000 spectrophotometer.

DNA Vaccination with Targeted Constructs. The Au beads were loaded with 1-3ug DNA using 50mM spermidine and 1M CaCl. Tube for bullets were washed with EtOH anhydrous 200 proof (Sigma) and after DNA loading Au the beads were washed and resuspended in 99.5+% EtOH. The suspension was inserted into washed Tefzel® tubing and allowed gold to settle for 10 minutes. EtOH was removed from tubes and air dried with inert Argon gas. After drying, tubes were shaken to resuspend dried gold powder and then cut into bullets for gene gun. DNA content was measured by adding 10uL of water and using a NanoDrop 1000 spectrophotometer to measure DNA concentration by measuring absorbance at wavelength 260nm ($c=[A*\varepsilon]/b$). Mice were anesthetized with Avertin and shot once per ear with the gene gun using nitrogen gas (psi 350). Mice were bled by tail vein before initial vaccination and once per week until day 35.

Peptide-Ovalbumin Protein Conjugation. Monomeric peptide was synthesized and purified through HPLC. Crude peptides were purified by reverse phase HPLC using a Vydac PR-C18 column (250mm ×22mm, 10 μm) on a BreezeTM HPLC (Water Inc.) with eluents of H2O/0.1% TFA (eluent A) and acetonitrile/0.1%TFA (eluent B). The following elution profile (referred to as Method A) was utilized: 0-1 minute, 90%A, 10%B; 1-100 minutes, eluent B was increased from 10-60% at a flow rate of 10 mL /minute. (Where A = H20 w/ TFA & B = acetonitrile w/ TFA). Elution of the peptides was monitored by UV absorbance at 220 nM. Expected mass was 3142.51 da, actual was 3141.11 da. The peptide fraction was verified through MALDI mass spectrometry. For peptide-antigen conjugation, OVA (Sigma) in PBS was incubated with varying molar amounts of sulfo-SMCC for 30 minutes at RT. Excess sulfo-SMCC was removed using a Amicon centrifugal filter column (15 kDa). Maleimide activated OVA was incubated

with 50 fold molar excess peptide containing a reactive thiol group for 30 minutes at RT. Samples were then prepared for SDS-PAGE and stained with coomassie blue.

Intracellular Cytokine T-cell Flow Cytometry. Mice were vaccinated with antigen and then splenocytes and draining lymph nodes were isolated on day 10 post vaccination. Draining lymph nodes were identified by tracking the injected Evans blue dye near the original injection site to nearby lymph nodes. Splenocytes and lymph nodes were made into single cell suspension and incubated with either Leukocyte Activation Cocktail (LAC, BD Pharmingen) or with 0.5ug/mL of correlating phage (endotoxin purified). Leukocyte activation cocktail contained PMA and Ionomycin plus Brefeldin A. After incubation, cells were blocked with FC block, stained for CD markers, and then fixed with 4% PFA in Staining Buffer (BD Pharmingen) for 20 minutes on ice. Cells were stored in Staining Buffer until further flow cytometric analysis. After fixation, cells were permeabilized with Wash/Perm buffer (BD kit) and stained for intracellular cytokines. Samples were analyzed using a FACsCalibur 4 color flow cytometer. Cells were gated on live cells based of forward scatter *vs*. side scatter dot plot.

CHAPTER III

PEPTIDE-ANTIBODY TARGETING OF LUNG CANCER

INTRODUCTION:

In addition to isolating DC peptides, the Brown Lab has extensive experience in isolating lung cancer peptides utilizing the panning protocol described previously. The goal is to isolate novel ligands in a quick and unbiased fashion that will allow specific targeting of lung cancer cells. The panning protocol has been used to isolated peptides that bind specifically to different lung cancer cells lines but not normal epithelial lung cell lines. In addition, the isolated peptides have shown wider binding to multiple different cancer cell lines. Binding profiles do not segregate based on histological characterization. To develop novel immunotherapeutics for cancer treatments, the goal of this second project is to test the function of these lung cancer peptides when placed on an antibody scaffold for rapid production of peptide-antibodies. The benefits of an antibody is its long serum half-life and Fc mediated function, while the cons of antibody usage is that it is difficult to produce and the target needs to be known prior to production. The benefits of peptides isolated through phage display are easy isolation and production, versatility, modifiability, and that fact that no prior knowledge of the targeted receptor is needed for identification. Peptides however have a short serum half-life and no known immunomodulatory effector function. A peptide-IgG conjugate could provide a platform to take advantage of the properties of each molecule (Table 5).

Table 5: Benefits of Developing Peptide-Antibody Immunotherapy		
	Antibody	Peptide
Pros	• Long serum half-life • Fc mediated function	• Easy isolation and production • Versatility (monomer vs tetramer)
Cons	• Difficult to produce • Need known target	• Short serum half-life • No known function
Benefit of combination	• Ability to isolate and produce targeting ligands without prior knowledge of receptor • Ability to modify with monomeric or tetrameric • Ability to target immune function (CDC, ADCC)	

Previous development of peptide-antibody conjugates

The main reason for producing these ligands is to develop a method to create targeting peptides without the need for individual receptor identification and antibody production required for traditional antibody therapies. In essence, the ideal situation would be to have one antibody on which multiple peptides could be attached in the Fab region to easily create a variety of targeting antibodies. Amgen has the claim to the first FDA approved peptide-antibody conjugate Nplate®. This peptibody serves as a thrombopoietin mimetic that stimulates platelet production as a treatment for immune thrombocytopenia [195]. The peptide for this conjugate was isolated through phage display. Amgen is currently working on other peptide-antibody conjugates for the treatment of cancer and other diseases.

Carlos Barbas and colleagues produced Cov-X, a unique antibody scaffold [196]. The Cov-X antibody is a catalytic antibody that allows for site specific conjugation of peptides on the Fab portion of the antibody. The antibody catalyzes the aldol reaction, a basic carbon-carbon bond-forming reaction, by mimicking the reaction used by natural class I aldolase enzymes. Mice were immunized with a reactive compound covalently trapped a Lys residue in the binding pocket of the antibody. The antibody uses the amino group of Lys to form an enamine with ketone substrates and use this enamine to form a new carbon-carbon bond with an aldehyde [197]. The adol for this reaction can be incorporated into the peptide of interest. Dr. Barbas and colleagues have tested this antibody using an RGD peptide that binds to αvβ3and αvβ5 integrins in a murine tumor model [198]. In this study, tumor reduction occurs when animals are treated with the peptide-antibody and this anti-tumor effect is dependent on ADCC. They have also done

work that involves vaccinating the animal so that they can produce the catalytic antibody themselves so that the peptide can be added afterwards for *in vivo* conjugation of the peptide-antibody. The serum half-life of the peptide also is increased to 5 days, which is more than a typical peptide but still less than what is expected for an antibody molecule. The serum half-life for IgG is 7-23 days depending on the subclass while peptides are cleared by renal filtration within minutes. The benefits of creating a peptide-antibody conjugate are increased serum stability of the peptide and easy manipulation of the targeting ligand while incorporating the immune functions of an antibody. Dr. Barbas and colleagues were able to quickly make a targeting antibody to markers that are relevant for cancer therapy. This was accomplished because of the unique antibody scaffold that he developed that allowed for facile conjugation to peptides. An antibody scaffold that allows for easy and quick conjugation with peptide would increase an antibody's range of targeting ability. The ultimate benefit of developing a peptide-antibody with isolated lung cancer peptides would be the rapid and facile development of therapeutically relevant targeting antibodies. The only deficit of this antibody scaffold is that only monomeric versions of the peptide have been utilized. The restriction of multimerization could potentially reduce the affinity these ligands are capable of acquiring. The proposed project will utilize tetrameric forms of NSCLC-targeting peptides and will increase the multimerization of targeting ligand that does not occur with the Cov-X antibody chemistry.

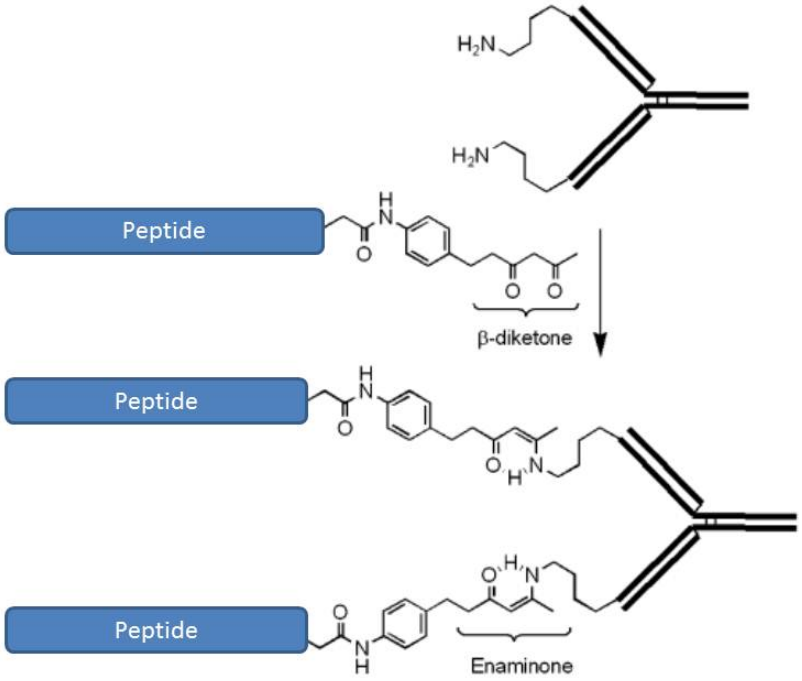


FIGURE 18 – SCHEMATIC OF CHEMISTRY USED FOR COV-X PEPTIBODY PRODUCTION
Formation of Cov-X antibody. Figure modified from Barbas et al. [196]

Preliminary data with lung cancer targeting peptides

Lung cancer targeting peptides were isolated by the Dr. Brown Laboratory through panning on eight different NSCLC cell lines that were produced in Dr. John Minna's laboratory. Eleven different targeting peptides were isolated. In addition to selectivity (the measure of targeted peptide binding normalized to control), specificity was also measured by taking the ratio of the selectivity value for the cancer cell line versus the BEAS-2B cell line (TABLE 6). This cell line is a normal bronchial epithelial control cell line to determine that the eleven peptides can target lung cancer cells specifically. The NSCLC cell lines are representative of different histological phenotypes, including adenocarcinoma, squamous, and large cell carcinoma cancers. As previously mentioned, anything with a selectivity of above twenty is considered specific to the cell line on which it was isolated. For some cell lines panning resulted in two different peptides.

Comparative binding assays of the eleven peptides on several different cell lines show that these peptides do bind to other NSCLC cell lines. In addition, H2009.1 was shown to have the widest binding profile. In addition, these peptides have binding affinity for cancer cells that were derived from organs other than the lungs. This includes breast, ovarian, and liver cancer cell lines. This demonstrates the potential utility of these peptides for targeting multiple cancer types. Multimerizing the peptide on a tetrameric scaffold increases the binding affinity of the peptides to half-maximal binding affinities that compete with antibodies. Various tetrameric peptides have been utilized in current

studies for drug deliver in direct conjugate and liposomal doxorubicin formats. The peptide has the exquisite ability to be produced with different reactive groups that make it versatile to different functions.

Of all the peptides, H2009.1 is the most heavily studied in the laboratory. The receptor for this peptide has been identified to be integrin αvβ6 through sequence homology with foot and mouth virus binding component to the receptor. The αvβ6 integrin has also been associated with early dysplasia and increases in expression throughout tumor progression [199]. This makes the integrin an ideal candidate to be a cancer biomarker for early detection of lung cancer. For initial studies with the peptide-antibody conjugate, the H2009.1 peptide will be used since it is the most powerful of the isolated lung cancer peptides.

Table 6: NSCLC Binding Peptides				
Peptide name	**Cell Line Used For Selection**	**Peptide Sequence**	**Selectivity[1]**	**Specificity[2]**
H1229.1	H1299 (LC)	VSQTMRQTAVPLLWFWTGSL	190	45
H1299.2	H1299 (LC)	YAAWPASGAWTGTAPCSAGT	83	29
H2009.1	H2009 (AD)	RGDLATLRQLAQEDGVVGVR	300	140
H460.1	H460 (LC)	EAMNSAEQSAAVVQWEKRRI	120	400
A549.1	A549 (AD)	MTVCNASQRQAHAQATAVSL	21	7.8
HCC15.1	HCC15 (SQ)	ATEPRKQYATPRVFWTDAPG	44	34
HCC15.2	HCC15 (SQ)	FHAVPQSFYTAP	220	73
HCC95.1	HCC95 (SQ)	MRGQTGKLPTEHFTDTGVAF	20	69
H1155.1	H1155 (LC)	MTGKAAAPHQEDRHANGLEQ	55	39
H1155.2	H1155 (LC)	MEKLPLSKTGRTVSEGVSPP	61	25
TP H661.1	H661 (LC)	TNSCRGDWLCDAVPEKARV	26	90

Panning protocol was done on 8 different Non-Small Cell Lung Cancer (NSCLC) cell lines and 11 different NSCLC targeting peptides were isolated. [1]Selectivity is defined as the output phage/input phage normalized to a control phage. [2]Specificity is the ratio of the selectivity value for the cancer cell line and a normal bronchial epithelial control cell

line, BEAS-2B. LC = Large Cell Carcinoma, AD = Adenocarcinoma, and SQ = Squamous Cell Carcinoma.

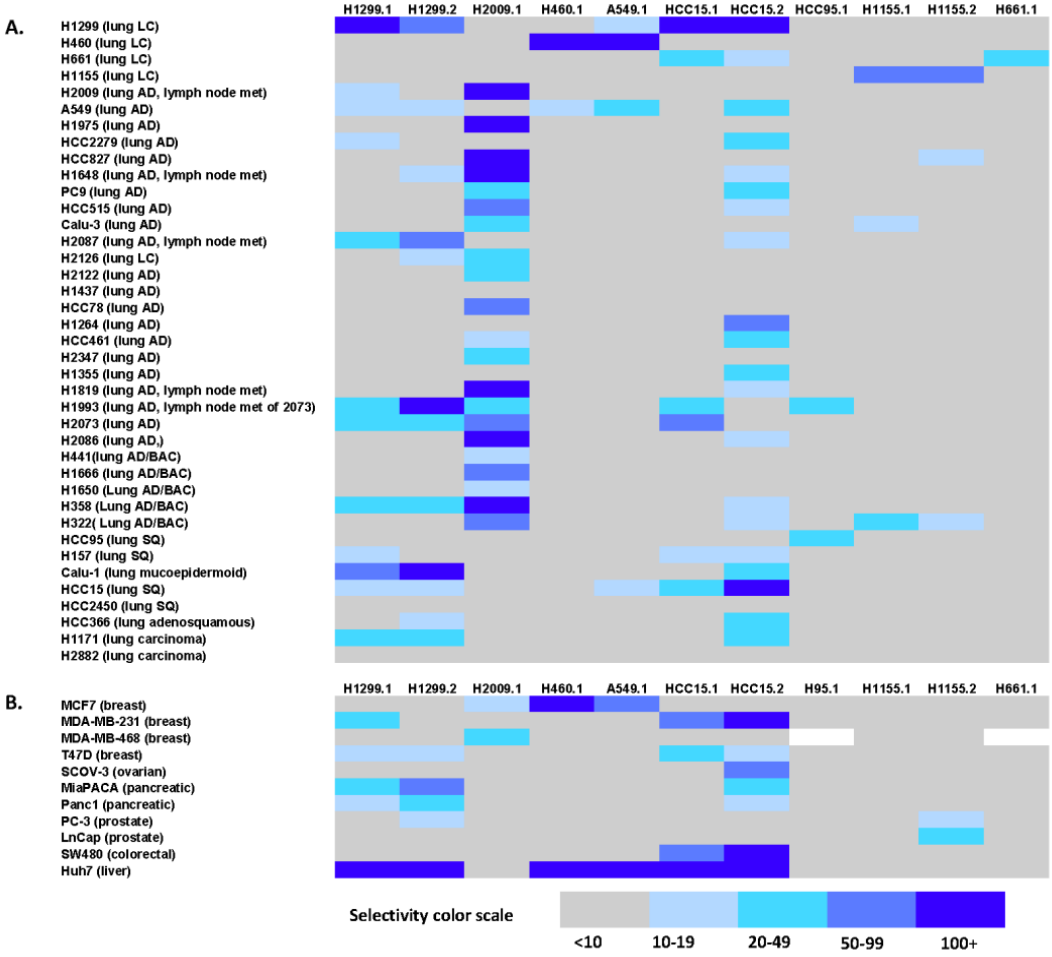


FIGURE 19 - NSCLC PEPTIDE BINDING PROFILE:
Binding of 11 peptides on a panel of different lung cancer cell lines. BAC = Bronchioloalveolar Carcinoma. H2009.1 peptide is seen to bind to most lung cancer cell lines. B. Binding of peptides on cancer cell lines from other sites. Of note is H460.1 binding of MCF7 breast cancer cells. Further work with H460.1 peptibody will be done on MCF7 cells.

RESULTS:

A secondary project to compliment the main DC project was chosen to further explore the uses of targeting peptides isolated through phage display in cancer immunotherapy. For this project, instead of focusing on stimulating an immune response against TAAs, the focus was placed on redirecting exogenous antibodies to a cancer biomarker. This would greatly increase the portfolio of therapeutic antibodies available for cancer treatments in the creation of an interchangeable peptide-antibody system.

Initial experiments for this project were meant as a proof of concept that could potentially justify further efforts into creating an interchangeable peptide-antibody system. Conjugation of the peptide to the antibody consisted of using tetrameric NSCLC peptides with a biotin moiety and a commercially available anti-biotin antibody. An initial imaging experiment visualized binding of the peptide-antibody to its target cell. The H2009.1 peptide was tested first since it is the peptide that binds the most lung cancer cells lines and its receptor is known. Peptide-antibody conjugates were made at either a 1:1 or 2:1 peptide/antibody ratio to test whether further multimerization of the peptide on the antibody scaffold would lead to increased binding to the H2009 cells. Imaging experiments following binding of the H2009.1-body in the polyclonal format showed increased binding to the H2009 cells compared to the control Scrambled-body. This was determined visually by increased fluorescent in cells incubated with the targeted peptide-antibody vs scrambled. The Scrambled control showed that the sequence of the peptide is important for binding and was not merely dependent on amino acid composition.

Binding of the H2009.1-body was further quantified through flow cytometry which clearly showed that the H2009.1-body binds greatly compared to Scrambled-body control. In addition, there is no benefit in having the 2:1 ratio compared to the 1:1 ratio. No difference in signal was seen between the two ratios with the targeted peptide-antibody, and the scrambled saw a little higher background with the 2:1 ratio. Previous results showed increased affinity of the H2009.1 peptide in the tetrameric format compared to the monomeric format, but an octomer on the antibody scaffold did not have an increased benefit to binding. To prove that this system was easily interchangeable, the H460.1-body was produced and tested on MCF7 cells. These cells were used in place of the original H460 cells because their larger size allows for easier visualization of peptide-antibody binding. The H460.1-body also showed increased binding compared to Scrambled-body control, though preliminarily it seemed like there is higher background with the Scrambled-body at the 2:1 ratio on these cells as well as increased binding of the H460.1-body at the 2:1 ratio. Increased fluorescence signal from the targeted peptide-antibody was not detected through flow cytometry with these cells, though a control experiment using the biotinylated peptide with SA-PE showed no increased signal. This is a characteristic of the H460.1 peptide and not a deficiency of the peptide-antibody format.

A complication arose when the peptide-antibody was switched onto the monoclonal antibody format. Initial imaging experiments on H2009 cells with the monoclonal H2009.1-body showed no strong binding to the cells. Flow cytometric analysis revealed that indeed the monoclonal format was not binding to the same capacity as the polyclonal. It was unknown whether the complex was unable to form

appropriately, or if the complex was forming correctly but then the biotin present in the media was competing with the peptide for binding. To discriminate between these scenarios, flow cytometry was used to compare binding of the monoclonal H2009.1-body when incubated in fully supplemented media, as had been done with the polyclonal analysis, or with PBS+ which lacks biotin as well as other proteins. The results showed that while the monoclonal peptide-antibody had decreased binding in media, binding was maintained if the monoclonal antibody conjugate was incubated in PBS+. This indicates that the complex is able to form, but competition of the biotin present in the media competes for peptide binding. Monoclonal antibodies tend to have lower affinities than their polyclonal counterpart. Therefore, the lower affinity associated with monoclonal anti-biotin antibody interferes with the stability of the peptide-antibody complex, hence it would be difficult to pursue further experimentation of the monoclonal format for *in vivo* studies. Once the monoclonal antibody format was utilized in targeting experiments, the imaging showed increased binding of the H2009.1-body onto H2009 cells compared to Scrambled-body, with further verification through flow cytometry (FIGURE 20-2). Even in the monoclonal format, no increased benefit was seen in utilizing the 2:1 ratio compared to the 1:1 ratio. As had previously been seen with the H2009.1 peptide outside of the antibody scaffold, the targeting peptide-antibody was seen to accumulate into cells over time. Internalization was verified through 3D modeling using confocal images of the cell. The behavior of the H2009.1 targeting peptide maintained the same whether on the antibody scaffold or with other conjugates previously tested, therefore the antibody did not interfere with peptide binding and internalization.

To test the potential of the peptide-antibody to be used for *in vivo* studies, a preliminary time-course experiment was performed to verify the stability of the polyclonal peptide-antibody conjugate. The H2009.1-body was incubated in media for 24, 48, and 72 hours at 37°C. The conjugate maintains stability at all-time points. Since the monoclonal format could not be used for experiments inquiring on ADCC effect on tumors, the polyclonal format would be used to look at *in vivo* tumor targeting through NIR imaging. A polyclonal antibody was purchased that was conjugated to the IR800CW licor dye, which had previously been utilized in the lab for *in vivo* imaging. The peptide-antibody conjugate was prepared as before and its binding to H2009 cells was verified through flow cytometry. Mice bearing dual H2009/H460 tumors were then injected with either the 1:1 ratio of the H2009.1-body or the Scrambled-body through tail vein. After 24 hours images were taken of the mice to trace peptide-antibody migration. Initial experiments showed promising results with the H2009 tumor showing higher H2009.1-body signal compared to H460 tumor, as well as H2009.1-body having a higher signal than Scrambled-body. However, several complications prevent further analysis. Of the 5 mice that were injected 2 died, indicating either toxicity of the compound or a negative reaction to a non-murine antibody.

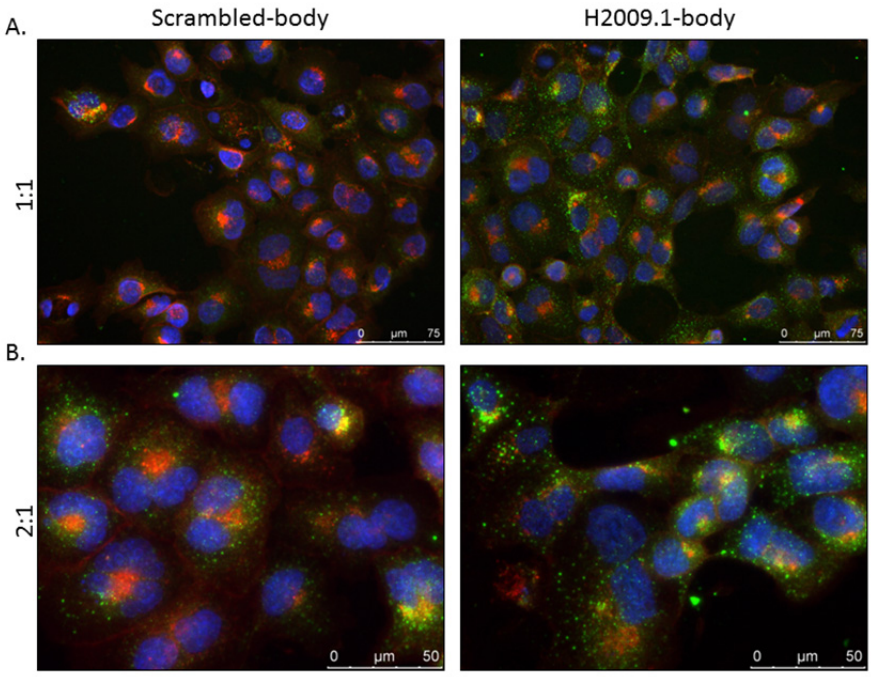


FIGURE 20-1 - H2009.1-BODY BINDING TO H2009:
A and B: H2009 cells were cultured overnight in 8-well chamber slides. Peptibody was prepared by mixing biotinylated tetrameric peptide with anti-biotin polyclonal rabbit antibody (complex final concentration = 10nM) at either a 1:1 peptide/antibody ratio or at a 2:1 ratio. Peptibody was added to cells for 1 hour at 37°C and then washed 4 times with PBS+0.1%BSA. Cells were fixed, permeabilized and incubated with goat anti-rabbit AF488 secondary antibody for 30 minutes. WGA TxRED was used to stain cell membranes. A. shows 20x magnification and B. shows 40x magnification. Images were taken on Leica DM5500 upright fluorescence microscope

C.

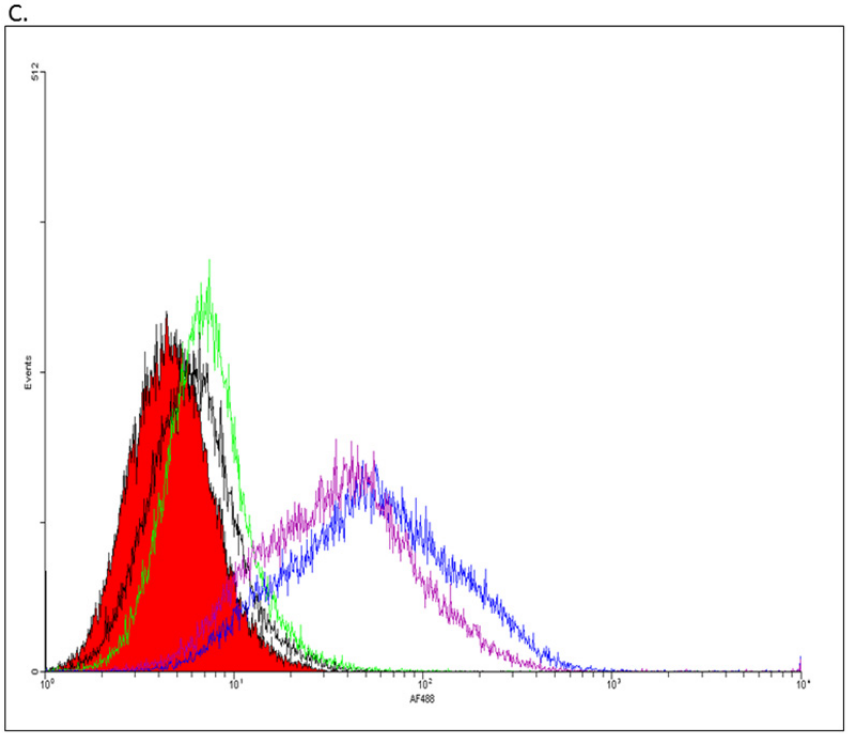


FIGURE 20-2 - H2009.1-BODY BINDING TO H2009 THROUGH FLOW CYTOMETRY:
C: For flow cytometry, 1:250 dilution of GAR488 was added to mixture to be incubated with peptibody. After washes the cells were incubated in cell dissociation buffer for 5 minutes and then scraped off. Flow cytometry was done with live cells in this buffer on the FACScan. Red=Cells with nothing, Black=cells incubated with Scrambled-body 1:1, Green=Scrambled-body 2:1, Blue=H2009.1-body 1:1, and Purple=H2009.1-body 2:1.

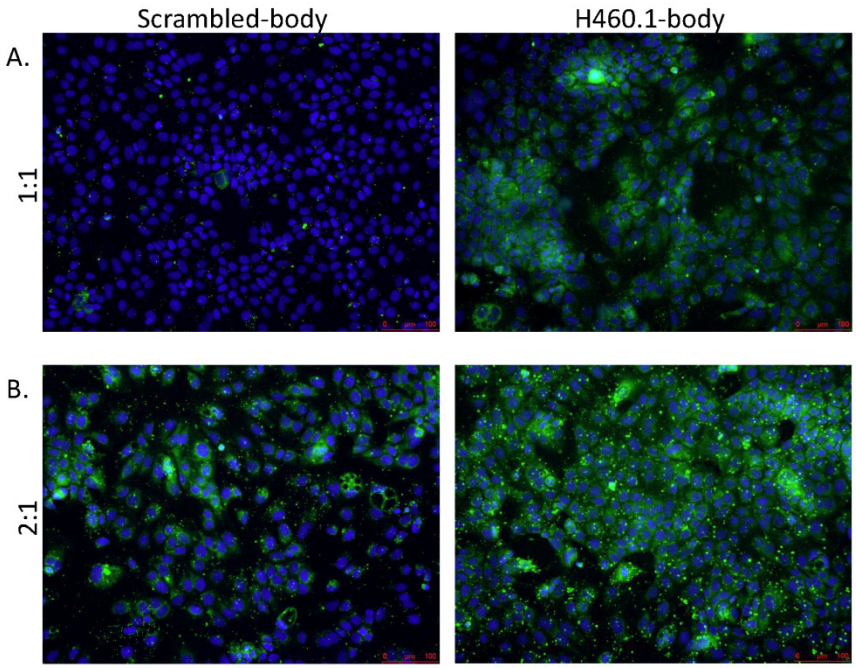


FIGURE 21 - H460.1-BODY BINDING TO MCF7:
MCF7 human breast cancer cells were cultured overnight in 8-well chamber slides. Peptibody was prepared by mixing biotinylated tetrameric peptide with anti-biotin polyclonal rabbit antibody (complex final concentration = 10nM) at either a 1:1 peptide/ antibody ratio or at a 2:1 ratio. Goat anti-rabbit AF488 conjugated antibody was also added at a 1:250 dilution. All components were incubated together with 500uL PBS for 30 minutes. 500uL of RPMI with 10% FBS (R5) was added, and the mixture was incubated on cells for 1 hour at 37°C. After incubation, the cells were washed 4 times with PBS+0.1%BSA. Cells were then fixed with 3.7% PFA in PBS for 10 minutes and prepared for imaging. Images were taken with a Leica DM5500 upright fluorescent microscope at 20x magnification. Flow cytometry analysis was not possible, since no fluorescence signal was seen, even with peptide-SA/PE control (data not shown). Cells were incubated in cell dissociation buffer for 5 minutes and then scraped off. Flow cytometry was done with live cells in this buffer on the FACScan. Red=Cells with nothing, Black=cells incubated with Scrambled-body 1:1, Green=Scrambled-body 2:1, Blue=H2009.1-body 1:1, and Purple=H2009.1-body 2:1.

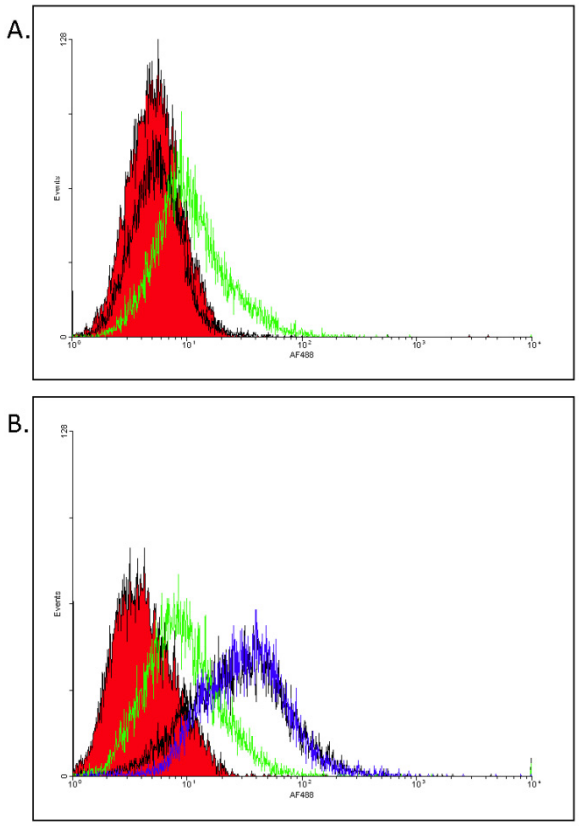


FIGURE 22 - H2009.1-BODY COMPARISON BETWEEN POLYCLONAL AND MONOCLONAL:
A: Flow cytometry was performed as previously described in R5 media on H2009 cells except monoclonal anti-biotin antibody was used. Red=Cells alone, Black=Scrambled-body, and Green=H2009.1-body. Monoclonal H2009.1-body showed decreased cell binding compared to polyclonal, with a slight increase of binding above Scrambled-body. B: Red=Cells alone, Black=H2009.1-body polyclonal in R5, Green=H2009.1-body monoclonal in R5, and Blue=H2009.1-body monoclonal in PBS+. Washes were done 4 times with PBS alone instead of PBS+0.1%BSA .

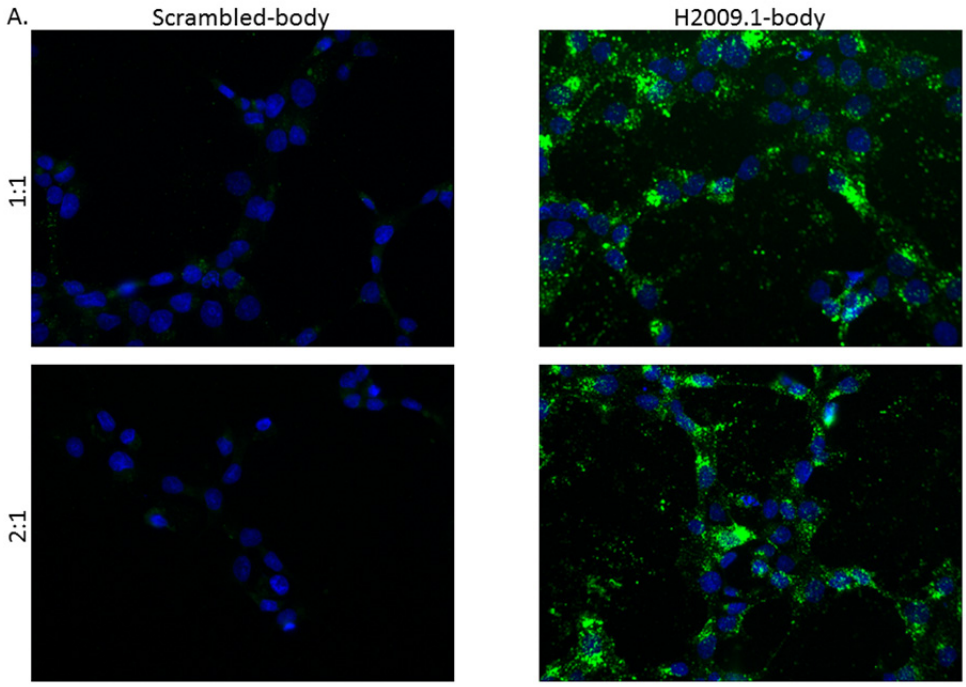


FIGURE 23-1 - PEPTIBODY MONOCLONAL CHARACTERIZATION IN PBS+: A. H2009 cells were cultured overnight in 8-well chamber slides. Peptibody was prepared by mixing biotinylated tetrameric peptide with anti-biotin monoclonal IgG2a murine antibody (complex final concentration = 10nM) at either a 1:1 peptide/antibody ratio or at a 2:1 ratio. GAM AF488 was added at 1:250 dilution, and solution was made in PBS+ instead of media. Cells were incubated with complex for 1 hour at 37°C. Cells were washed 4 times with PBS+0.1%BSA and fixed. Images were taken on Leica DM5500 upright fluorescence microscope

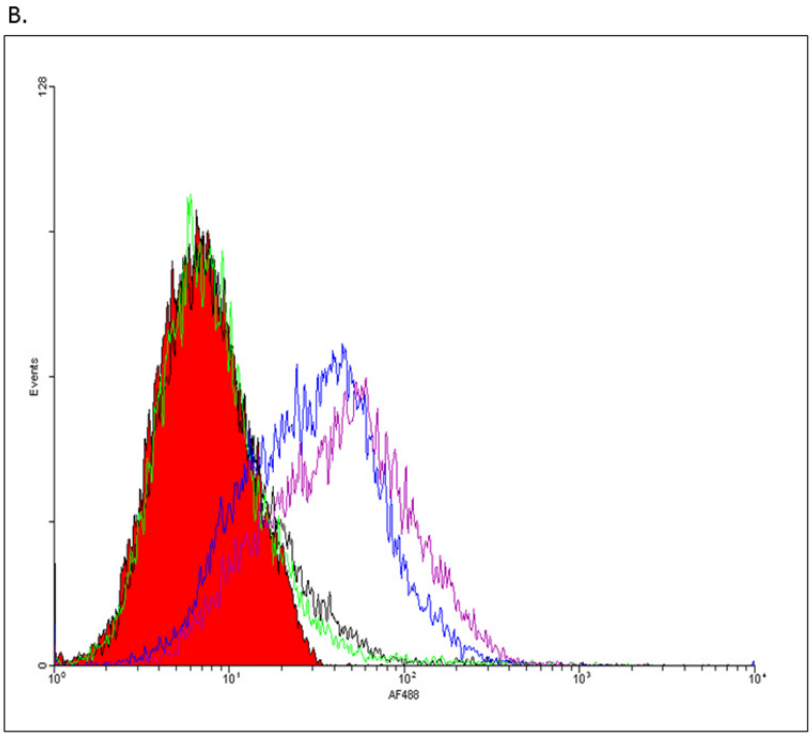


FIGURE 23-2 - PEPTIBODY MONOCLONAL CHARACTERIZATION IN PBS+: B: For flow cytometry, peptibody mixture was made the same as for imaging. After washes the cells were incubated in cell dissociation buffer for 5 minutes and then scraped off. Flow cytometry was done with live cells in this buffer on the FACScan. Red=Cells with nothing, Black=cells incubated with Scrambled-body (monoclonal) 1:1, Green=Scrambled-body (monoclonal) 2:1, Blue=H2009.1-body (monoclonal) 1:1, and Purple=H2009.1-body (monoclonal) 2:1.

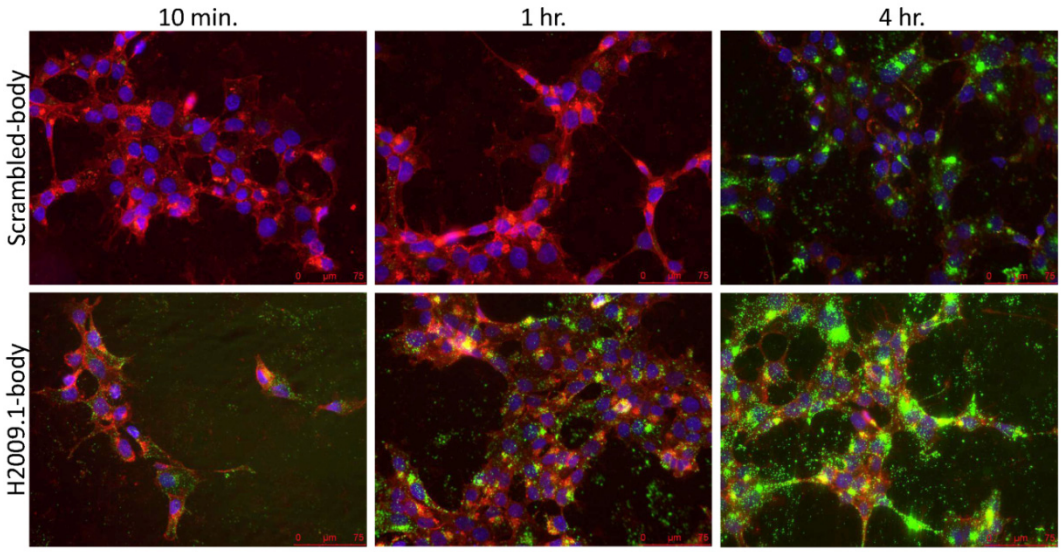


FIGURE 24 - TIME COURSE IMAGING OF H2009.1-BODY (M):
H2009.1-body (monoclonal) was produced in same fashion as previously mentioned (in PBS+). Cells were incubated in 8-well chamber slides with either Scrambled or H2009.1-body in PBS+ for indicated amounts of time. Cells were then fixed and stained with WGA TX red (1:100 in PBS for 10 minutes). Images show 1:1 peptide/antibody ratio. Binding of H2009.1-body is seen starting at 10 minutes, and binding increases up to 4 hours. Background signal from Scrambled-body is seen at 4 hours, but targeted is still significantly brighter.

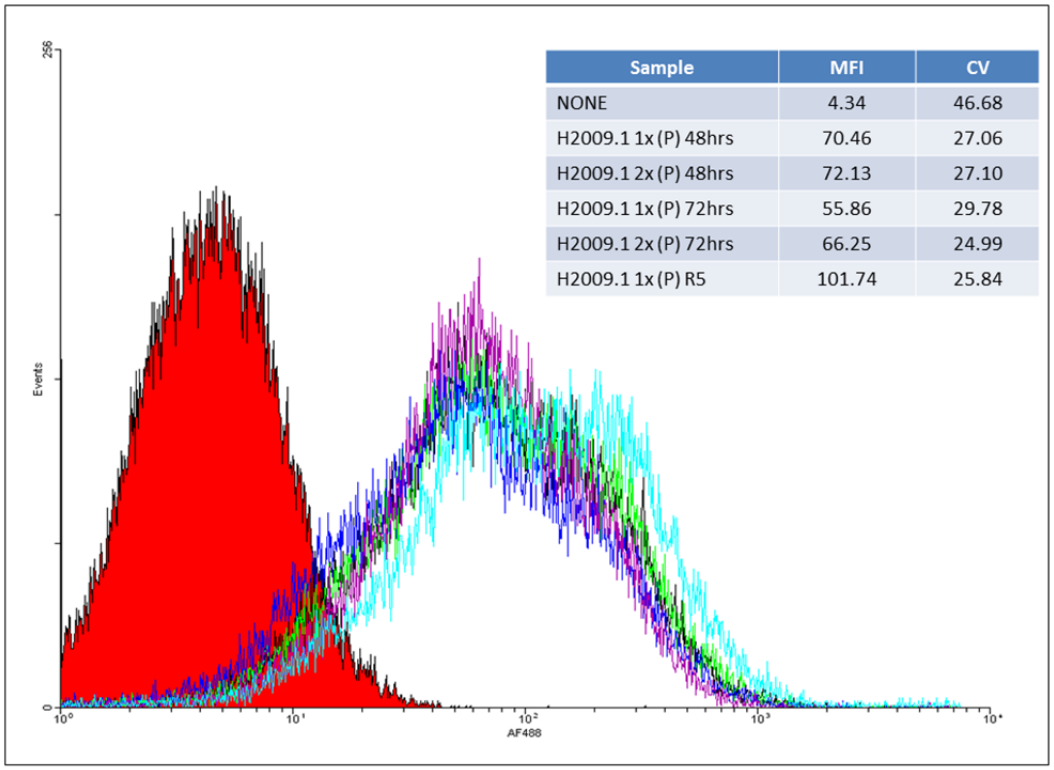
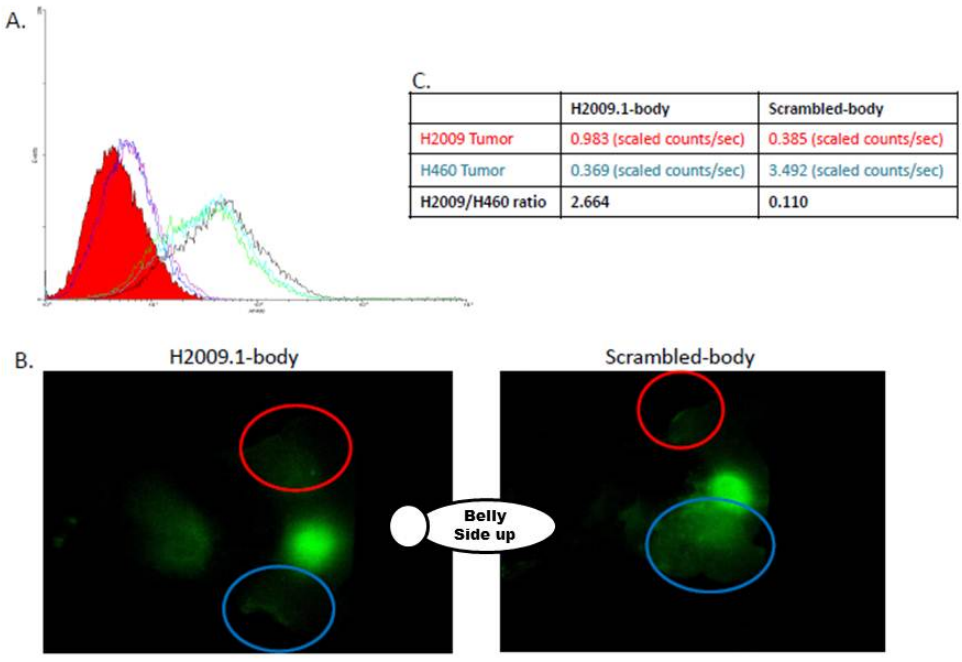


FIGURE 25 - TIME COURSE H2009.1-BODY (P) STABILITY):
H2009.1-body (polyclonal) is made in PBS and incubated in R5 for indicated time (48 hours and 72 hours). Red: None, Black: H2009.1-body (1x) 48 hours, Green: H2009.1-body (2x) 48 hours, Blue: H2009.1-body (1x) 72 hours, Purple: H2009.1-body (2x) 72 hours, and Light Blue: H2009.1-body (1x) R5. H2009.1 R5 was incubated onto cells after 30 minutes incubation in PBS (R5 added immediately before incubation). 1x indicates 1:1 peptide/antibody ratio, and 2x indicates 2:1 peptide/antibody ratio. 1:250 dilution of GAR AF488 was added after 48-72 hours and incubated for 30 minutes before being placed on H2009 cells. Cells were analyzed live through flow cytometry for analysis of peptibody binding. H2009.1-body was still seen to bind after incubation in media, indicating that the complex is stable for several days.



	H2009.1-body	Scrambled-body
H2009 Tumor	0.983 (scaled counts/sec)	0.385 (scaled counts/sec)
H460 Tumor	0.369 (scaled counts/sec)	3.492 (scaled counts/sec)
H2009/H460 ratio	**2.664**	**0.110**

FIGURE 26 - IN VIVO PEPTIBODY TUMOR TARGETING WITH TARGETED AND SCAMBLED PEPTIDE-ANTIBODIES:
INITIAL FUNCTIONAL ASSAY (A): Peptibody complex with Goat anti-biotin polyclonal antibody was made as previously described for other antibodies. Complex was incubated on H2009 cells for 1 hour in R5 complete media. RED=None, BLACK= H2009.1-body (IR800) 1x, GREEN=H2009.1-body (IR800) 2x, BLUE=Scrambled-body (IR800) 1x, PURPLE=Scrambled-body (IR800) 2x, LIGHT BLUE=H2009.1-body (P) 1x. Targeting to H2009.1 cells was still seen with IR800 antibody. B. 50ug of peptide-antibody was injected through tail vein into mice bearing H460 and H2009 dual tumors (on opposite flanks). After 24 hours, signal was imaged using the Maestro In Vivo Imaging system. C. Fluorescent quantification of images

DISCUSSION:

The benefit of an antibody-peptide conjugate is the combination of capabilities exerted by each separate molecule. The results indicate that previously isolated NSCLC peptides can be utilized for a peptide-antibody conjugate that can bind lung cancer cells *in vitro* and *in vivo*. The conjugate was able to go through initial tests for binding and *in vivo* functionality using the biotinylated peptide and an anti-biotin antibody. The polyclonal format of the conjugate binds its target cells as analyzed through imaging and flow cytometry. The conjugate also had long term stability when incubated in media up to 75 hours. Increased valency of the peptide-antibody conjugate by adding a 2:1 molar ratio of peptide to antibody did not increase binding of H2009.1 on H2009 cells. The control Scrambled peptide-antibody conjugate demonstrated higher background binding at the 2:1 ratio. The H460.1 peptide-antibody preliminarily showed increased binding to MCF7 cells at the 2:1 ratio, but there was also increased background binding from the Scrambled peptide-antibody. This indicates that increased valency on the antibody scaffold may not lead to increase binding for all NSCLC targeting peptides. All peptides would have to be tested to determine which peptides benefit from increased valency on the antibody scaffold.

Problems arose when the peptide was placed on the monoclonal IgG2a format. Initially no binding was seen, but upon further investigation it was seen that binding occurred in PBS+, which suggests that the biotin present in media was competing for binding with the antibody. This indicates that the monoclonal antibody affinity is less than the polyclonal. Regardless, lack of functionality of the monoclonal peptide-antibody in fully supplemented media inhibited to ability to further study its functionality. Of

particular interest was the ability of the peptide-antibody to direct an ADCC or complement response against the tumor cells for direct lysis. This cannot be tested until a more stable IgG2a peptide-antibody conjugate is developed. Initial studies that attempted to measure cell death during complement/ADCC did not show positive results with monoclonal and polyclonal antibody formats.

The peptide-antibody (polyclonal) conjugate was able to be utilized for *in vivo* tumor imaging. The anti-biotin IR800CW conjugate was used for this experiment. This dye had shown positive results in previous tumor studies using the H2009.1 peptide direct dye conjugate. To control for targeting, mice bearing the H2009 tumor and the non-targeted H460 tumor were produced by the Brown lab. The results indicate preliminarily that the peptide-antibody conjugate can be visualized during *in vivo* tumor targeting. Increased near-infrared signal was seen in the H2009 tumor in the mouse injected with the H2009.1 peptide-antibody when compared to the H460 tumor in the same mouse and the H2009 tumor in the mouse injected with Scrambled peptide-antibody. However, several complications prevented this study to continue further. This includes the high level of toxicity to the mice presumably from either the compound or from an air bubble during injection. An experienced senior lab member performed these injections; therefore the introduction of air bubbles is most likely due to solution viscosity. In addition, increased signal that would indicate targeting of the H2009 tumor was not seen when organs were imaged *ex vivo*. Parallel studies with the peptide-dye conjugate showing no targeting suggests that either a different dye or a different *in vivo* imager needs to be used for optimal visualization. Previous experiments utilized Licor dyes with the Licor instrument, but this instrument is currently unavailable for use. This instrument/dye

combination worked for initial tests of peptide tumor targeting *in vivo* because of the sensitivity and clean image that allowed for comparable results. Consistent results were obtained even with *ex vivo* organs, something that was not transferable to the instrumentation used of the peptide-antibody experiment. There are dyes that would be optimal for use with the Maestro and other available instruments, but they are currently not available on an anti-biotin antibody. Without further optimization our ability to truly assess the ability of the conjugate to target comparatively to the scrambled peptide, especially *ex vivo*, was inhibited. These problems should be addressed during planning of future experiments.

The H460.1 peptide was tested on MCF7 cells for further testing of peptide-antibody functionality. The peptide-antibody polyclonal conjugate bound to different target cells, solidifying the idea of using the NSCLC peptides to create a peptide-antibody interchangeable system. The H460.1 peptide was tested because it binds the cell surface and is not internalized. The target may be to either an extracellular marker or to a secreted factor. Previous imaging experiments with various peptide-dye conjugated did not show peptide-dye internalization. Further analysis of this peptide-antibody conjugate was inhibited by the inability to analyze binding through flow cytometry. This seems to be an inherent characteristic of the peptide, since control experiments using the standard peptide-dye did not show fluorescence through flow cytometry. This could be because the receptor is being shed during preparation, or as previously mentioned the target is a secreted factor. Surface bound peptide may also dissociate from the cell, making flow cytometry analysis difficult. Further imaging studies using fluorescence quantification would provide a better analysis of conjugate binding.

The peptide-antibody conjugate was created with different valencies to determine if increased peptide quantity correlated with increased binding. Interestingly no enhanced binding was seen with the H2009.1 2:1 ratio antibody. They may be from binding saturation of the target receptor. When a new format is made it would be interesting to test different antibodies that vary in peptide valency. Flow cytometry could still be used for comparative analysis, and peptide-antibody variations (for example, having the tetrameric on one antibody arm versus having both arms having a dimer) could be more closely analyzed. It would also be interesting to see if this translated into variations with *in vivo* imaging and potentially during antibody therapy with a murine tumor model. This is important because one of the benefits of using these peptides versus the Cov-X peptibody is the flexibility in valency. Further analysis could elucidate which peptide-antibody ratio is best suited for different applications.

Another potential area of research is the development of bispecific antibodies. Different lung cancer or immune cell targeting peptides could potentially be utilized for different immune functions. Dr. Barbas has already started looking into this question of multi-valency and production of bispecific antibodies with his Cov-X antibody [200]. In addition, several other studies have been done with bispecific antibodies, especially where one Fab binds to a cancer antigen and the other binds to an immune cell [201]. Bispecific antibodies tested in clinical trials include the MDX-210 (targeting Her2 and CD64), MDX-H210 (humanized version of MDX-210) and MDX-447 (targeting EGFR and CD64), where CD64 (FcγR1) antibody portion is meant to bind immune cells and bring them into proximity with cancer cells [202-205]. These studies saw biological effects but not consistent anti-tumor activity. Further development with this design,

especially with peptide-antibodies, could potentially increase ADCC and immunological anti-tumor function.

While there are benefits to utilizing the NSCLC peptides on an antibody scaffold, it would be optimal to have a means of chemical conjugation of peptides directly onto the antibody without the need to use genetic engineering. Direct conjugation of the peptide could potentially resolve issues with binding stability and facilitate production of the IgG2a subclass. A full analysis is warranted of ADCC and CDC capability with the new conjugate, since ADCC has been implicated as the main mechanism of action for antibody mediated tumor clearance. The conjugation method proposed by Dr. Barbas and colleagues could be made possible by addition of a β-diketone moiety on the tetrameric peptide scaffold. This moiety would facilitate conjugation with the Cov-X antibody. The development of a peptide-antibody conjugate with this chemistry would allow further analysis of anti-tumor immunological function for future cancer antibody treatments.

In order to understand the utility of this conjugate for cancer treatment, the ADCC/CDC functionality of the NSCLC peptide-antibodies would have to be tested. These peptides have been isolated for internalization for drug delivery, so it is still to be seen whether these peptides would be useful for induction of ADCC. If internalization interferes with antibody-mediated immune function, the H460.1 peptide could serve as an interesting alternative since it is the one lung cancer peptide that is not internalized. Wash conditions during panning could also be changed to bias the selection towards peptides that remain bound on the extracellular receptors. In addition, peptides could be isolated with the protocol changed to isolate those ligands that are present in the acid wash. As a reminder, and acid wash is performed to remove surface bound phage during panning. If

other peptides are to be isolated, peptides that target murine cancer cell lines would also be under consideration. This would facilitate murine anti-tumor studies in immune competent mice, where the full effect of ADCC/CDC should be seen.

The work that has been done on this project has been preliminary and should be regarded as a proof of concept to serve as a foundation for future work. Further investigation into development of peptide-antibodies for cancer treatments would greatly expand potential ligands available for anti-tumor studies.

MATERIALS AND METHODS:

Peptibody Imaging and Flow Cytometry. Anti-biotin antibody (rabbit polyclonal from Abcam, Cambridge MA, mouse IgG2a monoclonal from Novus Biologicals, Littleton CO) was incubated with biotinylated tetrameric peptide for 30 minutes at RT with either a 1:1 or a 2:1 peptide/antibody ratio for a final concentration of 10nM complex. For flow cytometry and H460.1 imaging data, 1:250 dilution of secondary AF488 conjugated antibody (either GAR or GAM) was incubated with peptide and antibody in 500µL of PBS. After incubation, 500uL of either R5 or PBS+ was added and immediately placed on cells. Cells were incubated with peptibody for 1 hour at 37°C. Cells were washed 4 times with PBS+0.1%BSA and then either prepared for imaging (fixed with 3.7% PFA in PBS on 8-well chamber slides, treated with DAPI fluoromount G) or prepared for flow cytometry. For flow cytometry, after incubation and washes, cells were incubated for 5 minutes in cell dissociation buffer (Gibco) and then scraped off for live cell flow cytometry analysis on a FACScan cytometer.

Peptide-Antibody In Vivo Imaging. Anti-biotin antibody (goat polyclonal) with a 1:1 conjugated IR800CW licor dye was conjugated at a 1:1 peptide/antibody ratio for a final amount of 50ug peptide-antibody for each NOD/SCID mouse. Conjugate was injected via tail vein and imaged on the Maestro *in vivo* imager after 24 hours. After imaging *in vivo*, organs and H2009/H460 subcutaneous tumors were immediately resected and imaged for fluorescence.

CHAPTER FOUR

FUTURE DIRECTIONS AND CONCLUSIONS

Overall, there are several barriers that need to be overcome in both projects which will be discussed in this section. Experiments to surpass these barriers were not performed due to time constraint on the predoctoral project, but nonetheless these future experiments would be worth performing to further the use of peptides in cancer immunotherapy.

Future Directions for DC peptides

The data collected from this project lays the groundwork for further studies into the utility of the DC peptides in a tumor vaccine model. The main focus of future experiments should be the development and testing of a new vaccination format in murine tumor models.

DC-liposomal delivery of antigen utilizing DC targeting peptides

In the Brown Lab, liposomes are being constructed to target therapeutic agents to lung cancer cells because of the ability to deliver high drug load and its clinical relevance with Doxil. DC targeted liposomes are a flexible platform that will be tested for the ability to deliver high antigen load *in vivo* (FIGURE 27). Doxorubicin loaded liposomes (Doxil) have been approved for clinical use in ovarian cancer, and targeted liposomes using CD11c and DEC 205 antibodies have been tested in mouse models for antigen delivery to DCs [206, 207]. Liposomes can be loaded with antigenic protein, a chemically synthesized epitope peptide, or a genetic construct for antigenic expression *in vivo*. A liposomal DC targeted vaccine that has been tested is in a murine B16F-OVA

tumor model is an OVA loaded liposome with anti-CD11c and DEC205 antibody fragments [208]. This study saw induction of antigen specific CD8+ T-cells and tumor protection in mice that received the vaccine with IFNγ or LPS. Glycan-modified liposomes that target DC-SIGN also boost antigen specific CD4+ and CD8+ T-cell responses [209]. In the proposed future directions, the OVA protein loaded liposomes (100 nm in size) will have DC targeting peptides conjugated to their surface. While the ability to prevent antigen degradation and clearance and have increased uptake by professional APCs have marked liposomes as useful vaccination vehicles, they have also been noted for their adjuvant effect. In particular, research on the use of phospholipids for increased immunogenicity has been explored [210-212]. Liposomes can serve as an alternative to other adjuvants that display local or systemic hypersensitivity.

The quantity of maleimide-modified phospholipid is used to increase the amount of exposed maleimide groups on the liposomes for peptide conjugation. This formulation grants the ability to optimize peptide valency and density on the liposome surface. Peptide density can be modified with varying amounts of maleimide-modified phospholipids, while valency can be changed by using monomeric, dimeric, or tetrameric peptide. After the lipids are mixed together, the organic solvent in which they are stored in is removed. The lipids are re-hydrated with an aqueous solvent containing the cargo that will be loaded into the liposomes. The liposomes are then processed through an extruder to obtain liposomes of uniform size. The resulting liposomes contain exposed maleimide groups on the outside. These maleimide groups will form chemical bonds with cysteine through basic maleimide chemistry. The targeting peptides will be synthesized with a cysteine carboxy-terminal that will allow this reaction to occur and

conjugate the peptides around the liposomes. The PEG group functions to create stealth liposomes to limit non-specific uptake *in vivo*. The resulting product will be liposomes loaded with a cargo of antigen and modified with DC targeting peptides on the surface. Peptides that are to be conjugated to the surface of liposomes can be made as either a monomer or a tetramer. These liposomes will be administered by intradermal injection into mice. The time points measured for serum collection will be the same, every two weeks with booster administrations at the 3rd and 5th serum collection.

Liposomes have already been used in the lab to target DCs (Figure 28) Previous experiments in the Brown Lab have shown that liposomes conjugated with the first 16 amino acids of the XS52.1 peptide can specifically deliver a plasmid containing the luciferase gene and allow expression of luciferase in DCs *in vivo* [174]. Liposome-based immunization will be tested by collecting serum from the mice to be tested for a humoral immune response. If liposome-based immunization does not produce robust immune responses, adjuvants will be added to determine if this induces heightened immune responses. There is a high level of clinical relevance to these experiments since liposomal vaccination against the MUC1 tumor antigen is currently being tested in phase III trial for treatment of NSCLC [213]. Phase IIB trials with this vaccine showed minimal toxicity but only had a survival difference of 4.4 months that did not reach statistical significance [214]. However, a subgroup of patients with stage IIIB disease had a strong trend in 2-year survival, illustrating the potential of vaccines to be used for long term tumor protection. Adding a targeting moiety to this format could potentially make a difference when it comes to eliciting the immune system enough to see therapeutic benefit in a clinical setting.

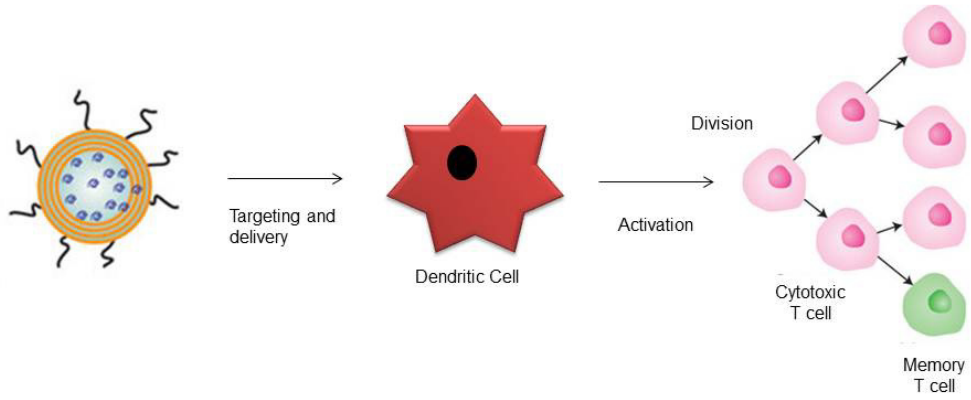


FIGURE 27 - IN VIVO LIPOSOMAL DC TARGETING SCHEMATIC:
Figure modified from Acharya et al [215]. Activated DC that have received the antigen load from liposomes with targeting peptides are able to activate naïve T-cells. T-cells will then proliferate, giving rise to memory T-cells that will mediate long term immunity while activated cells can directly lyse tumors cells.

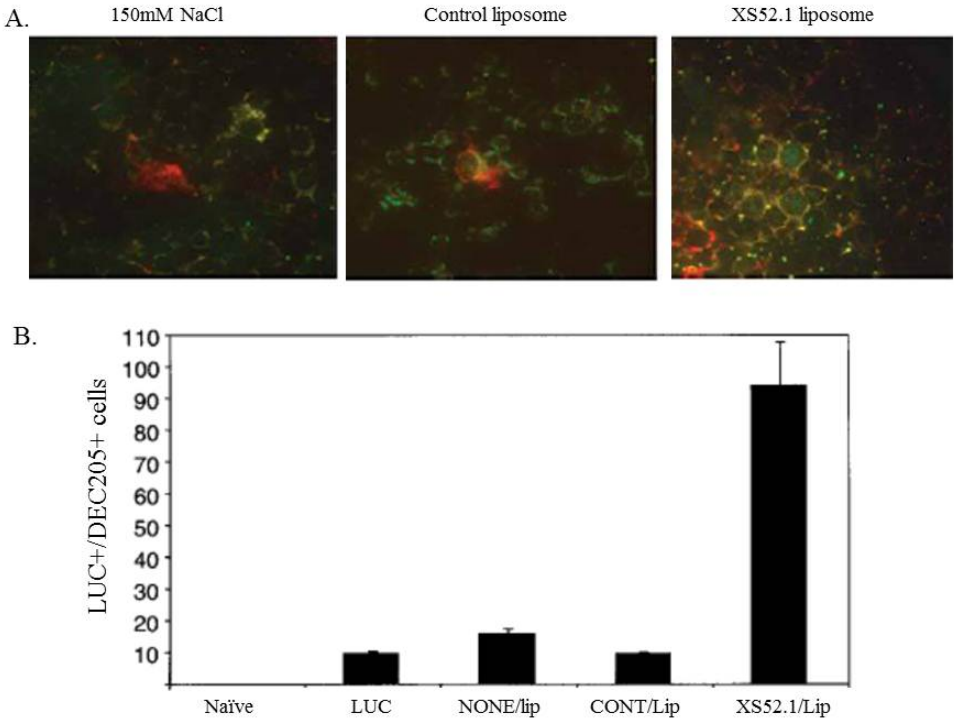


FIGURE 28 - XS52.1 PEPTIDE MEDIATED GENE DELIVERY:
Modified from McGuire et al. The XS52.1 targeting peptide can deliver an active gene outside the context of the phage. Luciferase expression was detected in migratory DC following Biojector injection of peptide-targeted LUC plasmid (LUC) liposomes. (A) Mice were administered liposomes containing LUC and targeting peptide XS52.1 or control random peptide. Control mice were injected with 150 mM NaCl. After 1 day, lymph nodes were extracted and cells were spun onto slides. Cells were immunostained for luciferase (green) and DC-marker Dec205 (red). (B) Quantification of luciferase expression in lymph node DCs following administration of the lipid formulations XS52.1 (XS52.1/Lip) and control (CONT/Lip), and compared to a LUC lipid formulation without peptide (NONE/lip), naked plasmid DNA (LUC), or saline and naïve. Luciferase positive, DC marker-positive cells (Luc+/DEC205+) were separately counted in the LNs of each mouse. The averages and error bars (SEM) are shown from five samples for each of the three mice per group.

Tumor model studies for vaccine efficacy

If successful, the liposomal vaccination strategy and delivery mechanism will be assessed in a tumor model. In order to study immune responses to a TAA, we will use tumor cells expressing ovalbumin (OVA) as the antigen. We will transfect full-length OVA mRNA into 4T1 cells, which is a BALB/c derived breast cancer cell line. Other cancer cell lines could be utilized depending on the desired tumor model for study. The genetic construct will have the OVA antigenic sequence. Cancer cells will be injected in the flank subcutaneously. The cells of choice will be OVA-transfected 4T1. This system will undergo prophylactic and therapeutic treatments. The immune response will be measured by collecting sera every two weeks and measuring by ELISA. When an immune response to the antigen is observed, the mice will be injected with cancer cells subcutaneously in the flank. The tumor mass growth between the two groups will be observed to see if tumor growth is impeded by the vaccination. For the therapeutic experiment, the tumors will be induced prior to injections. The tumors will be formed with subcutaneous injection of OVA-4T1 cells. When a palpable mass is observed, vaccination will occur in similar groups as the prophylactic, where 2 groups of mice will either receive the vaccine or will go without. The reduction of tumor mass will hopefully be observed. If effective immunization occurs, there should be elimination of the subcutaneous tumor in the mouse. Humoral immune response will be measured through ELISAs and western blots. The cell-mediated response will be determined by measuring antigen-specific MHC-restricted cytotoxicity through *in vitro* killing assays with incubation of OVA presenting tumor cells and isolated CD8+ T-cells. The appropriate controls will be usage of non-OVA transfected cells and MHC class I and II blocking

antibodies. T-cell activation will also be measured with ELISPOT to determine how many cells secrete cytokines in the presence of OVA. Though OVA is not a native protein in the mouse model, the vaccination format can be switched to test a more relevant antigen, such as Her2/neu in Her2 genetic breast cancer mouse models. In a recent study using antigenic Her2 peptide, liposomes that targeted TLRs showed anti-tumor efficacy in Her2 expressing murine tumor model [216]. The determination of the best performing vaccination format will lead to further developments and studies in cancer therapy.

Developing a vaccination strategy using the DC targeting peptides has immense relevance to clinical use. PowderMed, now owned by Pzifer, has DNA vaccines against infectious agents such as influenza as well as a non-small cell lung cancer vaccine that are undergoing clinical trials [217]. Their method is a ballistic approach similar to the one tested for the genetic immunization project. Any protein/peptide antigen based vaccine, not just those directed against cancer, can benefit from a DC targeting agent that mediates a heightened immune response.

Future directions for peptide-antibody project

As mentioned in the discussion, the work that has been done with this project laid the foundation for future studies to be done with this conjugate. Before further analysis is done for functionality, alternative means of antibody production should be investigated to produce a more stable peptide-antibody conjugate

Development of stable peptide-antibody conjugate

In this project, difficulties were seen in the stability of the monoclonal format of the peptide-antibody conjugate. While this peptide-antibody conjugate could potentially inhibit tumor growth through signaling on the polyclonal format, the initial strategy was to induce tumor death through targeted ADCC mediated by the Fc portion of the antibody. Towards this goal, development of a stable IgG2a peptide-antibody conjugate would be ideal.

The two means through which to develop the stable conjugate would be through chemical conjugation or with a genetic construct. The chemical conjugation of the peptide with the antibody would be heavily modeled according to the Cov-X antibody. The murine catalytic antibody is commercially available and the sequence of the Fab portion is published if further genetic manipulation is required for mass production. The only requirement would be for the appropriate reactive group to be placed on the peptide. In this aspect our laboratory has an advantage over the Barbas group, in that the ability to modify both the monomeric and the tetrameric peptide with the reactive group is possible. The Barbas group has been able to make a maximum of 4 peptides presented off of the Fab region, but we have to ability to present up to 8 peptides. Initial studies using the 1:1 versus the 2:1 peptide/antibody ratio did not show improved benefit of the extra peptide presentation. This would be more accurately tested however in the direct conjugate with the antibody.

The other means of making the conjugate would be through genetic means. Already available in the laboratory is a genetic construct that encodes for the Fc portion of an IgG isotype. The Fc portion is produced and dimerized and the genetic construct

would allow for peptide presentation off of the Fc portion. The caveat is that only one peptide would be presented per chain, making at most a dimeric presentation of the peptide. The advantage of this method would be the ability to produce the conjugate in large quantities for facile purification through a Protein A/G column.

Conclusions

The hypothesis for this project state that peptides isolated through phage display can be utilized to improve cancer immunotherapeutics. The first aim was to demonstrate that the DC peptides are capable of a heightened antigen-specific immune response. In addition to showing that the DC peptides bind preferentially to immature DCs, both *in vitro* and *ex vivo,* it was also demonstrated that the DC peptides do elicit a heightened humoral response against phage proteins. There were initial attempts to measure T-cell responses from vaccination, but the current consensus is that the data acquired is not reliable due to lack of appropriate positive controls. The initial experiments were unable to detect increased cytokine production (IFNγ, IL-10, IL-4, and TNFα) in T-cells from vaccinated mice. While direct experiments to follow DC *in vivo* uptake of targeting phage were never performed, the conclusion reached is that these peptides are capable of mediating increased humoral function. One crucial component that is missing from the project however is tests for anti-tumor efficacy. This work remains to be done.

The second aim of the project was to demonstrate the ability of a peptide-antibody conjugate to utilized the specificity of the peptide to target cells. The lung cancer peptides were seen to maintain binding specificity to their target cells on the

antibody scaffold. In addition, preliminary data shows that the conjugate binds to tumor cells *in vivo.* This specific aim is more of a direct measurement of the ability of peptides to improve cancer immunotherapy. However, further testing in a mouse tumor model would be needed to ultimately determine the anti-cancer properties of the conjugate.

In conclusion, the project has been able to accomplish its original aims in determining peptide ability in two different immunotherapeutic approaches. This work is a foundation for future studies utilizing peptides in cancer immunotherapy.

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