EFFICACY IN A MOUSE MELANOMA MODEL OF A DENDRITIC CELL-TARGETING THERAPEUTIC DNA VACCINE AND ENHANCEMENT OF ITS ACTIVITY BY CO-TREATMENT WITH ANTIBODIES TO NEUTRALIZE INTERLEUKIN-10

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ABSTRACT

Introduction: Vaccines fusing Macrophage Inflammatory Protein-3α (MIP-3α) to an antigen have shown efficacy in malaria, melanoma (prophylactically), and lymphoma models. The MIP-3α component targets nascent peptides to immature dendritic cells by interaction with CCR6, leading to processing, cross-presentation, and the induction of strong immune effector responses. Other studies have provided evidence that IL-10 is integral to the maintenance of the tolerogenic melanoma microenvironment. The therapeutic efficacy and immunogenicity of a DNA vaccine fusing MIP-3α to melanoma differentiation antigen gp100, and the additional effect of neutralizing IL-10 in the tumor, were analyzed in this study.

Methods: The B16F10 mouse spontaneous melanoma syngeneic transplantable mouse model system was utilized, with a standard therapeutic protocol: challenge with lethal dose of B16F10 cells ($5x10^4$) on day 0; vaccinate by intramuscular electroporation with 50μg vaccine on days three, 10, and 17; and (if used) administer 150μg doses of IL-10 neutralizing antibody (αIL-10) starting day five for every three days intratumorally. Vaccine controlling for MIP-3α contains a mutation abrogating chemokine functionality and is termed dMIP-3α-gp100 or antigen-only vaccine. Efficacy assessed by analyses of tumor size, tumor growth, and mouse survival. Immunogenicity was assessed primarily by flow cytometric methods, including intracellular cytokine staining to assess vaccine-specific T-cell responses. Mechanism of αIL10 was elucidated by gene expression analyses and a knockout mouse model.

Results: With this therapeutic protocol, it was demonstrated that MIP-3α-gp100 vaccine significantly slows tumor growth and increases mouse survival compared to antigen-only

vaccine. Both CD4+ and CD8+ effector T-cells play a role in protection as determined by T-cell depletion studies, and the vaccine-specific CD8+ tumor infiltrating lymphocyte (TIL) profile correlates with protection. Combining this vaccine with αIL-10 led to further decreases in tumor size and increases in overall survival compared to vaccine alone. Mechanism of effect of αIL10 therapy was shown to be independent of the measured TIL profile. Analysis of tumor lysate transcription levels show significant upregulation of IFN-α4, which is known to have anti-tumor effects. The mechanism was confirmed by observing no therapeutic benefit of αIL-10 in a mouse model with IFNα-receptor knocked out.

Conclusions: Efficient targeting of antigen to immature dendritic cells with a chemokine fusion vaccine offers a potential alternative approach to the *ex vivo* dendritic cell antigen loading protocols currently undergoing clinical investigation. The flexibility and ease of construction of the vaccine make it an excellent platform for inducing immunity to tumor-specific neoantigens. Combining this approach with agents able to modulate the tolerogenic tumor microenvironment offers promise as a novel melanoma therapy.

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PREFACE

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Dedication

This dissertation is dedicated to my past and my future. To my parents for all their love and support, and especially to my father who unfortunately did not live to see this day. He is likely still explaining to others in the afterlife that I'm busy "making Frankensteins." This dissertation is also dedicated to my son, RJ. You have provided brand new perspectives on life and a love that just doesn't make rational sense. I am very excited to watch you grow and learn. Maybe one day you will even read this. I hope so.

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CHAPTER 1

INTRODUCTION TO THE FIELD

Section 1: Melanoma

Clinical Characteristics

Melanocytes are cells located in the basal layer of the epidermis that produce the pigment melanin, which gives the skin its tan or brown color, and which also accounts for the darker color of benign common nevi (moles) [1]. Melanin protects the deeper layers of skin from the harmful effects of ultraviolet light [2, 3]. Cutaneous melanoma occurs when these skin melanocytes turn cancerous. Melanoma can be divided into four primary subtypes: superficial spreading (SSM), lentigo maligna (LMM), nodular (NM), and acral lentiginous melanoma (ALM). SSM is the most common and all references to melanoma will be referring to this subtype unless otherwise specified. LMM is primarily found in elderly populations with chronic sun exposure. NM is rare, but more invasive and more difficult to detect as it doesn't follow the ABCDEs cited below. ALM is rare (2-3% of all melanomas), is the primary type of dark-skinned patient groups, is not related to ultraviolet radiation (UVR) exposure, and has a very poor prognosis. Melanoma can often times be detected by phenotypic changes to a patient's nevus. Early detection can be facilitated by observing the classical signs of malignant melanoma, the ABCDE's: Asymmetry, Border (irregular), Color (non-uniform), Diameter (greater than 6mm), and Evolving characteristics [1, 2].

Melanoma staging is based on lesion thickness, mitotic rate, lymph node involvement, and distant metastasis as determined by physical exams, skin biopsies, and/or imaging tests. Generally, the thicker the lesion, the higher likelihood of eventual spread. The lesion's mitotic rate represents the proportion of cells undergoing mitosis, with more growth correlated with cancer progression and spread [1, 2]. Stages I and II

include progressing thickness, mitotic rate, and ulceration of lesions without lymph node involvement. Stage III is characterized by spread to regional lymph nodes, and stage IV includes metastasis of tumor cells to distant tissues (Table 1.1). The survival rates by stage are listed in Table 1.1, and are progressive with a generally good five-year survival rate for stages IA – IIB (70-97%), with the rate declining all the way down to 15-20% for stage IV metastatic melanoma [1, 2]. Of note, the five year survival of stage IV has increased dramatically from a historic 5% to the current 15-20% due to improved therapies [4].

Melanoma is treated based on stage by either surgery, chemotherapy, radiation, immunotherapy, or by a targeted therapy as outlined in Table 1.1. Surgery is the primary option and is usually curative for early stage melanomas. If Stage III is proven, regional lymph nodes will be excised in addition to the primary tumor. In cases of metastases, surgery is no longer curative but may still be helpful to patient longevity or quality of life. Chemotherapies such as alkylating agents (Dacarbazine), taxanes that affect cell division (Paclitaxel), and platinum-based DNA crosslinking drugs (Cisplatin) can be utilized in metastatic cases. However, "targeted" therapies that affect cancer-specific pathways (such as BRAF discussed below) and immunotherapies have better efficacy and so are used more frequently [1, 2]. Radiation is generally only used in specialty cases of melanoma or for palliative care if melanoma has metastasized to regions such as brain or bone that cannot be surgically removed but cause the patient a great deal of pain or discomfort [1, 2].

Epidemiology

Melanoma is one of the few cancers whose incidence has been steadily increasing in most developed countries since the 1950's [5, 6]. In 2016, projections predict 76,380 incident cases of melanoma in US, with 10,130 deaths. Melanoma is the fifth leading cause of cancer among men in the US and the seventh leading cause among women. Through 2012, incidence was steadily increasing in both males and females [7]. Taken together, it is the sixth most commonly diagnosed cancer across both genders [8]. Although melanoma accounts for less than 1% of skin cancers, it is by far the leading cause of deaths from skin cancer [1, 2]. Incidence of non-melanoma skin cancers are not required to be reported to cancer registries, but amongst those reported, melanoma accounts for 74% of skin cancer deaths [2]. One person dies every hour from melanoma in the US, and, occurring at a median age of 52 years, melanoma strikes individuals in the prime of their lives, almost a decade before most solid tumors arise [6].

The primary risk factor for melanoma has been well established: ultra-violet radiation (UVR). The magnitude of the risk depends on patterns of sun exposure, with intermittent intense exposures more highly correlated than chronic exposure. Risk also depends on nevus counts, inherited susceptibility traits, and individual characteristics such as fair skin and freckles [9–11]. UVR exposure early in life elevates the risk of skin cancers, including melanoma [12]. Up until the past five years, the estimated annual increase in melanoma incidence rate has been approximately 3-7% per year for Caucasians worldwide [13]. A proportion of that increase has been attributed to enhanced detection and public awareness [5], but it has been also been shown that increased UV exposure plays a more significant role in the increase in incidence [9, 14], with tanning beds also being implicated [15]. Variabilities in the ozone layer due to human causes or sunspot

activity are also hypothesized to increase the incidence of skin cancers, including melanoma [16–18].

Importantly, the incidence rate has leveled off in the past five years in the high risk countries of USA, Canada, Australia, and New Zealand. The reductions in incidence have been found to be directly related to birth cohorts and not calendar period, leading to the hypotheses implicating differences in generational UV exposure, likely precipitated by public health and awareness efforts. Despite these improvements, incidence is still increasing in much of Europe [19, 20].

Genetics, Molecular Biology, and Targeted Therapies

A familial history of melanoma does lead to an increase in risk, even accounting for the fact that 90% of melanomas are sporadic and not directly due to germline genetic errors. One's risk is increased 2.62-fold if a parent and 2.92-fold if a sibling has had melanoma [1]. Of the 10% that are directly familial, several genetic loci are involved in the susceptibility. The primary ones are cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase inhibitor 2A (CDKN2A). *CDKN2A* gene encodes for two tumor suppressors that are cell cycle inhibitors: p16 and p14. Protein p16 inhibits CDK4/6, leading to cell cycle arrest in G1 phase. Protein p14 directly interacts with the master tumor suppressor p53 and plays a role in induction of apoptosis. If *p16* is mutated or silenced, CDK4/6 can phosphorylate and inactivate the tumor suppressor Retinoblastoma protein (Rb), allowing the cell to progress in the cell cycle. If *p14* is also, the cell has diminished capacity to apoptose by action of p53. Similarly, *CDK4* can be mutated to disallow binding of p16, but this mutation is rarer. If a person carries the *CDKN2A*

mutation, the risk of developing cutaneous melanoma is at 30% by age 5 and 67% by age 80. Up to 10% of all patients with multiple primary melanomas also carry this mutation [1, 3, 21]. In sporadic melanomas, *CDNK2A* is often silenced by epigenetic promoter methylation, amplification of *CDK4*, or amplification of *cyclin D*, among others [1]. Loss/silencing of *p16* is associated with a poor prognosis [22].

Furthermore, the red hair color phenotype (red hair, fair skin, freckles) increases melanoma risk with UVR exposure, and is associated with variants in the melanocortin-1 receptor gene (MC1R). MC1R signals in an important pathway within melanocytes to control the relative proportions of eumelanin (brown/black pigment) and pheomelanin (red/yellow pigment). *MC1R* genetic variants (and rarely null mutants [23]) cause a reduction of eumelanin that directly leads to UVR sensitivity and higher risk of melanoma and other skin cancers, and these mutations are also associated with *BRAF* mutations [1, 24, 25]. Xeroderma pigmentosum, a rare heritable disorder in which DNA repair mechanisms are dysfunctional, results in extremely high rates of melanoma and all skin cancers at young ages [1].

The mitogen-activated protein kinase (MAPK) pathway is a signaling pathway involved in regulating cell growth and survival, and it includes some key kinases such as RAS, ERK, MEK, and BRAF. This signaling pathway is aberrantly regulated in many cancer types. In melanoma, mutations in the *BRAF* and *NRAS* genes are very common, with about 80% of cutaneous melanomas on skin that is not chronically sun damaged have mutations in either *BRAF* (59%) or *NRAS* (22%). The vast majority (~80%) of *BRAF* mutations are the V600E single amino acid mutation that renders the kinase

constitutively active. Most of the other mutations are at the same locus, defined by V600K. Germline mutations in *BRAF* have not been identified [1, 26, 27].

All currently licensed targeted therapies for melanoma affect the MAPK pathway, especially BRAF. Vemurafenib selectively inhibits V600E *BRAF* mutant melanoma and has been FDA approved since 2011 after phase III trials showed a 63% reduction in risk of death compared to chemotherapy [1, 28]. Dabrafenib inhibits all V600 *BRAF* mutations to different extents and was FDA approved for treating V600E *BRAF* melanoma in 2013 after the phase III trial showed enhanced efficacy over chemotherapy [1, 29]. The MEK inhibitor trametinib has also shown efficacy as a monotherapy in patients who have not utilized other BRAF inhibitors[30], and has been shown to enhance efficacy in a combination therapy with Dabrafenib [31].

The phosphatidylinositol-3 kinase (PI3K) signaling pathway, also involved in general cell growth and survival, can be affected a few different ways in melanoma. The pathway is activated by NRAS among others, includes Akt as the primary effector protein, and PTEN as a tumor suppressor that inhibits Akt. As mentioned, mutations in *NRAS* are relatively common, as are functional losses of *PTEN* and overexpression of *Akt* [1, 32]. In addition, the receptor tyrosine kinase KIT (CD117) undergoes activating mutations or copy number amplifications in 28% of melanomas caused by chronic sun exposure. KIT is involved in melanocyte development, proliferation, differentiation, migration, and survival [1, 33]. Transcription factor MITF is likewise involved in melanocyte development and pigment formation. *MITF* has been found to be a melanoma oncogene, with genetic amplification found in 10-20% of primary melanomas and not found in benign nevi [1, 34]. Finally, apoptotic pathways are dysfunctional in most

melanomas. Melanomas can evade both extrinsic and intrinsic apoptotic pathways by downregulating death receptors (Fas, TRAIL) and the cytochrome c-associated factor Apaf-1, respectively [1, 35]. Drugs directed at mitigating the effects of many of these specific mutations are currently under investigation, but none are yet available in the clinic[1].

Section 2: Melanoma Immunology

Anti-tumor effector responses

Before analyzing the immunology of melanoma, a few immunological concepts require clarification. T-cells recognize antigen in the context of Major Histocompatibility Complex (MHC) molecules – class I, recognized by CD8+ T-cells and expressed on all somatic nucleated cells, and class II, recognized by CD4+ T-cells, and expressed on specialized antigen presenting cells (APCs). Human Leukocyte Antigen (HLA) proteins are the human equivalent of the mouse H2 proteins, and they have identical functionality. For purposes here, the protein will be called MHC. T-cells require processing of proteins into immunogenic peptides that can fit into the groove of the MHC molecule that is then presented on the surface of the cell to meet potential matching T-cell receptors. In order for a T-cell clone to be able to generate a response against a protein, the protein must have peptide segments compatible with the presentation process. Furthermore, with tumors that are derived from self-tissues, issues of tolerance arise. During T-cell development, most T-cells specific to self-antigens are either eliminated or changed into natural regulatory T-cells (nTregs) in the thymus. Peripheral tolerance is discussed on its own later. In summary, in order to elicit a natural or vaccine anti-tumor response, the

antigens must be immunogenic to T-cells and not restricted by tolerance mechanisms [36].

Melanoma is the primary example of an immunogenic tumor. As such, a great deal of work in understanding tumor immunity has been tested in melanoma patients and model systems. Clinically, the productive involvement of the immune system is apparent from better prognoses alongside immune-mediated vitiligo development and from primary melanomas sometimes having halos of depigmentation, regressing spontaneously, and exhibiting infiltration of specific lymphocytes[37–39]. It has also been shown that melanomas, likely due to their genesis from DNA-damaging UVR, is the most mutagenized of all human cancers [40, 41]. The plethora of mutated protein antigens, also termed neoantigens, that T-cells can target are hypothesized to be the reason for the tumor's potential immunogenicity[40]. These observations, among others, provided strong evidence that T cells were the primary players in melanoma immunogenicity. Initial studies found antigens on patient melanomas that specifically interacted with CD8+ T-cells [42]. Studies went on to show that patient tumor infiltrating lymphocytes (TILs) that were isolated and expanded *in vitro,* or monoclonal CD8+ T-cell lines originating from patients, were able to recognize, react to, and kill melanoma cell lines and cultured melanocytes [37, 43–47].

The above research points to not only T-cells being the primary immune effector mechanism for tumor control, but also to CD8+ cytotoxic T lymphocytes (CTL) as the most important against melanoma [37, 48]. While CTLs are essential to an anti-tumor response, CD4+ helper T-cells (Th) have also been shown to play a role. In order for Th cells to have a direct effect on tumor cells, the interaction must include MHC-II, which is

usually not present on non-immune cell types. However, expression does occur on several tumor types, including melanoma, even as MHC-I expression is being lost [49]. Furthermore, it has been shown for many cancer cell lines that MHC-II expression can be induced by the T cell pro-inflammatory cytokine interferon gamma (IFN-γ), which is the primary effector cytokine of CD4+ helper type-1 (Th1) T cells [50]. It has also been shown that several melanoma antigens are successfully presented by MHC-II, and it has been hypothesized that successful vaccines may need to induce both CD4+ and CD8+ T-cells [45].

CD4+ T-cells can also "help" other cell types fight the cancer. It has been established that effective CD8+ T-cell anti-tumor responses rely on CD4+ T-cell help during the immune induction/activation phase. The cytokine IL-2 produced by CD4+ T-cells enhances CD8+ T-cell proliferation and activation. Further, CD8+ T-cell memory responses require CD4+ T-cell help [51]. Early vaccines reported that both T-cell types played a significant role[52]. A series of studies by the Greenberg group provided direct evidence for a CD8+ T-cell-independent role for CD4+ T-cells, hypothesized to be due to a CD8+ T-cell-independent immune reaction induced by cytokines secreted by CD4+ T-cells leading to activation and recruitment of APCs and innate cells, especially macrophages[53–56].

Antibody responses have also been found to play a role in anti-tumor immunity. Antibodies specific to gangliosides induced by a vaccine correlated positively with patient survival in one study[57]. It has also been shown that vaccinating dogs against human tyrosinase provides therapeutic efficacy and induces strong antibody responses in the dogs[58]. Serum screens routinely found melanoma-specific antibodies[59, 60]. One

specific glycoprotein 75 vaccination protocol induced protection that was mediated by antibody-dependent cellular cytotoxicity by inflammatory cells, specifically requiring FCγ I and III receptors[61]. It was also found later that immune inhibitory FCγ receptors such as FCγRIIB modulated these immune responses[62]. On the whole, however, it is still believed that CTLs are the most important effector arm of the immune response against tumors.

NK cells were first discovered due to their ability to non-specifically kill tumor cells[63]. Human melanoma cell lines have been shown to have reduced surface expression of MHC-I. Lack of MHC-I can remove inhibitory signals effectively activating an NK cell attack, and autologous NK cells have been found to kill melanoma cell lines in cases of both an absence or reduction of MHC-I surface expression[37, 64]. These cells can prevent melanoma outgrowth in severe combined immunodeficient (SCID) mice given NK cells simultaneously with tumor challenge[65]. Having higher innate cytotoxicity in peripheral blood results in lower overall risk of developing cancer[66]. However, first attempts at creating an adoptive cell transfer based NK therapy failed to show efficacy[67, 68]. This was attributed to poor homing ability of transferred cells or to immunosuppression from the tumor microenvironment [37, 69]. Overall, NK cells are known to play a role in cancers and melanoma but have not yet been successfully harnessed into an immunotherapy.

Tumor Associated Antigens – gp100

In order for healthy tissue to be transformed into cancerous tissue, errors in the regulation of cellular pathways must accumulate. One primary way this occurs is by the

mutation or overexpression of proteins involved in those pathways. Any mutation or genetic alteration that emerges as the result of selective pressure during tumorigenesis are "driver" mutations [70]. Driver mutations are at least partially responsible for the cancerous phenotype. In the course of tumorigenesis, the more mutagenic tumors have unstable genomes that lead to the mutation or aberrant expression of other proteins not involved in tumorigenesis. These are called "passenger" mutations [70]. Driver mutations were of clinical interest because of the theoretical potential for arresting cancer progression by blocking them with a targeted drug. This is the case in melanoma with BRAF inhibitors [27]. However, driver mutations in melanoma are difficult to distinguish from the abundance of UV-induced passenger mutations[71].

In immunotherapy, the goal is to induce immunity against a driver mutation or a uniform passenger mutation. These mutations and/or aberrantly expressed proteins are termed tumor-associated antigens. Tumor-associated antigens can be divided into three basic groups. The first group is comprised of the mutated neoantigens. A neoantigen is formed when protein-coding genes are mutated directly from carcinogenesis or indirectly from cancer-associated genetic instability. Neoantigens can provide "new" epitopes that are recognized as foreign. T-cells specific for these antigens are not subject to central tolerance, as the epitopes appear foreign and not as "self" [72]. However, these mutations are not always immunogenic [73].

The second group is made up of the cancer/testis antigens. These proteins are normally only expressed in germline cells but are aberrantly expressed in cancer cells. T-cells specific to these antigens are not generally affected by central tolerance, as germline cells do not express MHC molecules and are located in immune-privileged sites in the

body. The Melanoma-Antigen Gene (MAGE) family are prime examples of cancer/testis antigens [73].

The third group consists of the tumor-differentiation antigens. These proteins are specific for the cancerous tissue type and are overexpressed or aberrantly expressed in cancer. While T-cells specific to these antigens are restricted by central tolerance mechanisms, it is still possible to generate immune responses against them, both naturally and by vaccinations. In melanoma, gp100 is in this category, among others [73]. Table 1.2 lists most of the known shared melanoma antigens, including the antigen used for the studies herein: gp100.

Gp100 is synthesized initially as a 70 kDa protein that then undergoes immediate-early glycosylation in the endoplasmic reticulum and processing in the *cis*-Golgi. Most gp100 is quickly sorted to stage I early melanosomes prior to *trans*-Golgi processing, resulting in a 100 kDa sized protein[74]. Figure 1.1 shows the potential processing events of the protein. Proteolytic cleavage and refolding of gp100 has been found to be essential to the progression of amorphous and immature stage I melanosomes into fibrillar, ovoid, and organized stage II melanosomes[75]. Melanosome stages III and IV further mature to produce and deposit melanin[76]. Importantly, gp100 is expressed primarily in melanocytes, with some expression found in the retina and substantia nigra, but not found in any other tissue type tested. Also, gp100 transcripts were found in high abundance in specimens representing all possible stages of melanoma, contrasting with much lower amounts detected in normal melanocytes[77]. Gp100 is also present in the B16 mouse melanoma model. In B16, the protein is 626 amino acids in length, with 79.7% sequence homology as compared to the human gp100. Expression as detected by Western and

Northern blots shows melanocyte lineage specificity consistent with the human gp100[78].

Peripheral Tolerance/Tumor Microenvironment

Cancerous melanocytes are just one of many cell types within a melanoma tumor mass. Other abundant cell types include keratinocytes, fibroblasts, endothelial cells, and immune cells, all of which play a role in tumor progression[79]. Immune cells in the microenvironment are able to suppress natural and induced immune responses by mechanisms of peripheral tolerance, summarized in figure 1.2. Some main mechanisms that have been shown to play a role in melanoma include the extrinsic suppression of CTLs by CD4+ CD25+ FoxP3+ regulatory T-cells (Treg), metabolic dysregulation by indoleamine-2,3-dioxygenase (IDO), and engagement of the T-cell inhibitory receptor Programmed cell Death -1 (PD-1) with its ligand PD-L1[80–84]. Interestingly, it has been shown that these factors are not intrinsic to the tumor but are driven as a counter-response to anti-tumor inflammatory immune responses. This represents a normal component of immune regulatory pathways that have evolved to reduce excessive inflammation in response to immune stimuli. One study showed that IDO and PD-L1 expression and Treg accumulation were due to CD8+ CTL presence and secretion of IFN-γ[80]. The primary method of T-cell immune evasion by melanomas is thought to be through the loss or down regulation of class I MHC expression, which can be detected in up to 67% of metastatic melanoma cases[85, 86]. Natural Killer cells, normally activated when class I MHC is down-regulated, are suppressed in melanoma microenvironments by action of immune mediators IDO and prostaglandin E2 and by

inhibition of NK activating receptors such as NKG2D[87]. In addition to these, other immune suppressive mechanisms include down regulation of peptide transporters and antigen processing machinery, down regulation of tumor antigens, and local synthesis of immunosuppressive cytokines such as IL-10 and TGF-β by tumor infiltrating cells[85, 88, 89].

IL-10

Interleukin-10 (IL-10) is a heterodimeric cytokine belonging to a family that includes IL-22, IL-27, and others. The receptor for IL-10 is in the type II cytokine receptor family, consisting of two chains (IL-10R1/2) that associate with Janus family kinases JAK1 and TYK2, which activate the STAT3 transcription factor [90]. IL-10 is produced by a variety of immune cell types: activated macrophages, activated dendritic cells, Tregs, regulatory B cells, Th1, and T helper type 2 (Th2) cells. IL-10's primary function is thought to be a negative feedback regulator of innate and cell-mediated responses, as it is produced by either activated cells or regulatory cells functioning to repress effector cells, including activated macrophages and dendritic cells.

Specifically, IL-10 inhibits production of the Th1-skewing cytokine IL-12, which is also a critical stimulus for IFN-γ secretion. IFN-γ plays an essential role in Th1 effector function, responsible for cell-mediated immune reactions against intracellular insults. IL-10 suppresses these functionalities (ability to inhibit IFN-γ production led to IL-10's initial identification) [90]. IL-10 also inhibits the expression of costimulatory molecules and class II MHC molecules on dendritic cells and macrophages, thus inhibiting T-cell activation or persistence of ongoing effector responses [90]. IL-10 also curbs production

of tissue damage mediators like reactive oxygen species and matrix metalloproteinases by activated macrophages [91]. IL-10 has also been found to inhibit expression of MHC-I [92] and inflammatory mediators, such as TNF-α[93], interferon response genes[94], and type-I interferons themselves (i.e. IFN-α) [95, 96].

In addition, IL-10 is thought to be especially important for controlling prolonged immune reactions. IL-10 knockout mice develop colitis, and a mutation in the human IL-10 receptor results in severe colitis that develops in infancy[90]. IL-10 plays a significant role in several immune-mediated diseases. In cases of IL-10 deficiency, persistent immune activation can cause diseases such as psoriasis, rheumatoid arthritis, and the aforementioned inflammatory bowel diseases such as Crohn's disease[97]. IL-10 deficient mice develop lethal intestinal inflammation under normal growth conditions caused by inflammatory immune reactions to normally benign commensal bacteria[98]. These mice also succumb by septic shock to normally non-lethal challenges with lipopolysaccharide[99]. Overproduction of IL-10 is associated with active lesions of cutaneous leishmaniasis[100] and the progression of melanomas[101] and EBV-associated lymphomas[102].

Conversely, IL-10 has been shown to also elicit pro-inflammatory responses by enhancing granzyme and IFN-γ capabilities of CTLs and NK cells under certain conditions, and it has been well established that IL-10 promotes proliferation, differentiation, and antibody production by B-cells [91]. Consequently, IL-10's ability to enhance antibody production plays a key role in lupus etiology. IL-10 is significantly enhanced in murine models of lupus and in the human disease, and neutralizing IL-10 has

shown clinical efficacy in treating lupus symptoms[103]. IL-10 is a complex cytokine with varied effects depending on the cell type and the environmental context.

Section 3: Applied Immunology

Mouse Model

The model utilized in the following studies is exclusively the B16F10 (B16) mouse melanoma model. B16 was a spontaneous tumor that arose in the C57Bl/6 genotype of mice. The cells have been continuously in culture since then, and implantation in syngeneic mice provides a robust model without issues of xenotransplantation. A standard dose in the range of $5x10^4$ to $3x10^5$ cells administered subcutaneously to C57Bl/6 mice will result in consistent melanoma growth in these mice with fully intact immune systems. Untreated mice will succumb to the tumor within a few weeks. Due to the mutagenic nature of the cell line, each laboratory strain may have slight genotypic differences that can influence growth rate, so it is essential to establish a laboratory-standardized protocol.

Table 1.3 highlights the similarities and differences between this model and the typical human melanoma. In general, it is more difficult to elicit an effective immune response to the mouse tumor compared to the human cancer due to lower MHC-I expression and higher resistance to IL-2 treatment. Logically, an immune response seen in the B16 model will likely be stronger in humans. Another difference is the course of progression. Even with Stage IV metastatic cancer, humans can survive for months to a few years, whereas mice will succumb reliably in well under one month, usually by three weeks. Importantly, the model has similar patterns of expression of antigens that can be

recognized by T-cells. The B16 melanoma model is therefore a more restrictive but reasonable model for studying immunological interventions against melanoma [104].

Melanoma Clinical Immunotherapies

The idea of cancer immunotherapy is not new, and has its roots in 1890 when William Coley directly injected streptococcal bacteria (termed Coley's toxins) into inoperable tumors in an attempt to stimulate immune-mediate tumor regression. This intervention resulted in highly variable and difficult to reproduce results, but it also had some real successes [105]. Similarly, Ehrlich in 1909 proposed the first formulation of the cancer immune surveillance hypothesis, now widely supported [106]. However, these hypotheses were widely disregarded until further evidence of tumor immune reactivity emerged in the 1980's and 90's [107]. Some landmark studies include the findings that immunocompromised mice were more likely to develop carcinogen-induced tumors [108], that immunogenic cancer antigens exist [48], and that dendritic cell activation and reinfusion can induce T-cell mediated regression of melanoma metastases [109].

The first wave of melanoma immunotherapies included treatment with high-dose IL-2, which had been shown in preclinical studies to expand tumor-specific T-cells [107]. Clinical studies showed 6-7% complete remission and 10% partial remission rates in metastatic melanoma patients [110, 111], and the monotherapy was approved by the FDA in 1998 [107]. However, high-dose IL-2 monotherapy carried only moderate results combined with high toxicity resulting in mild benefit without significant improvement in overall survival [107, 112]. In the few patients that did achieve complete remission, durable remission was common, a hallmark of immunotherapies [107].

The second and more efficacious treatment of this first wave of immunotherapies was interferon adjuvant therapy. Adjuvant therapy is given to patients without metastases after surgical resection of primary tumor to prevent recurrence or the emergence of undetected metastases. IFN-α2b treatment has shown benefit in early stage II/III melanomas [113], and clinical studies elucidated that adjuvant IFN therapy significantly increased disease free survival and sometimes overall survival [107] . IFN adjuvant therapy became the first immunotherapeutic agent to show significant benefits in survival in a phase III randomized controlled trial, and is to this day a part of the standard of care for patients with advanced stage II or stage III melanoma [2, 107].

The second wave of immunotherapies focused on targeting T-cells. As mentioned above, T-cells play an essential role in anti-tumor immune responses, but are restricted by mechanisms of tolerance. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a negative regulator of activated T-cells that competitively interacts with the costimulatory B7 molecules on APCs, displacing the primary and essential second signal for T-cell activation: CD28. This results in reduced activation and attenuated clonal expansion [114]. By blocking CTLA-4 with a specific antibody (clinical name: ipilimumab), the negative pathway is blocked, allowing for continuing activation of tumor-reactive T-cells. This ‘releasing the brakes’ therapy was termed an immune checkpoint inhibitor. A large Phase III trial including patients with unresectable stage III/IV melanoma showed that median overall survival was increased from 6.4 to 10 months, but treatment was associated with severe immune-mediated adverse events [115].

A second immune checkpoint inhibitor revolutionized cancer therapy: anti-programmed cell death protein 1 (PD-1). Anti-CTLA-4 works on the level of T-cell

activation, but anti-PD1 works on activated and differentiated T-cell effector cells that are on the periphery interacting with the tumor cells. PD-1 is expressed only on activated cells. When PD-1 on T-cells interacts with its ligand, PD-L1, expressed on tissue exposed to inflammation, especially tumors, the effector T-cell's functions are restrained. This serves as a direct feedback loop to curb chronic inflammation at peripheral sites [114]. Blocking this interaction allows effector T-cells on the periphery of the tumor to continue their anti-tumor activities.

There are two currently approved anti-PD-1 drugs: pembrolizumab and nivolumab. A summary of clinical trial results can be seen in Table 1.4. All measures of efficacy for either version of anti-PD-1 were much higher than ipilimumab. In a first-line therapy setting, objective response percentage increases from 12-19 (ipilimumab) to 34-40 (pembrolizumab) or 25-40 (nivolumab). A trial combining nivolumab and ipilimumab pushed the objective response up to 40-61%. Median survival increased from 11 months (ipilimumab) to 17 months (nivolumab) to 40 months if you combine the two[116]. Overall survival is also enhanced with anti-PD-1 drugs compared to ipilimumab. Ipilimumab has a three-year survival of 25%. Nivolumab treatment results in a 43% two-year survival rate, and combining nivolumab with ipilimumab leads to 79% survival after two years[116]. In summary, immune checkpoint inhibitors have produced exciting clinical results, and studies are actively ongoing to try and improve on these results.

Melanoma Vaccines

In order to have a successful vaccine, one needs to consider the antigens utilized and the context in which they administered. Cancer vaccines carry different requirements

than those for infectious diseases (ID). First, the proper antigen(s) need to be identified to target with a vaccine. While this is true of all vaccines, it is more difficult to find targetable antigens in the cancer context due to factors such as tolerance, mutagenicity, and population heterogeneity. Second, cancer vaccines require stimulation of T-cells, especially CTLs, and, with the exception of neoantigens or oncogenic viral antigens, must be able to elicit immune responses to antigens that are poorly immunogenic. Third, adjuvants must recruit and activate APCs and especially dendritic cells within the proper inflammatory cytokine context to elicit the necessary downstream effector responses. Alum and other approved adjuvants utilized in ID vaccines elicit strong antibody responses but poor CTL responses. Finally, tumor immune suppression represents a major barrier to the development of effective anti-tumor vaccines. Therefore, new strategies are necessary and have been under development[117]. Figure 1.3 summarizes these concepts and provides specific examples of tools currently in development.

First, researchers and clinicians attempted to create peptide/protein vaccines against known melanoma associated antigens discussed earlier, including gp100, TRP-2, tyrosinase, MAGE-1/3, and others. None of them so far have shown significant improvement of overall patient survival[118]. A meta-analysis of small, single arm studies demonstrated an objective response rate across all studies of only 2.9% (9 out of 323). This rate was only slightly improved to 5-10% when enhanced by multipeptide strategies, multiepitope strategies, and inclusion of immune adjuvants such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-2[118]. Larger, comparative phase II and III trials confirmed the marginal efficacy of these vaccines. A phase II trial investigated a multi-epitope vaccine combined with either GM-CSF or IFNα

adjuvants. T-cell responses were seen in 35% of patients, and if stratified, the immune responders did have improved survival from 13.4-21.3 months. However, only six objective responses were observed among 121 patients[119]. A phase III trial tested a modified gp100 vaccine combined with IL-2 versus IL-2 alone. The trial showed modest increases in the objective response rate (6 to 16%) and progression-free survival (1.6-2.2 months), but only an increased trend in survival (11-17 months) [120]. A lack of specific T-cells and lack of correlation between gp100 specific T-cells and clinical response suggested lack of efficacy[118]. This was confirmed by more phase III trials where gp100 vaccine showed little additive effect when combined with a different schedule of high dose IL-2 [121] or with anti-CTLA-4 treatment[115].

Another method under investigation is the use of whole cancer cell-based vaccines. These carry a theoretical advantage because they are not restricted to a few pre-determined epitopes. All tumor-associated epitopes, including mutated neoepitopes, are a part of the vaccine[118]. Irradiated, autologous metastatic melanoma cells engineered to secrete GM-CSF provided clinical benefit to patients[122], and potential synergy with anti-CTLA-4 therapy[118]. The M-Vax vaccine idea takes autologous irradiated melanoma tumor cells and adds treatment with a hapten to augment immune responses. A phase II trial showed enhanced survival between responders and non-responders, and a phase III trial is ongoing[123]. In comparison, a phase III trial of an allogeneic whole-cell vaccine originating from three melanoma cell lines (Canvaxin) was stopped early due to lack of efficacy[118].

One way to elicit strong CTL responses is to utilize viral vectored vaccines. In melanoma, a herpes virus engineered to express GM-CSF and to lyse tumor cells,

OncoVex now T-VEC, is licensed for use. Promising phase II results with an overall response rate of 26%[124] led to a recent phase III trial comparing T-VEC to GM-CSF. Durable response rates (2.1-16.3%) and overall response rates (5.7-26.4%) were significantly higher, but with only marginal increase in overall survival (18.9-23.3 months). Differences in durable response rates were more pronounced in patients of stage IIIB/C (0-33%) and stage IVa (2-16%) but were indistinguishable in stage IVb-c patients (3-7% vs. 3-4%)[125]. The benefits were enough to get FDA approval, and this treatment is a second line therapy utilized in patients who do not respond to checkpoint inhibitors or other special circumstances[126].

Many other novel vaccine modalities and modifications are in development, aimed at enhancing each of the three facets of figure 1.3. In this dissertation, a novel melanoma vaccine will be analyzed. This vaccine utilizes a promising mode of delivery that acts as its own adjuvant: electroporation [127]. The novelty of the DNA vaccine lies in the chemokine macrophage inflammatory protein 3α (MIP3α) fused to antigen of choice, gp100, as diagrammed in figure 1.4. MIP3α is also known as CCL20 and is a chemokine that binds to CCR6 located primarily on immature dendritic cells. The chemokine acts to target nascent vaccine protein to infiltrating immature dendritic cells that will then mature and initiate both CD4+ and CD8+ T-cell responses, as is summarized in figure 1.5 [128–130]. This dissertation will further also assess the interaction between vaccination and blocking of a major immunosuppressive cytokine in the tumor microenvironment: IL-10. Finally, this dissertation will conclude with a look into the future, with a special focus on the incorporation of neoantigens into this vaccine platform.

Chapter 1: Tables

Table 1.1: Clinical characteristics of melanoma, adapted from [1, 2]

Stage	Description	Common Treatments	5-year survival %	10-year survival %
IA	The melanoma is less than 1.0 mm thick. It is not ulcerated and has a mitotic rate of less than 1/mm^2.	Surgery	97	95
IB	The melanoma is less than 1.0 mm thick and is ulcerated or has a mitotic rate of at least 1/mm2, OR it is between 1.01 and 2.0 mm and is not ulcerated.	Surgery	92	86
IIA	The melanoma is between 1.01 mm and 2.0 mm thick and is ulcerated, OR it is between 2.01 and 4.0 mm thick and is not ulcerated.	Surgery with sentinel node biopsies	81	67
IIB	The melanoma is between 2.01 mm and 4.0 mm thick and is ulcerated, OR it is thicker than 4.0 mm and is not ulcerated.	Surgery with sentinel node biopsies and option of adjuvant therapy	70	57
IIC	The melanoma is thicker than 4.0 mm and is ulcerated. It has not been found in lymph nodes or distant organs	Surgery with sentinel node biopsies and option of adjuvant therapy	53	40
IIIA	The melanoma can be any thickness, but it is not ulcerated. It has spread to 1 to 3 lymph nodes near the affected skin area, but the nodes are not enlarged and the melanoma is found only when they are viewed under the microscope	Surgery with lymph node dissection. Adjuvant therapy or other trial therapy to help prevent recurrence	78	68
IIIB	One of three possibilities: 1) The melanoma can be any thickness and is ulcerated. It has spread to 1 to 3 lymph nodes near the affected skin area, but the nodes are not enlarged and the melanoma is found only when they are viewed under the microscope.	Surgery with lymph node dissection Imlygic viral therapy, BCG vaccine, adjuvantal therapy, IL-2, radiation,	59	43

	2) The melanoma can be any thickness, but it is not ulcerated. It has spread to 1 to 3 lymph nodes near the affected skin area. The nodes are enlarged because of the melanoma. 3) The melanoma can be any thickness, but it is not ulcerated. It has spread to small areas of nearby skin (satellite tumors) or lymphatic channels (in-transit tumors) around the original tumor, but the nodes do not contain melanoma.	immunotherapies, targeted therapies, chemotherapies, and combinations thereof are all options at the doctor's discretion		
IIIC	One of three possibilities: 1) The melanoma can be any thickness and is ulcerated. It has spread to 1 to 3 lymph nodes near the affected skin area. The nodes are enlarged because of the melanoma. 2) The melanoma can be any thickness and is ulcerated. It has spread to small areas of nearby skin (satellite tumors) or lymphatic channels (in-transit tumors) around the original tumor, but the nodes do not contain melanoma. 3) The melanoma can be any thickness and may or may not be ulcerated. It has spread to 4 or more nearby lymph nodes, OR to nearby lymph nodes that are clumped together, OR it has spread to nearby skin (satellite tumors) or lymphatic channels (in transit tumors) around the original tumor and to nearby lymph nodes. The nodes are enlarged because of the melanoma.	Surgery with lymph node dissection Imlygic viral therapy, BCG vaccine, adjuvantal therapy, IL-2, radiation, immunotherapies, targeted therapies, chemotherapies, and combinations thereof are all options at the doctor's discretion	40	24
IV	The melanoma has spread beyond	Primary tumors,	15-20	10-15

	the original area of skin and nearby lymph nodes to other organs such as the lung, liver, or brain, or to distant areas of the skin, subcutaneous tissue, or distant lymph nodes. Neither thickness nor spread to nearby lymph nodes is considered in this stage, but typically the melanoma is thick and has also spread to the lymph nodes	enlarged lymph nodes, and metastases are surgically removed where possible. Targeted therapies and immunotherapies are primary options and can be combined with chemotherapy		

Table 1.2: Shared melanoma antigens recognized by T-cells, adapted from Castelli, C., et al.[73]

Antigen	Expression	Mechanism of activation
Tyrosinase	Melanomas and melanocytes	Overexpression
Melan-A/MART-1	Melanomas and melanocytes	Overexpression
TRP1	Melanomas and melanocytes	Overexpression
TRP2	Melanomas and melanocytes	Overexpression
Gp100	Melanomas and melanocytes	Overexpression and abnormal transcription
NA17-A	Melanomas but not melanocytes	Abnormal transcription
TRP2-INT2	Melanomas but not melanocytes	Abnormal transcription
MAGE1	Many tumors and testis	Ectopic expression of a normal gene
MAGE2	Many tumors and testis	Ectopic expression of a normal gene
MAGE3	Many tumors and testis	Ectopic expression of a normal gene

Table 1.3: B16 as a model for human melanoma: similarities and differences, adapted from Overwijk and Restifo[104]

Similarities	Differences
Human melaanomas express at least five different melanoma differentiation antigens, including gp100. All of them can be recognized by CD8+ T-cells from melanoma patients. The mouse homologs of these genes are all expressed in B16 melanoma.	Human melanomas express variable levels of MHC-I. B16 melanomas regularly express low levels of MHC-I
It has been shown that mouse CD8+ T-cells can react to melanoma differentiation antigens, including gp100.	Growing human melanoma biopsies in IL-2 will yield melanoma-specific CD8+ T-cells in ~50% of cases. This will rarely happen in B16
Vitiligo is correlated with favorable clinical prognosis in human melanoma patients. In some treatment models, induced vitiligo in mice can protect from B16 tumor challenge.	Adoptive transfer of TILs can reduce subcutaneous melanoma burden in humans but does not significantly impact B16
Adoptive transfer of gp100-specific CD8+ T-cells can reduce tumor burden in humans. Similar protocols in mice can reduce pulmonary B16 metastases.	Humans can survive for months or years despite melanoma growth. B16 will kill untreated mice within weeks.
Human melanomas can often be induced to express MHC-II by IFN-γ treatments. This occurs regularly in B16.	

Table 1.4: Clinical trials with checkpoint inhibitors. Adapted from Marquez-Rodas, et al. [116]. The following are defined abbreviations of the table: Objective Response (OR); Overall Survival (OS); and Progression-Free Survival (PFS). '(m)' denotes months.

Drug	Study	N	Phase	OR(%)	OS(m)	OS(%)	PFS(m)
Pembrolizumab	KEYNOTE001(B1)	135	1	38			
	KEYNOTE001 (B1+B2+D)	411	1	40			
	KEYNOTE002	540	II	21-25			2.9
	KEYNOTE006	834	III	33-34		68-74 (1yr)	4-5
Nivolumab	Weber	90	I	24			
	Hodi	107	I	30-40	17	40 (3yrs)	
	CheckMate037	405	III	32			4.7
	CheckMate066	418	III	40		73 (1yr)	5
Nivolumab + ipilimumab	Wolchok	53	I	40-53	40	79 (2yrs)	
	CheckMate069	142	II	61			
	CheckMate067	945	III	58			11.5
Ipilimumab	MDX010-20	676	III	11	10	25 (2yrs)	2.7
	D24	502	III	15	11.2	21 (2yrs); 18 (5yrs)	2.4

Chapter 1: Figures

Figure 1.1: Processing and epitope mapping of gp100 as melanosome matures from stage I to stage II, adapted from Yasumoto, et al [74]. The black box denotes signal sequence, black dots are potential N-glycosylation sites, and gray dotted lines are proteolytic cleavage sites. Vaccine peptide spans amino acids 25-235, which includes several glycosylation and cleavage sites, indicating that nascent vaccine peptides may have variable sizes.

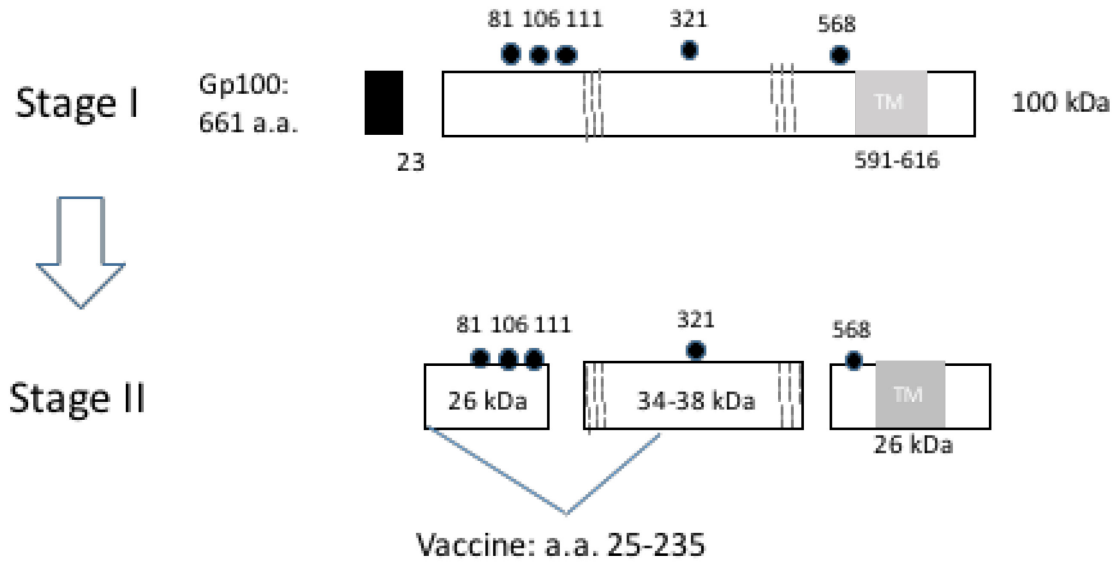


Figure 1.2: Tumor tolerogenic microenvironment, adapted from Croci, et al. [131]. This figure highlights the primary methods of immunosuppression and immune escape utilized by tumors. As noted in the text, melanoma has been shown to utilize each of these methods. The following are defined abbreviations: Indoleamine 2,3-dioxygenase (IDO); regulatory T-cells (Tregs); myeloid-derived suppressor cells (MDSC); transforming growth factor-beta (TGF-β); prostaglandin E2 (PGE_2); cytotoxic T-lymphocyte associate protein 4 (CTLA-4); and programmed death receptor 1 (PD-1).

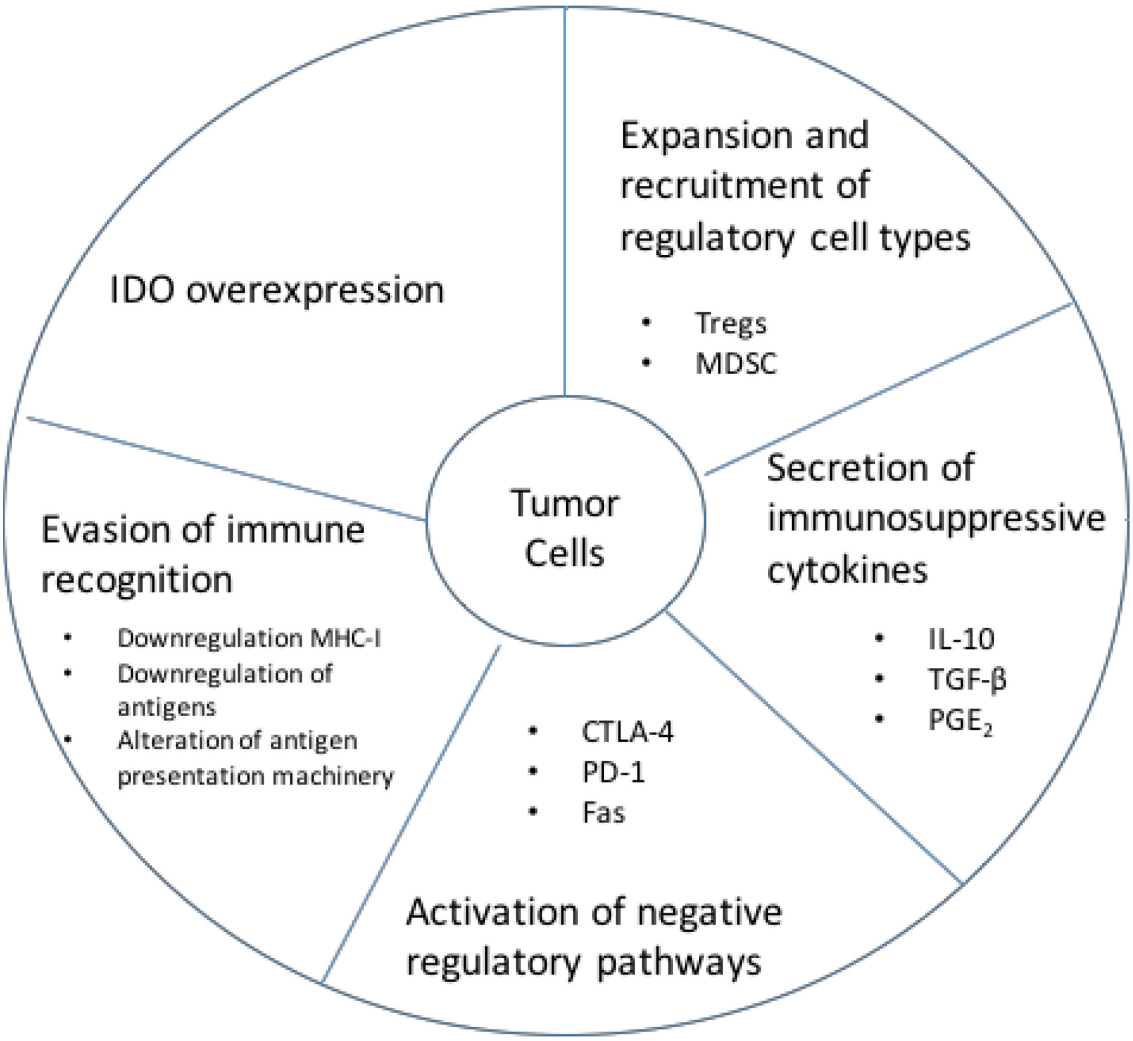


Figure 1.3: Characteristics of successful therapeutic melanoma vaccines, adapted from Ott, et al [118].

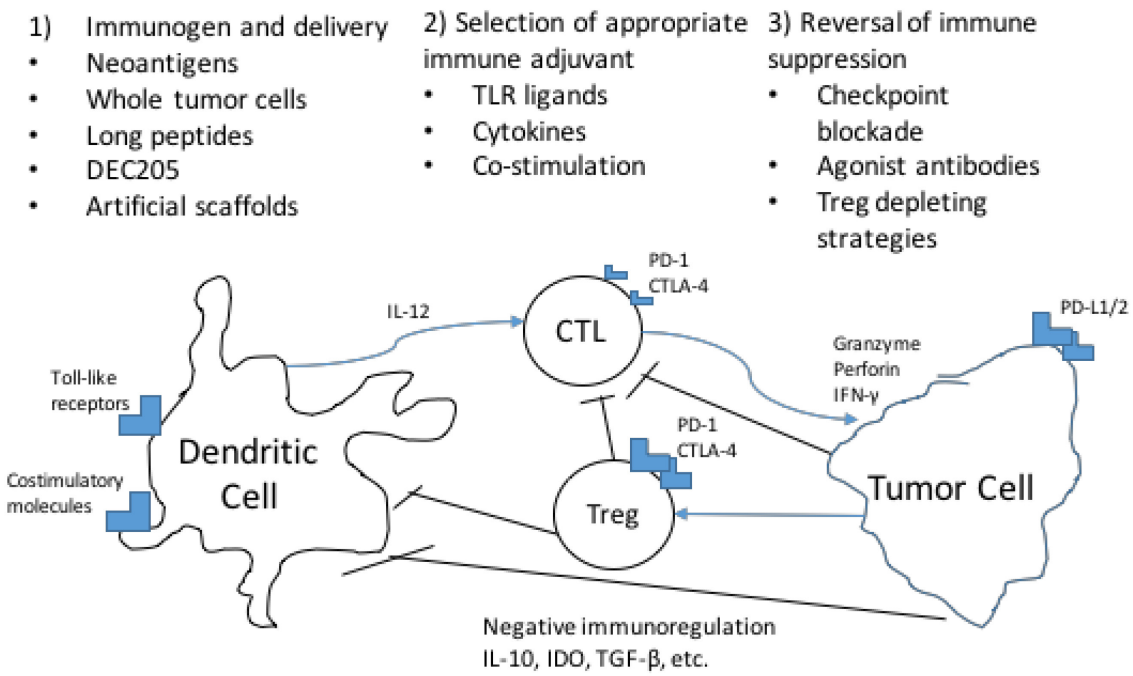


Figure 1.4: Vaccine construct. mMIP3α designates the mouse form of the protein. Sp is a short spacer region of 14 amino acids. The antigen utilized is the human form of gp100, amino acids 25-235. Gp100 is followed immediately by myc and histidine tags for *in vitro* analyses. Bottom construct is dMIP3α-gp100, also known as antigen-only construct. Only alteration is the noted C6S mutation that abrogates chemokine functionality. All possible antigens remain intact. Constructs are inserted into a modified pCMV mammalian expression vector.

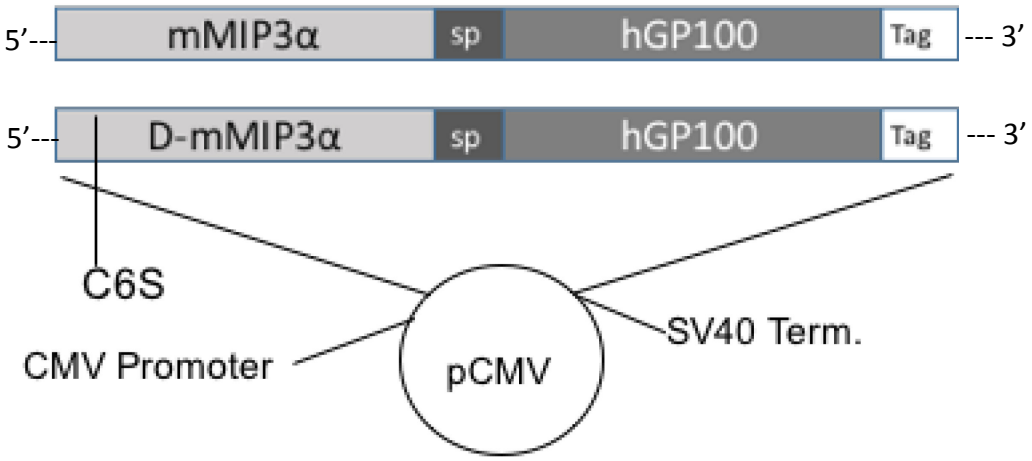
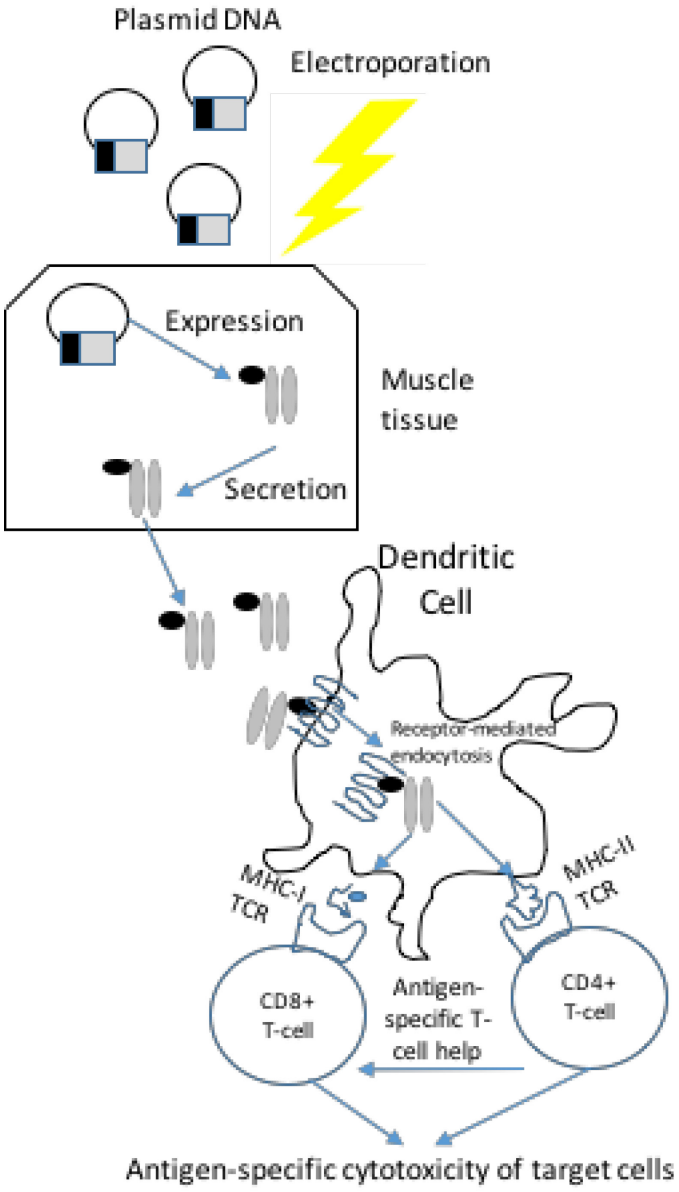


Figure 1.5: Chemokine-fusion DNA vaccine mechanism of action, adapted from Biragyn and Kwak [132]. Black color is representative of MIP3α chemokine and gray is representative of gp100 antigen. The chemokine receptor on the dendritic cell is CCR6.



CHAPTER 2

FUSION OF THE DENDRITIC CELL-TARGETING CHEMOKINE MIP-3 ALPHA TO MELANOMA ANTIGEN GP100 IN A THERAPEUTIC DNA VACCINE SIGNIFICANTLY ENHANCES IMMUNOGENICITY AND SURVIVAL IN A MOUSE MELANOMA MODEL

Introduction

The recent therapeutic successes with checkpoint blockade therapy (e.g. αCTLA-4 and αPD-1) [133] and the identification of cancer neoantigens as potential therapeutic targets[72, 134] have generated renewed interest in the field of cancer immunotherapy. Although only one therapeutic cancer vaccine is currently FDA-approved (Sipuleucel-T[135]), hypothesized synergies between current and future immunotherapies[136] have increased the need for new vaccine platforms that can best address the new immunotherapeutic opportunities.

DNA vaccines offer many advantages as cancer therapies. They generate effector immunity from all three arms of the adaptive immune response, particularly including CD8+ T-cells[137]. They avoid the inclusion of extraneous and possible deleterious antigens that may be components of bacterial or viral-based vaccines[137]. They stimulate innate immunity and avoid issues of safety and practicality associated with various vectors[137]. They can also be readily adapted to novel or mutating antigenic targets, are stable at room temperature, and can be constructed quickly[137]. Clinical trials with a variety of antigens have demonstrated safety and immunogenicity of clinical DNA vaccines [138, 139]. However, initial trials for therapeutic DNA cancer vaccines have all shown limited effectiveness [140]. More recent advances in DNA vaccination modalities have rekindled interest in their potential efficacy for cancer therapy [141, 142]. Of note, DNA vaccines have shown efficacy in animals, with three licensed for veterinary use[143–145].

One of the primary hurdles for DNA vaccines has been their limited potency in the clinical setting [137]. Novel approaches to *in vivo* DNA delivery are being developed

to address this issue. *In vivo* electroporation has been shown in animal models to enhance the breadth and potency of elicited immune response [146–149]. Mechanistic studies have shown electroporation increases DNA uptake, stimulates local inflammation at the vaccination site, and enhances amount of vaccine antigen produced *in situ*[127, 150, 151]. *In vivo* electroporation is currently being utilized in the veterinary clinic as a mode of introducing a hormone into pregnant sows[152] and is currently undergoing clinical trials[153, 154].

Additionally, investigators have been taking advantage of the inherent flexibility of DNA to add immunomodulators to the vaccine construct in order to enhance the efficiency of initiating a specific immune response. Many studies have focused on increasing productive contact of nascent vaccine antigens to antigen presenting cells (APCs), especially dendritic cells (DCs). One approach is to fuse antigens to cytokines such as GM-CSF that can stimulate the development, proliferation, and maturation of DCs and monocytes[155–157]. Another approach is to fuse antigens to chemokines or similar molecules such as CCL5 [158, 159], CCL19 [160], defensins [161, 162], monocyte chemotactic protein 3 (MCP3) [163], interferon inducible protein 10 (IP-10) [163], viral chemokines [164], macrophage inflammatory protein 3α (MIP3α, also known as CCL20) [128–130, 165–167], and others [168] that can recruit and/or target nascent peptides to APCs. MIP3α fusion vaccines have been shown to direct antigen to immature DCs via CCR6 and mediate antigen uptake in a fusion-dependent manner, after which antigens are cross presented by both MHC class I and II, activating significant responses from both CD4+ and CD8+ T cells [128–130].

In the current studies, a DNA vaccine administered by intramuscular electroporation with a construct fusing MIP3α to the melanoma tumor-associated antigen gp100 has been analyzed in a therapeutic vaccination protocol utilizing the B16F10 melanoma mouse model system. MIP3α-antigen DNA vaccine constructs have shown efficacy in a prophylactic melanoma model [130], a therapeutic lymphoma model[128], and a prophylactic malaria model [165]. Here we compare therapeutic MIP3α-gp100 vaccination to a construct with a mutated MIP3α sequence that abrogates its function, effectively providing a gp100 antigen-only vaccine. These experiments show that inclusion of functional MIP3α in the vaccine construct used in the therapeutic protocol enhances immunogenicity, slows tumor growth, and significantly extends survival compared to antigen-only vaccination.

Materials and methods

Animals and Tumor Model

5-6 week old female C57BL/6 (H-2b) mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a pathogen-free micro-isolation facility in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals. All experimental procedures involving mice were approved by the IACUC of the Johns Hopkins University (Protocol number MO13H219). B16F10 mouse melanoma cells were a generous gift from Dr. Arya Biragyn (NIH, Baltimore, MD). Upon giving mice differing doses of our specific line of B16F10, it was determined that $5x10^4$ cells would be our standard dose. 6-8 week old mice were challenged in the left flank subcutaneously with a lethal dose ($5x10^4$ cells) of B16F10 melanoma. Tumor size was recorded as square mm, representing tumor length × width (opposing axes) measured by calipers every 1-3 days. Mice were kept in the study until one of the following occurred: mouse death, tumor size eclipsing 20mm in any direction, or extensive tumor necrosis resulting in excessive bleeding.

Plasmids and Vaccination

Vaccine consisted of purified plasmid DNA in endotoxin-free PBS. The plasmid encoded either MIP3α-gp100 or dMIP3α-gp100 fusion sequence as shown in figure 1.4 and as described[130]. dMIP3α-gp100 vaccine DNA (also called antigen-only) is identical except for a point mutation in the chemokine changing a structurally necessary cysteine to serine (C6S), which abrogates chemokine functionality[130]. Vaccination plasmid was extracted from *E. coli* using Qiagen® (Germantown, Md) EndoFree® Plasmid Maxi and

Giga Kits. Vaccine DNA purity, quality, and quantity were verified by gel electrophoresis, restriction enzyme analysis, Nanodrop® spectrophotometry, and full insert sequencing. Mock vaccinations comprised of endotoxin-free PBS only. DNA injections were administered into the hind leg tibialis muscle. Immediately following injection, the muscle was pulsed using an ECM 830 Electro Square Porator™ with 2-Needle Array™ Electrode (BTX Harvard Apparatus®; Holliston, MA) under the following parameters: 106V; 20ms pulse length; 200ms pulse interval; 8 total pulses. Vaccinations of 50ug/dose were delivered at days noted in figure legends. Prophylactic immunogenicity (Figure 2.1) and efficacy (Figure 2.2) of the vaccine was confirmed, replicating previously reported results in which DNA was delivered by gene gun[130] and establishing that the vaccine does induce a specific response. Immunogenicity here was analyzed by ELISpot as outlined below. As described by others, vaccination for the therapeutic model began on day three[169, 170].

ELISpot

96-well Nitrocellulose plates (Multiscreen® HTS; EMD Millipore, Billerica, MA) were coated with 10μg/ml anti-mouse IFN-γ monoclonal capture antibody (BD Biosciences, San Jose, CA) and incubated overnight at room temperature. Plate was washed, media supplemented with IL-2 was added, and then the plate was incubated at 37°C until ready for use. EL-4 C57Bl/6 lymphoma line was utilized as antigen presenting cells (APCs). $3x10^7$ EL-4 cells were incubated with 10^{-6} M peptide for 45 minutes at 37°C and were then irradiated for 40 minutes, washed, resuspended in media+IL-2, and added to plate at $1x10^5$ cells/well. Spleens were sterilely harvested, ground, filtered through a mesh,

treated to remove red blood cells, washed, and resuspended in media+IL-2. Splenocytes in suspension were counted and then added to the plate at two-fold dilutions, with final cell counts being $2.5x10^5$, $1.25x10^5$, and $6.25x10^4$ cells per well. Plate containing capture antibody, irradiated peptide-pulsed APCs, and splenoctyes was incubated at 37°C/5%CO_2 for 24 hours. Plates were washed and then given 2μg/ml biotinylated anti-mouse IFN-γ monoclonal detection antibody (BD Biosciences, San Jose, CA) and incubated for 2 hours at room temperature. The plates were washed, streptavidin-HRP (BD Biosciences, San Jose, CA) was added, and plates were incubated for 1 hour at room temperature. After washing, AEC Final Substrate Solution (BD Biosciences, San Jose, CA) was added, spot development was monitored and stopped by distilled water after approximately 30 minutes. Plates were dried and spots were counted manually.

Transfection and Protein Detection

Ability of construct to produce full-length protein was assessed by transfecting pDNA into HEK-293T cells by Lipofectamine® (Invitrogen; Carlsbad, CA) utilizing standard manufacturer's protocol. Cells were harvested after 48 hours of incubation and lysed. Western Blots were performed on the lysate using laboratory protocols utilizing a Bio-Rad (Hercules, CA) Trans-Blot® SD Semi-Dry Transfer Cell to transfer proteins onto nitrocellulose membranes. The primary antibody used was mouse anti-human Myc for construct visualization and mouse anti-human β-actin for loading control at 1:5000 dilution, and the secondary antibody used was alkaline-phosphatase conjugated goat anti-mouse antibody at a 1:1000 dilution (all antibodies from Jackson ImmunoResearch Laboratories; West Grove, PA). Vaccine DNA was confirmed to produce specific protein

products in a mammalian system as detected by Western blots against the myc tag present at the 3' end of the construct after transfection into Hek-293T cells (Fig 2.3).

In Cell ELISA

Humoral immune responses to the vaccine were tested by an In-Cell ELISA assay to detect overall response to native B16F10 proteins, including gp100. The studies utilized the standard protocol for In-Cell ELISA from Abcam® (Cambridge, UK). In brief, wells of tissue-culture treated 96-well plates were seeded with $5x10^4$ B16F10 cells and were allowed to adhere for 3-4 hours at 37°C. Adherence was verified by microscopy before proceeding. Cells were fixed, incubated with serum or primary control antibody (rabbit anti-gp100 ab137078 [Abcam, Inc.; Cambridge, UK]) at varying dilutions overnight at 4°C, blocked with 2% BSA, and then incubated with peroxidase-conjugated goat anti-mouse IgG (serum) or goat anti-rabbit IgG(control) (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:5000. Wells were developed for 1 hour using ABTS® ELISA HRP Substrate (KPL, Gaithersburg, MD). The data were collected using the Synergy™ HT (BioTek Instruments, Inc. Winooski, VT).

Extraction of Splenocytes and TILs

Spleen and tumor cell suspensions were prepared by grinding sterile excised tissue between the frosted ends of microscope slides and then passing the tissue through a sterile 60 μM mesh. Splenocytes were processed by lysing red blood cells and washing with sterile PBS. Tumor lysate was washed with sterile PBS, and tumor-infiltrating lymphocyte (TIL) fraction was enriched by Lympholyte®-M Cell Separation Media

(Cedarlane®, Burlington, NC) according to the manufacturer's protocol. Prior to use all cells were counted by a Z1™ Coulter Counter® (Beckman Coulter, Inc.; Brea, CA) and/or a hemocytometer with Gibco™ Trypan Blue solution 0.4% (Life Technologies, Carlsbad, CA).

Intracellular Cytokine Staining and Flow Cytometry

Enriched splenocytes or TILs were seeded onto Falcon® Multiwell 24-well tissue culture treated plates (Corning, Inc.; Corning, NY) at $1x10^6$ cells per well (or all cells if total is less) and stimulated for 3-4 hours at 37°C with known immunodominant $gp100_{25-33}$ (KVPRNQDWL) peptide or control HA (YPYDVPDYA) peptide (JHU School of Medicine Synthesis & Sequencing Facility; Baltimore, MD) combined with Protein Transport Inhibitor Cocktail and costimulatory anti-CD28 and anti-CD49d agonizing antibodies (eBioscience, Inc. San Diego, Ca). Cells were collected, washed, fixed, permeabilized, and stained using standard laboratory protocols for intracellular staining. Fixation and permeabilization buffers from Mouse Regulatory T Cell Staining Kit #2 (eBioscience, Inc. San Diego, Ca) were used. Stains utilized were the following anti-mouse mAbs: PercPCy5.5 conjugated anti-CD3, APC-conjugated anti-IFNγ, FITC-conjugated anti-CD8, and PE-conjugated anti-CD4 (eBioscience, Inc. San Diego, CA). Utilized FACSCalibur™ and LSRII™ Flow Cytometers (BD Biosciences, San Jose, CA). Flow Data analyzed by FlowJo Software (FlowJo, LLC Ashland, OR).

Lymphocyte Depletion

To deplete the CD4+, CD8+, or both T cell subsets, immunized mice were injected i.p. with anti-CD4 (GK1.5), anti-CD8 (2.43), or both mAbs, which were generous gifts from Dr. Fidel Zavala (JHSPH, Baltimore, MD). Negative control vaccinated mice received isotype Rat IgG2b antibody against KLH (LTF-2) purchased from BioXCell (West Lebanon, NH). 100μg of antibody was given to each mouse i.p. on days -1, 0, and 7 from tumor challenge. Depletion efficacy was tested on days 0 and 10 by two-color flow cytometry analysis of peripheral blood lymphocytes using a FACSCalibur™ cytometer (BD Biosciences, San Jose, Ca) with FITC conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD8 mAbs (eBioscience, Inc. San Diego, Ca).

Statistics

Tumor size, immunologic, and flow cytometric analyses were statistically tested by one-way anova with Bonferonni correction and/or Tukey's multiple comparisons test. Mouse survival studies were statistically tested by the log-rank test. Tumor time course regressions were analyzed by mixed effect models. STATA v11.2 (StataCorp, College Station, TX) and Prism 6 (GraphPad Software, Inc. San Diego, CA) were utilized for statistical analyses and figure creation. Significance level of $\alpha \leq 0.05$ was set for all experiments.

Results

Systemic Immune Response

To initially evaluate the immunogenicity of the DNA construct, systemic immune parameters were examined. Mice were vaccinated with either PBS, MIP3α-gp100, or dMIP3α-gp100 (termed antigen-only) three times at one week intervals and then analyzed two weeks after the third immunization. The MIP3α-gp100 vaccine elicited significantly higher levels of B16F10-specific antibodies than antigen-only vaccine ($p=0.004$) and mock vaccine ($p<0.001$) (Figure 2.4). Interestingly, antigen-only vaccine had significantly higher B16F10-specific antibody levels than mock vaccine ($p=0.044$).

As was the case for the antibody concentration, the antigen-only vaccine elicited a moderate vaccine-specific CD8+ T-cell response that significantly differed from the mock vaccination by both percentage (Figure 2.5 top panel; $p=0.030$) and total number (Figure 2.5 bottom panel; $p<0.001$) of CD8+ T cells reactive to the immunogenic $gp100_{25\text{-}33}$ peptide. The addition of MIP3α to the vaccine significantly increased the percentage of ($p=0.049$) and total number of ($p=0.026$) vaccine induced CD8+ T cells compared to the antigen only vaccine, increasing the CD8+ T cell numbers by 46% (Figure 2.5). The MIP3α-gp100 vaccine elicited significantly higher percentages and numbers of vaccine-specific CD8+ T cells compared to mock vaccination (Figure 2.5; $p<0.001$ for both).

Therapeutic Vaccination Model

The potential of this vaccine construct to be utilized in a therapeutic setting against a solid tumor was assessed. A therapeutic regimen was developed with mice

vaccinated on days three, 10, and 17 post challenge with a lethal dose of B16F10 cells. Utilizing statistical regression models, it was determined that the overall slope of the tumor growth regression line was reduced in the MIP3α-gp100 vaccinated group compared to the antigen-only vaccinated group by 48% ($p=0.029$) and to the mock vaccinated group by 63% ($p<0.001$), whereas mock and antigen-only vaccines showed no significant difference to each other in slope (Figure 2.6). Slower overall growth also provides evidence that the differences seen in these experiments are not due to blocks to tumor transplantation.

In addition to tumor growth, the tumor burden of MIP3α-gp100 vaccine recipients proved to be significantly lower than mock vaccination at most all time-points tested and significantly lower than antigen-only vaccine on the critical day 14 time-point – the last time point including all studied mice. On day 14 post challenge, the average tumor size was reduced in the MIP3α-gp100 group by 55% compared to the mock vaccination group ($p<0.001$) and by 51% compared to the antigen-only vaccination group ($p=0.001$) (Figure 2.7).

Survival analysis mirrored tumor growth and burden analyses. MIP3α-gp100 vaccination significantly enhanced survival as compared to antigen only ($p=0.017$) and mock ($p<0.001$) vaccines, whereas antigen only and mock vaccinations did not have significantly different survival curves (Figure 2.8). MIP3α-gp100 vaccination enhanced median survival by 24% and 10% and it enhanced the 25% survival by 33% and 20% compared to mock and antigen only vaccinations, respectively (Figure 2.8).

T-cell Subset Depletion

To determine if effector T-cells played a role in mediating this enhanced protection, and, if so, which subsets might be involved, groups of mice were vaccinated three times over three weeks to develop significant vaccine-specific effector responses and then challenged with tumor under differing depletion conditions: depleting CD4+, CD8+, both CD4+ and CD8+, and no depletion of T cells. Figure 2.9 shows representative flow cytometric analysis of depletion efficacy. In a mouse lymphoma model, a similar MIP3α-OFA vaccine showed the CD8+ T-cell effector response to be essential for protection with the CD4+ T-cell effector response being expendable[128]. However, in this melanoma solid tumor model, depleting CD4+ or CD8+ T-cells individually show a similar phenotype as the isotype depletion control. Single depletions have similar tumor growth rates, tumor sizes, and survival compared to isotype depletion (Figures 2.10-2.11). Importantly, depleting both subsets of T-cells simultaneously provided a phenotype similar to the unvaccinated control in those same analyses (Figures 2.10-2.11). Large tumor size outliers in both single depletion groups hint that some proportion of the mice are reliant on the depleted subset for protection, but the overall groups either utilize both effector subsets relatively equally or one is able to compensate for lack of the other when necessary.

Tumor Infiltrating Lymphocytes

It has been documented that presence and activity of tumor infiltrating lymphocytes (TILs) can correlate with anti-tumor responses in melanoma patients[171]. The intratumoral characteristics of MIP3α-antigen vaccine responses have not previously been documented. Utilizing a therapeutic vaccination protocol as outlined, total CD4+

and CD8+ TILs were harvested one week after the second vaccination, counted by flow cytometry, and normalized by tumor size. Surprisingly, both MIP3α-gp100 and antigen-only vaccines induced significantly higher CD4+ TIL ($p=0.048$; 0.049) and CD8+ TIL ($p<0.001$; $p=0.001$) responses compared to mock vaccine and were at similar levels to each other (Figure 2.12). However, antigen-only vaccine did not provide a clinically relevant response, not differing significantly from the negative control group in tumor growth, size, and survival (Figures 2.6-2.8). In this system, TIL levels themselves appear not to correlate with protection.

Finally, the levels of CD8+ TILs that secrete IFN-γ upon stimulation with immunodominant $gp100_{25-33}$ vaccine antigen were assessed. These results mirrored the systemic splenic CD8+ T-cell data (Figure 2.5). Antigen-only vaccine induced a moderate response, with significantly higher $gp100_{25-33}$-reactive CD8+ TILs by percentage ($p=0.002$) and normalized total numbers ($p=0.030$) compared to the PBS vaccinated negative control goup (Figure 2.13). MIP3α-gp100 vaccination significantly enhanced the percentage ($p=0.003$) and normalized numbers ($p=0.019$) of $gp100_{25-33}$-reactive CD8+ TILs compared to antigen-only vaccine (Figure 2.13). Although the two vaccines elicit a similar number of total TILs, the MIP3α-gp100 vaccine elicits a more robust vaccine-specific effector TIL response that correlates with the enhancement of tumor suppression and mouse survival seen.

Discussion

Our data demonstrate that the addition of the chemokine MIP3α to the gp100 DNA vaccine construct enhanced vaccine immunogenicity and therapeutic potential. Although the antigen-only vaccine elicited a significant immune response compared to the mock vaccine, when utilized as a therapy, only the MIP3α-gp100 vaccine slowed tumor growth and enhanced mouse survival. As has been shown *in vitro*[129, 130], MIP3α-gp100 vaccine directs the antigen in such a way that both CD4+ and CD8+ effector T-cells can be activated. In this study, depletion of either T cell population showed a protection phenotype similar to the non-depleted vaccine group, while depletion of both led to no protection, similar to that observed with mock vaccination. Finally, the data show that the therapeutic protection phenotype provided by MIP3α did not correlate with overall TILs, but did correlate with $gp100_{25\text{-}33}$ vaccine peptide-reactive CD8+ TILs, elucidating that the immune activity and not the quantity of tumor infiltrate correlates with therapeutic efficacy. The roles and mechanisms of tumor infiltrating effector CD4+ TILs are complex and still being defined[172], and therefore the intriguing finding of effector CD4+ T cells providing therapeutic efficacy in the absence of CD8+ T cells will be the subject of future work.

Vaccinations produce results from the combination of vaccine antigen(s), antigenic structure (DNA, peptide, etc.), route of administration, and adjuvants/immunomodulators that culminate in a specific immune response within the proper context. In DNA vaccines, addition of MIP3α to circumsporozoite protein (CSP) with vaxfectin adjuvant[165] creates a robust, protective antibody response against malaria, addition of MIP3α to oncofetal antigen (OFA) given by gene gun creates a

therapeutic response against lymphoma mediated by CD8+ T-cells[128], and as reported here, addition of MIP3α to gp100 given by intramuscular electroporation creates a therapeutic response against melanoma mediated by both CD4+ and CD8+ effector T-cells. All of these experiments have shown responses to be significantly enhanced by the chemokine in different contexts. Co-administration of MIP3α can enhance vaccine responses by enhanced DC recruitment[173]. However, our previous studies have indicated that in the context of a DNA fusion vaccine, MIP3α is acting by directing antigens to DCs, not by recruiting DCs *in vivo*[165]. Therefore, we hypothesize that in this context, adjuvantal effects of electroporation recruit DCs to the vaccine site, and then the MIP3α fused to gp100 increases efficiency of vaccine DNA uptake into infiltrating immature dendritic cells, resulting in enhanced downstream effector responses. This research provides further evidence for the utility of adding chemokine immunomodulators to vaccine constructs within any immunological context.

A primary strength of this DNA vaccine system is its modularity and ease of construction. This study shows that taking the gp100 antigen that induces a specific albeit not therapeutically relevant response on its own can become therapeutically relevant simply by fusing it to MIP3α. Therefore, logically, other more immunogenic and effective antigens could potentially be further enhanced by the addition of MIP3α. A burgeoning new field in cancer vaccinology is the utilization of cancer-specific neoantigens as better vaccine targets that are not subject to central tolerance restrictions[134]. Our modular DNA vaccine could easily and quickly be constructed to utilize neoantigens as they are discovered in real time. Testing the principle of this idea will be the subject of future studies, utilizing now delineated immunogenic neoantigens

found in the B16F10 cell line[174]. In addition to neoantigens, future studies could also examine the efficacy of this vaccine system with other solid tumor models, in combination with current treatments such as immune checkpoint blockade, and in combination with novel immunomodulators.

In conclusion, our data show that addition of MIP3α enhances the immunogenicity and efficacy of a therapeutic vaccine against the aggressive solid tumor model, B16F10 mouse melanoma. The addition of MIP3α to therapeutic vaccines could present a useful strategy to enhance the responses of currently studied vaccines. Furthermore, the modularity of the plasmid provides a realistic platform for creating neoantigen vaccines in a clinically relevant time frame. These findings show that MIP3α can be a plug and play addition to the cancer immunologist's vaccine toolbox that deserves further testing to determine the true potential of the novel design.

Chapter 2: Figures

Figure 2.1**:** Prophylactic vaccination protection confirmation. Mice were vaccinated three times over two week intervals with PBS or 50μg MIP3α-gp100 by i.m. electroporation. Mice were challenged with a lethal dose of B16F10 ($5x10^4$) two weeks after third immunization. Tumor time course was tracked, analyzed by linear regression models, and tumor growth was found to be significantly reduced ($p<0.001$), replicating prior published data. Data represent one experiment with 5-6 mice per group.

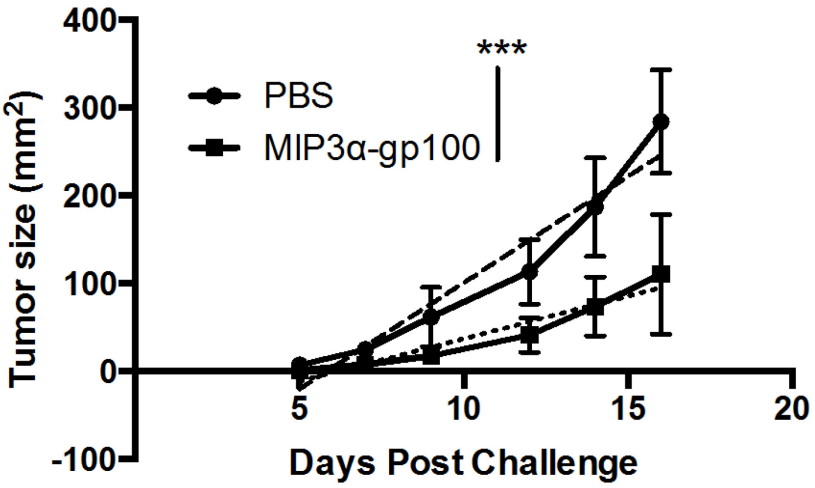


Figure 2.2: ELISpot confirmation of vaccine prophylactic immunogenicity. Mice were vaccinated three times at two week intervals. Two weeks after last immunization, mice were sacrificed, spleens were harvested, and isolated splenocytes tested by standard ELISpot protocol utilizing irradiated EL-4 cells as antigen presenting cells and $gp100_{25\text{-}33}$ immunogenic peptide (or HA peptide for control) as stimulus. Spots counted manually. Samples assayed in triplicate, with sample size of four mice per group. Analyzed by ANOVA with $p<0.01$.

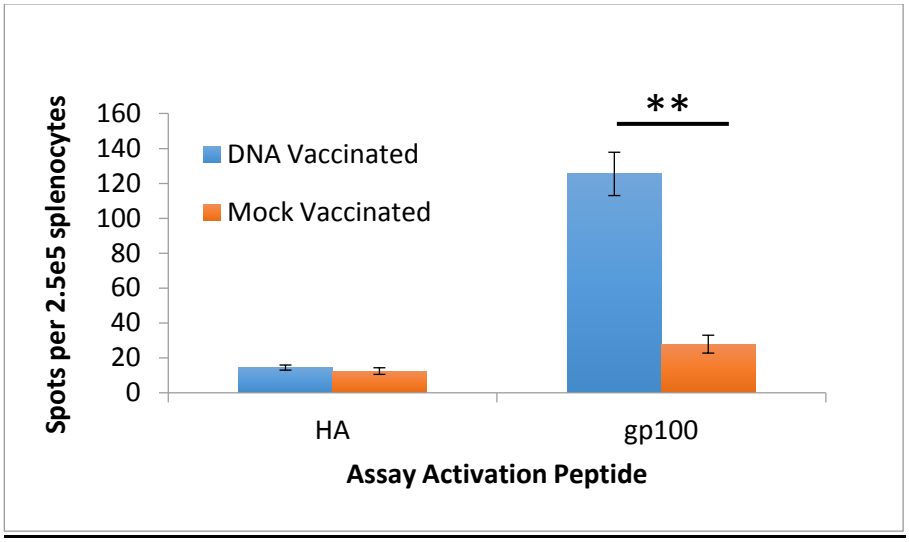


Figure 2.3: Vaccine peptide production in mammalian cell culture system. Different lanes represent different DNA preps, with Mock being untransfected HEK-293T cells. Weights in kDa of the ladder bands are noted. Full length construct is estimated to be 40 kDa, consistent with primary band below. Alternate bands the result of cleavage products and glycosylation[74].

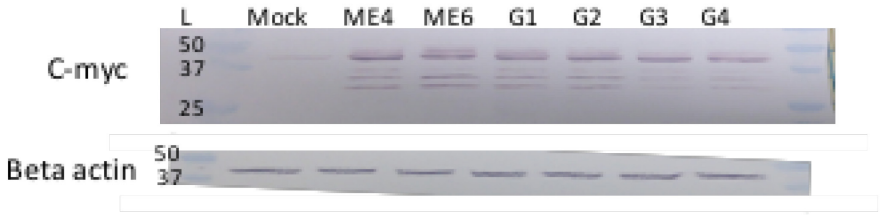


Figure 2.4: Anti-B16F10 antibody production in prophylactically vaccinated mice. Mice were vaccinated three times at one week intervals with endotoxin-free PBS, dMIP3α-gp100, and MIP3α-gp100 fusion vaccine. Analysis occurred two weeks post third vaccination. Data represent two independent experiments with 3-5 mice per group per experiment. In-Cell ELISA performed utilizing fixed B16F10 cells as antigens. Experimental data are shown at a 1:2000 serum dilution after 30-minute colorimetric development. Absorbance values from pre-immune mice were subtracted from post immune mice to obtain the delta absorbance. All groups were significantly different from each other by Anova. *p<0.05, **p<0.01, ***p<0.001

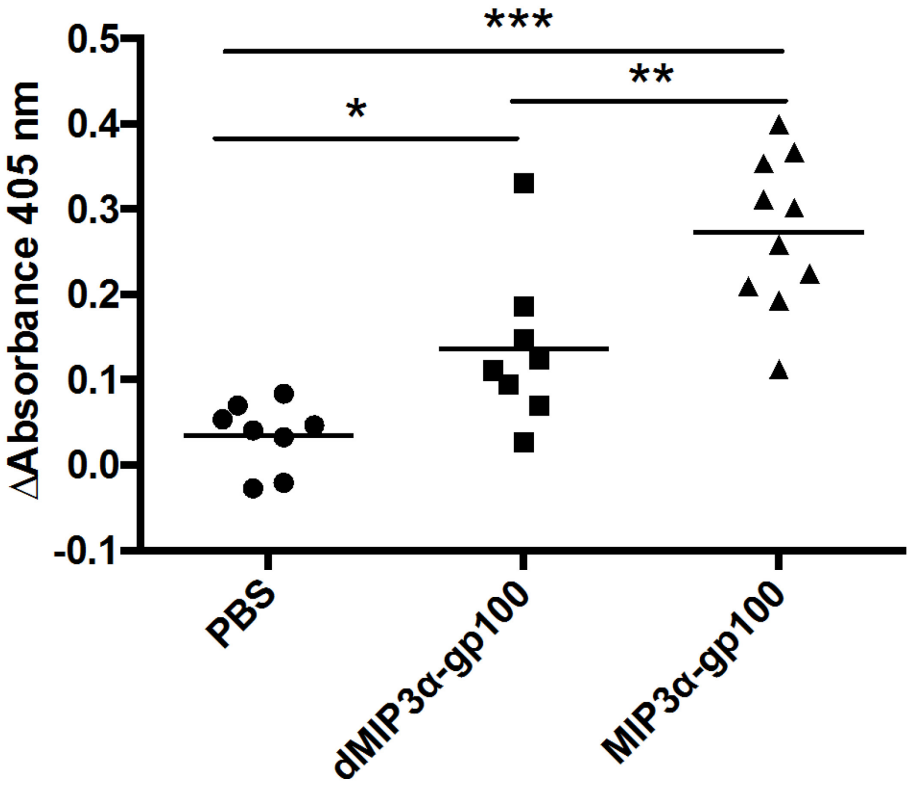
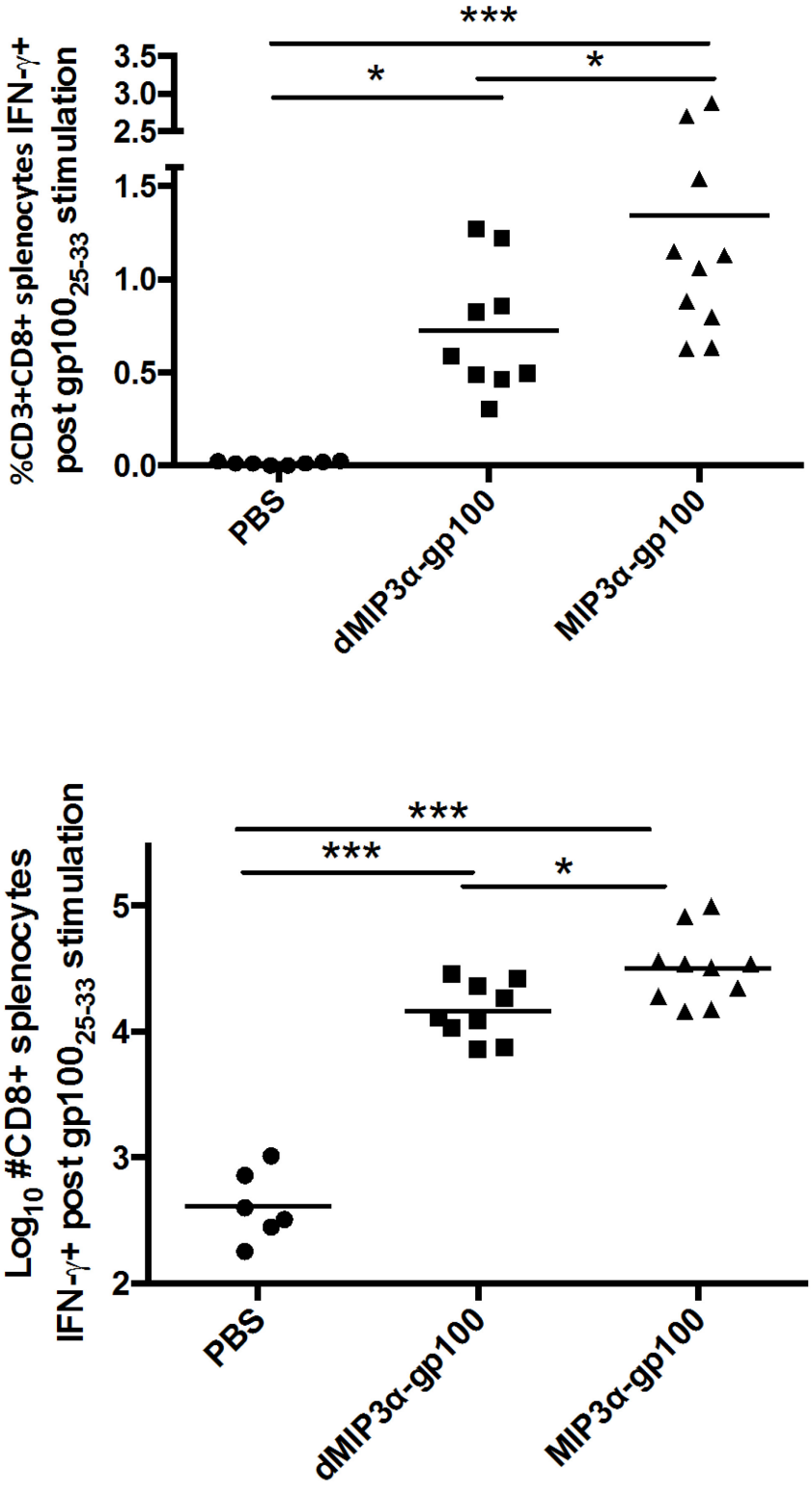


Figure 2.5: Percentage and number of vaccine-specific splenocytes in prophylactically vaccinated mice. The experimental design is the same as Figure 2.2, with this experiment analyzing splenic CD8+ T cells reactive to *ex vivo* stimulation by $gp100_{25-33}$ peptide. Activation was signaled by cytoplasmic IFN-γ accumulation as measured by Intracellular Cytokine Staining Flow Cytometry. Top panel shows the data as percentage of CD3+ splenocytes. The bottom panel estimates the total number of reactive CD3+CD8+ splenocytes by extrapolating flow cytometric data to measured splenic total cell counts. For both panels, all groups differ significantly from each other, as determined by by Anova, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 2.5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*
*
%CD3+CD8+ splenocytes IFN-γ+ post gp100$_{25-33}$ stimulation
3.5
3.0
2.5
1.5
1.0
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PBS
dMIP3α-gp100
MIP3α-gp100
***
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*
Log$_{10}$ #CD8+ splenocytes IFN-γ+ post gp100$_{25-33}$ stimulation
5
4
3
2
PBS
dMIP3α-gp100
MIP3α-gp100

Figure 2.6: Vaccine effects on tumor growth rate in therapeutic model. Vaccinations occurred on days 3, 10, and 17 post challenge. Tumor growth rate was assessed between days 10 and 16, from when the tumor growth of the negative control group began accelerating to the time when mice began to be censored due to endpoints being reached. The graph shows one representative experiment of two, five to seven mice per group and includes linear regression lines and slopes. MIP3α-gp100 vaccine has a significantly different slope from dMIP3α-gp100 and mock PBS vaccination, as evaluated using a statistical mixed effects regression model. The group receiving dMIP3α-gp100 did not differ significantly compared to the group receiving mock vaccination. *p<0.05, **p<0.01, ***p<0.001

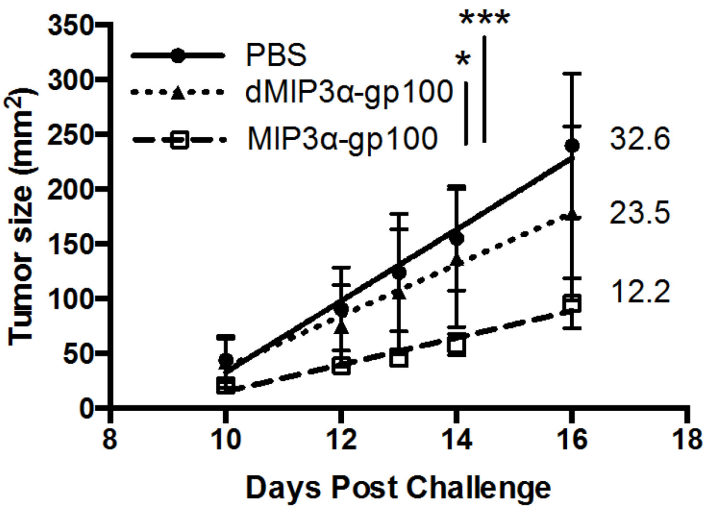


Figure 2.7: Vaccine effect on tumor burden in therapeutic model. Vaccinations occurred on days 3, 10, and 17 post challenge. Tumor size at day 14 post challenge, the last point before any mice were removed from experiments. The data are representative of two experiments, with 5-8 mice per group per experiment. MIP3α-gp100 vaccine recipients had a significantly smaller tumors compared to dMIP3α-gp100 and mock PBS vaccinated mice, as determined by Anova. dMIP3α-gp100 was not significantly different from mock *p<0.05, **p<0.01, ***p<0.001

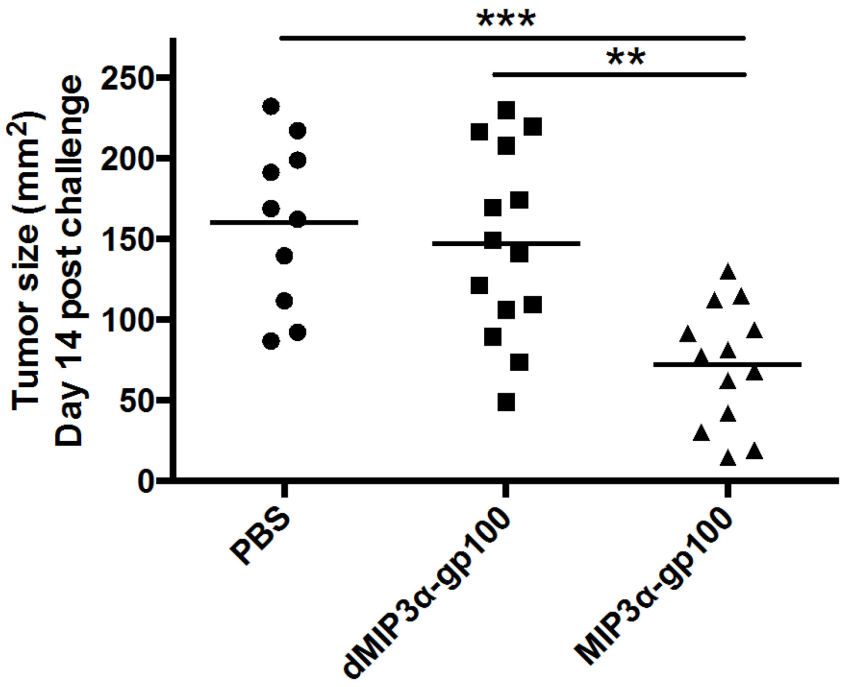


Figure 2.8: Vaccine effects on mouse survival in a therapeutic model. Vaccinations occurred on days 3, 10, and 17 post challenge. Mice were removed from the study at the following endpoints: death, tumor size surpassing 2cm in any dimension, or excessive tumor bleeding and ulceration. Data representative of two experiments, 5-8 mice per group per experiment. The MIP3α-gp100 vaccine group survives significantly longer compared to the dMIP3α-gp100 and mock PBS vaccination groups by the log-rank test. dMIP3α-gp100 did not differ significantly from mock. *p<0.05, **p<0.01, ***p<0.001

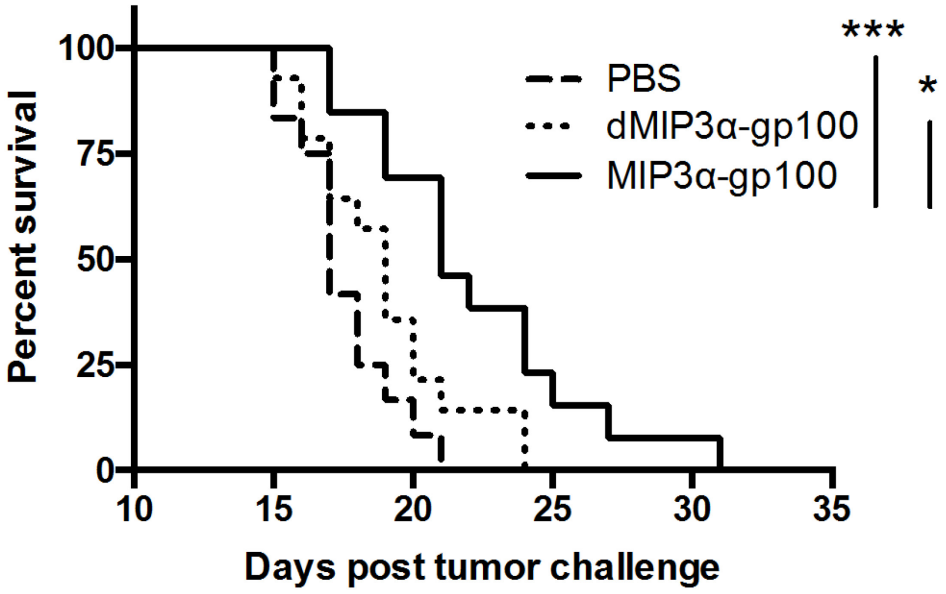


Figure 2.9: T-cell subset depletion confirmation. Mice were vaccinated three times at one week intervals and then challenged with a lethal dose ($5x10^4$) of B16F10 cells. T-cell subsets were depleted one day prior to challenge, on day of challenge, and 7 days post challenge. Quality of the depletions were assessed by flow cytometric analysis of peripheral blood lymphocytes on days 0 and 10. Representative flow cytometry plots shown depicting CD4 and CD8 expression gated on overall lymphocytes from blood collected at day 10 post challenge.

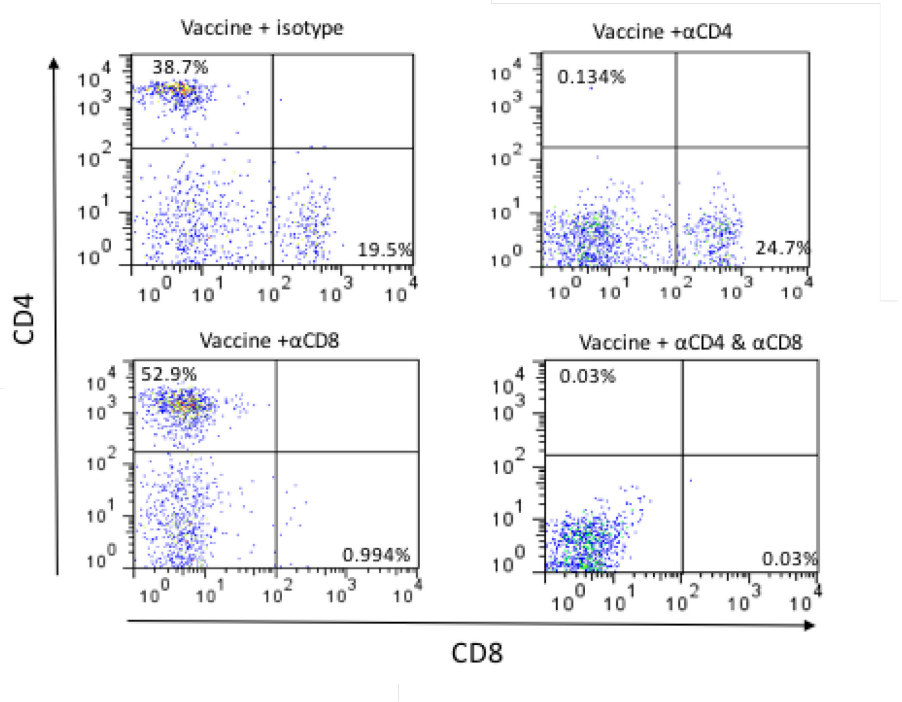


Figure 2.10: Tumor burden under T-cell subset depletion conditions. Mice were vaccinated three times at one week intervals and then challenged with a lethal dose ($5x10^4$) of B16F10 cells. T-cell subsets were depleted one day prior to challenge, on day of challenge, and 7 days post challenge. Tumor sizes at day 13 post challenge are shown here, with n=4 per group. Bars indicate comparisons where $p<0.05$

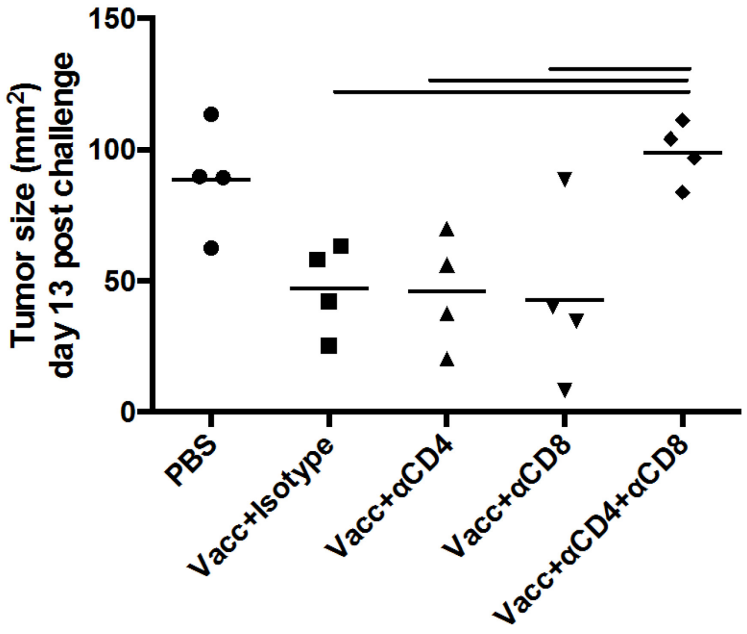


Figure 2.11: Tumor growth rate and mouse survival with vaccination under T-cell depleting conditions. Experiment set up the same as Figure 2.8. Top panel shows the tumor growth regression plot from day 8 to 17 post challenge. Bottom panel shows mouse survival. Both panels analyzed similarly as Figures 2.4 and 2.6 respectively. All data n=4 per group. Bars indicate comparisons where $p<0.05$

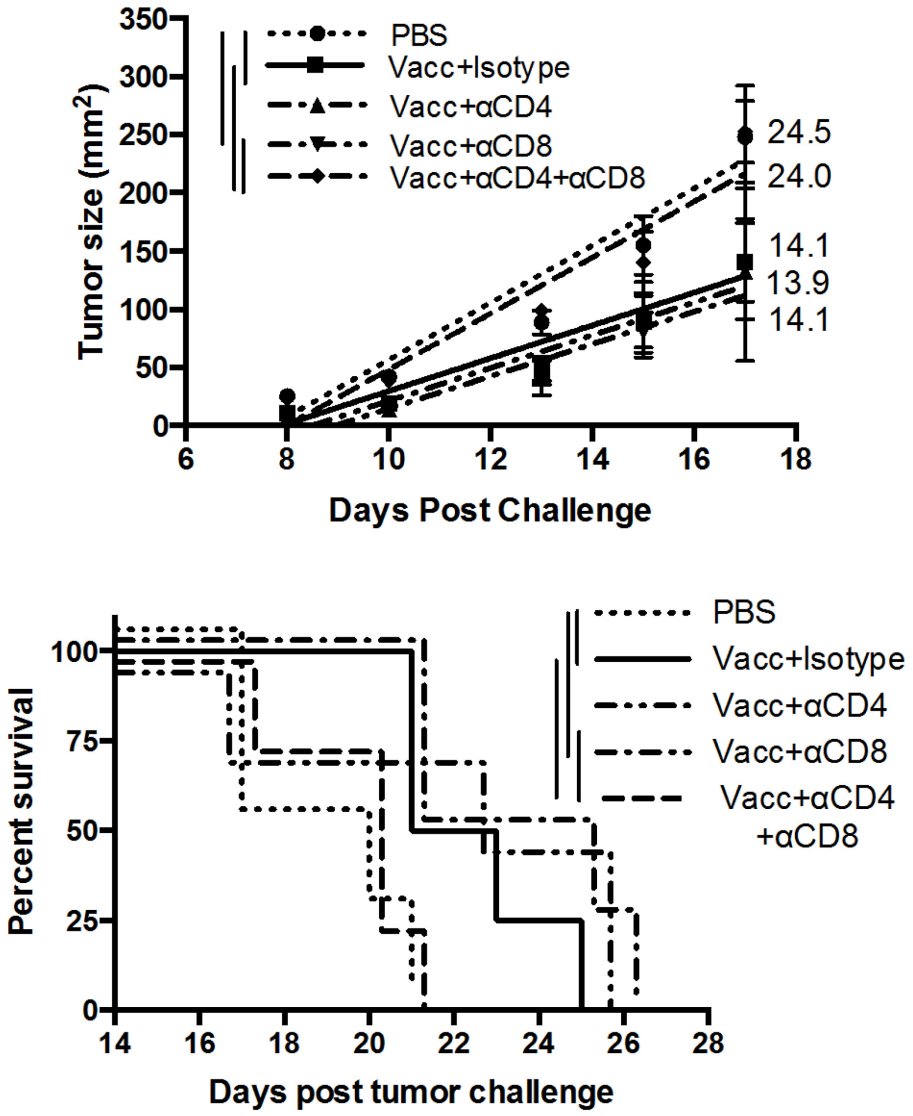


Figure 2.12: Analysis of vaccine effects on tumor infiltrating lymphocytes (TILs) in a therapeutic model. Vaccinations occurred on days 3 and 10 post challenge. Mice were sacrificed on day 17 and lymphocyte-enriched tumor suspensions were analyzed by flow cytometry. The top panel shows CD8+ TILs, and the bottom panel shows CD4+ TILs. Data from one representative experiment with 4-5 mice per group, and two independent experiments were performed with similar results. Both vaccine formulations have significantly higher CD4 and CD8 TILs compared to mock vaccination but not to each other, as assessed by Anova. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM

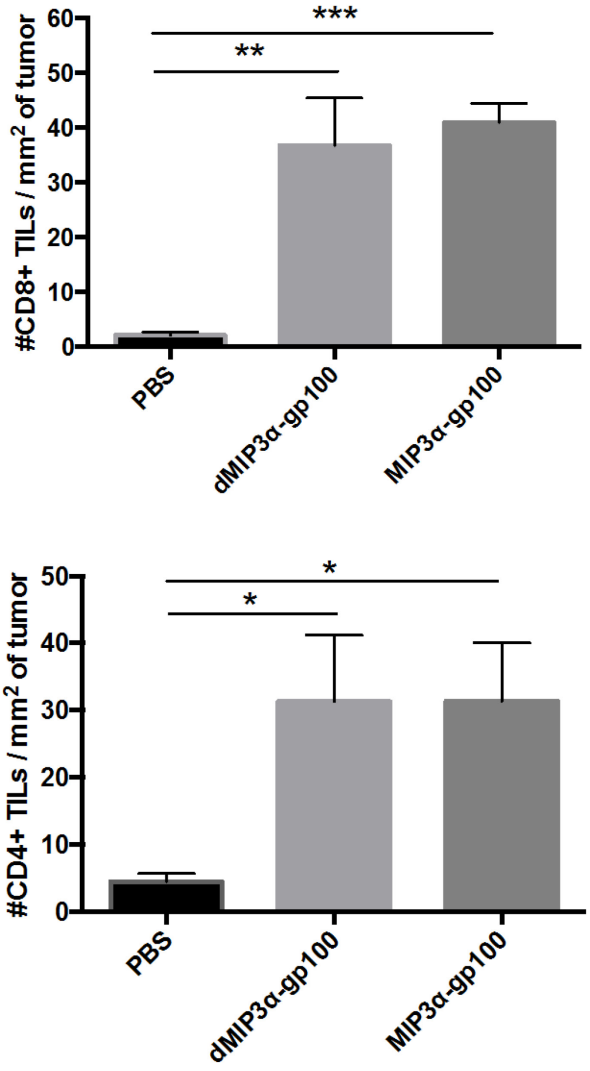
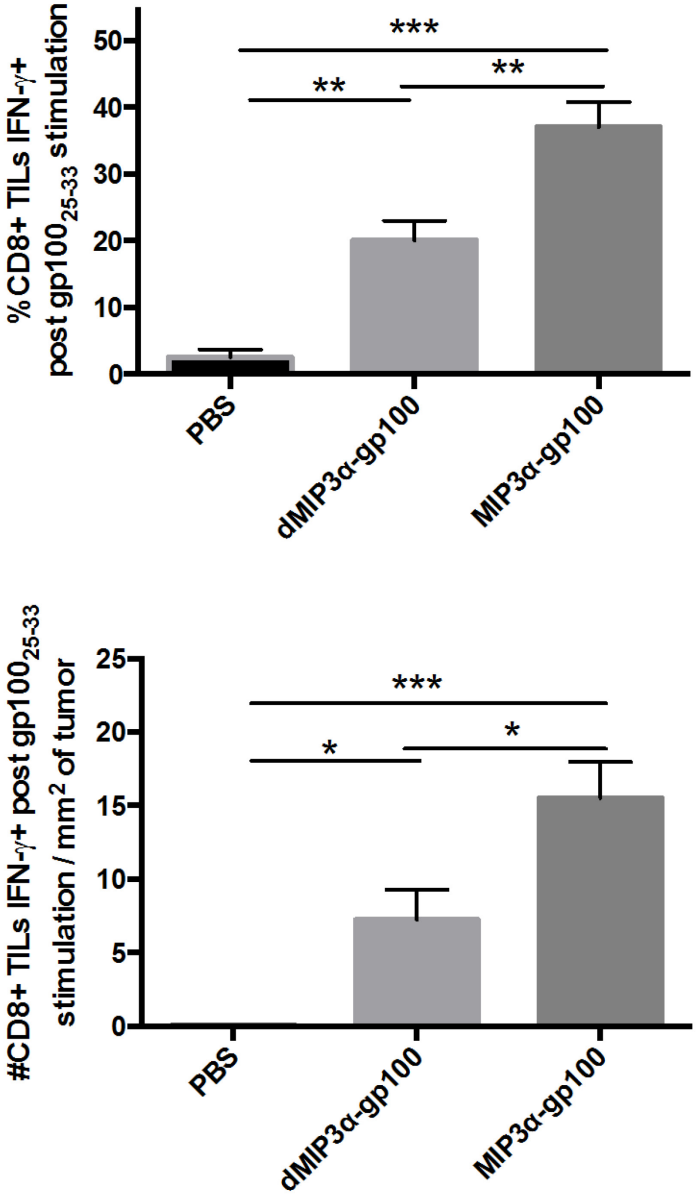


Figure 2.13: Analysis of vaccine effects on vaccine-specific TILs in a therapeutic model. Vaccinations occurred on days 3 and 10 post challenge. Mice were sacrificed on day 20 and lymphocyte-enriched tumor suspensions were collected. CD8+ TILs reactive to *ex vivo* stimulation by $gp100_{25\text{-}33}$ peptide were delineated by Intracellular Cytokine Staining Flow Cytometry measuring cytoplasmic IFN-γ accumulation post stimulation. Top panel shows percentage of CD8+ TILs reactive to antigen. Bottom panel represents the estimated total number of reactive CD8+ TILs normalized to tumor size. All groups were significantly different from each other by Anova. HA irrelevant negative peptide and PHA/ionomycin positive controls confirmed the protocol validity (data not shown). Data are from one representative experiment with 4-5 mice per group, and results were successfully repeated. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Error bars represent SEM

Figure 2.13:


% CD8+ TILs IFN-γ+ post gp100 25-33 stimulation
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CHAPTER 3

INTRATUMORAL INTERLEUKIN-10 BLOCKADE SIGNIFICANTLY ENHANCES VACCINE EFFICACY BY INCREASING INTERFERON-ALPHA EXPRESSION

Introduction

Chapter two characterized the efficacy and immunogenicity of a novel therapeutic melanoma vaccine platform. MIP3α-gp100 enhanced immunogenicity over antigen-only vaccination, and MIP3α-gp100 provided significant anti-tumor efficacy, whereas antigen-only vaccination did not. Despite enhanced efficacy, the vaccine was unable to induce tumor regression. It was hypothesized that the ultimate inability of the vaccine to induce tumor regression and clearance was due to tolerogenic factors in the tumor microenvironment [79]. The microenvironment is complex with many factors influencing tolerance, such as regulatory T-cells [80], immunosuppressive cytokines [131], T-cell checkpoint inhibitors [80], directed evolution of tumor cells [175], and others [131].

One generally immunosuppressive cytokine thought to play an essential role in melanoma immune tolerance is interleukin-10 (IL-10) [176]. Many activated immune cells, especially CD4+ T helper type-1 cells (Th1), regulatory T-cells (Tregs), and monocytes or macrophages [97, 177] produce IL-10. IL-10 reduces antigen presentation by inhibiting expression of MHC-II and costimulatory molecules [178, 179]. IL-10 also impedes expression of a variety of inflammatory mediators while increasing production of anti-inflammatory mediators [97]. Macrophage tolerogenic scavenging, where macrophages increase phagocytosis while decreasing all immunostimulatory pathways, is enhanced by IL-10 [97]. IL-10 inhibits differentiation and maturation of dendritic cells [180] and suppresses production of the T-cell activation cytokine IL-12 by APCs, further hindering their ability to initiate robust Th1 immune responses [97, 181]. Furthermore, IL-10 can act directly on CD4+ T-cells, inhibiting their proliferation and ability to produce effector cytokines such as IL-2 and interferon-γ, among others [182, 183].

IL-10 has been shown to be actively suppressive in many cancer systems. Tumor-induced IL-10 suppresses the ability of splenic dendritic cells to initiate T-cell responses[184]. This dendritic cell paralysis was reversed by an immunostimulatory oligonucleotide combined with blockade of the IL-10 receptor[185]. Similar tumor-induced monocyte dysfunction was reversed by neutralization of IL-10[186]. In bladder cancer, tumor-induced IL-10 inhibits type 1 immune responses at the tumor site[187]. IL-10 contributes to FasL-mediated endothelial cell barriers, where endothelial cells at sites of inflammation express FasL to apoptose circulating T-cells [188], and it regulates endothelial cell junction integrity and barrier function reciprocally with IFN-γ [189]. Both of these functions can prevent effector cells from reaching the tumor microenvironment.

IL-10 has thus become a target for anti-cancer therapies targeting many different cancer types. Blocking IL-10 enhanced the delayed-type hypersensitivity response of BCG anti-bladder cancer vaccination[190–192]. A combination of CpG, anti-IL10, and anti-IL10 receptor oligonucleotides that were targeted to tumor associated macrophages provided significant anti-tumor effect in a mouse hepatoma model [193]. An aptamer specifically neutralizing IL-10 provided significant protection against the CT26 colon carcinoma model [194]. Utilizing mice primed by either IL-10-deficient dendritic cells or wild type dendritic cells with neutralizing IL-10 antibody provided protection in a prostate cancer model[195].

IL-10 has been especially well studied in melanoma. IL-10 suppresses mechanisms of immune surveillance in melanoma [88] and promotes melanoma growth by inhibition of macrophage function and induction of tumor proliferation, both directly

and through enhanced angiogenesis [176]. IL-10 down-regulates MHC/HLA type 1 and antigen presentation machinery in melanomas [101, 196]. Some B16 cell lines produce IL-10 [197, 198], and, in human melanomas, tumoral production of IL-10 is a prognostic factor [199]. Neutralizing IL-10 enhanced IFN-γ production [200] and the efficacy of a dendritic cell vaccine in B16 melanoma [201], and knocking out IL-10 led to mice being more resistant to B16 tumors [200] and more sensitive to treatment with a B16-specific Fab-targeted superantigen [202]. Finally, tellurium-based compounds sensitize B16 tumors to chemotherapies by inhibiting IL-10 autocrine signaling [197, 203].

One effect of IL-10 especially important to this study is its ability to inhibit production of IFNα [94–96]. IFN-α2b treatment has shown benefit in patients with early stage II/III melanomas[113], and clinical studies elucidated that adjuvant IFNα therapy significantly increased disease free survival and sometimes overall survival[107]. IFNα adjuvant therapy became the first immunotherapeutic agent to show significant benefits in survival in a phase III randomized controlled trial and is a part of the current standard of care for patients with advanced stage II or stage III melanoma[2, 107].

IL-10 is a "late cytokine," and it is hypothesized that its primary physiological role is to prevent excessive immune reactions by contributing to peripheral tolerance in cases of inflammatory antigen persistence [97]. The above research points to this being pivotal in maintenance of tolerance in cancer systems, including melanoma. We therefore hypothesize that blocking IL-10 in the tumor microenvironment will help maintain more robust inflammatory local reactions that can allow vaccine-induced immune responses to provide prolonged anti-tumor efficacy. In this study, neutralizing IL-10 at the tumor site enhances the efficacy of the DNA MIP3α-gp100 therapeutic vaccine. The αIL10 therapy

does not appear to directly alter T-cell tumor infiltration, T-cell differentiation, or vaccine immunogenicity, as measured by vaccine peptide-reactive tumor infiltrating CD8+ T-cells. Instead, the mechanism by which αIL10 therapy enhances anti-tumor efficacy is associated with enhanced intratumoral expression of IFNα. Vaccinated mice with the interferon-alpha receptor knocked out did not derive any additional protection from αIL-10 treatment, confirming that type-1 interferons are responsible for the anti-B16 melanoma efficacy of αIL-10 treatment seen in MIP3α-gp100 vaccinated mice.

Materials and methods

Animals and Tumor Model

5-6 week old female C57BL/6 (H-2b) mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a pathogen-free micro-isolation facility in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals. 8-10 week old interferon-alpha receptor 1 knockout mice (*IFNαr1*$^{-/-}$) were a generous gift from the Charles Drake lab (JHMI, Baltimore, MD) and are B6.129S2-*IFNαr1*^{tm1Agt}/Mmjax genotype from Jackson Laboratories (Bar Harbor, ME). All experimental procedures involving mice were approved by the IACUC of the Johns Hopkins University (Protocol number MO13H219). B16F10 mouse melanoma cells were a generous gift from Dr. Arya Biragyn (NIH, Baltimore, MD). 6-8 week old wild type and 10-12 week old knockout mice were challenged in the left flank subcutaneously with a lethal dose ($5x10^4$ cells) of B16F10 melanoma. Tumor size was recorded as square mm, representing tumor length × width (opposing axes) measured by calipers every 1-3 days. Mice were kept in the study until one of the following occurred: mouse death, tumor size eclipsing 20mm in any direction, or extensive tumor necrosis resulting in excessive bleeding.

Plasmids, Vaccination, and αIL-10 Therapy

Vaccine consisted of purified plasmid DNA in endotoxin-free PBS. The plasmid encoded MIP3α-gp100 fusion sequence as described[130]. Vaccination plasmid was extracted from *E. coli* using Qiagen® (Germantown, MD) EndoFree® Plasmid Maxi and Giga Kits. Vaccine DNA purity, quality, and quantity were verified by gel electrophoresis,

restriction enzyme analysis, Nanodrop® spectrophotometry, and insert sequencing. Mock vaccinations comprised of endotoxin-free PBS only. DNA injections were administered into the hind leg tibialis muscle. Immediately following injection, the muscle was pulsed using an ECM 830 Electro Square Porator™ with 2-Needle Array™ Electrode (BTX Harvard Apparatus®; Holliston, MA) under the following parameters: 106V; 20ms pulse length; 200ms pulse interval; 8 total pulses. Vaccinations of 50ug/dose were delivered at days noted in figure legends. As described by others, vaccination for the therapeutic model began on day three[169, 170]. Antibody against mouse IL-10 (Clone JES5.2A5; BioXcell, West Lebanon, NH) was administered intratumorally at 150μg per dose. Typical time course of therapy begins on day 5 post tumor induction and is given every three days for a maximum of 6 doses.

Extraction of Splenocytes and TILs

Spleen and tumor cell suspensions were prepared by grinding sterile excised tissue between the frosted ends of microscope slides and then passing the tissue through a sterile 60 μM mesh. Splenocytes were processed by lysing red blood cells and washing with sterile PBS. Tumor lysate was washed with sterile PBS, and tumor-infiltrating lymphocyte (TIL) fraction was enriched by Lympholyte®-M Cell Separation Media (Cedarlane®, Burlington, NC) according to the manufacturer's protocol. Prior to use all cells were counted by a Z1™ Coulter Counter® (Beckman Coulter, Inc.; Brea, CA) and/or a hemocytometer with Gibco™ Trypan Blue solution 0.4% (Life Technologies, Carlsbad, CA).

Intracellular Cytokine Staining and Flow Cytometry

Enriched splenocytes or TILs were seeded onto Falcon® Multiwell 24-well tissue culture treated plates (Corning, Inc.; Corning, NY) at $1x10^6$ cells per well (or all cells if total is less) and stimulated for 3-4 hours at 37°C with known immunodominant $gp100_{25-33}$ (KVPRNQDWL) peptide or control HA (YPYDVPDYA) peptide (JHU School of Medicine Synthesis & Sequencing Facility; Baltimore, MD) combined with Protein Transport Inhibitor Cocktail and costimulatory anti-CD28 and anti-CD49d agonizing antibodies (eBioscience, Inc. San Diego, Ca). Cells were collected, washed, fixed, permeabilized, and stained using standard laboratory protocols for intracellular staining. Fixation and permeabilization buffers from Mouse Regulatory T Cell Staining Kit #2 (eBioscience, Inc. San Diego, CA) were used. Stains utilized were the following anti-mouse mAbs: PercPCy5.5 conjugated anti-CD3, APC-IFNγ, FITC-CD8, PE-CD4, FITC-PD-1, APC-Foxp3, PE-Granzyme, AF700-Foxp3 (eBioscience, Inc. San Diego, CA), Pacific Blue-CD8, PercPCy5.5-CD25, PE-Cy7-IFN-γ, Pacific Blue-CD44, PercPCy5.5-CD8, APC-CD62L (BioLegend, San Diego, CA), Pacific Orange-CD4, and AmCyan-Live/Dead Aqua (Life Technologies, Carlsbad, CA). FACSCalibur™ and LSRII™ Flow Cytometers were utilized (BD Biosciences, San Jose, CA). Flow Data was analyzed by FlowJo Software (FlowJo, LLC Ashland, OR).

RNA Extraction and RT-PCR

Samples were taken one day after the dose of αIL-10 where more than 80% of mice have harvestable tumors (size > $30mm^2$). Mice were sacrificed and portions of tumor harvested weighing less than 150mg. Tumor minced as finely as possible and added to Trizol®

(Ambion® by Life Technologies, Carlsbad, Ca) at 1ml Trizol® per 50mg tumor. RNA was extracted utilizing manufacturer's protocol and including a 75% ethanol wash step. Pellet was air dried and resuspended in nuclease-free water. The cDNA Reverse Transcription reaction was performed with 1μg extracted RNA and the High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems™ by Thermo Fisher, Halethorpe, MD) utilizing the manufacturer's protocol. Real-Time quantitative Reverse Transcription-PCR (qRT-PCR) performed utilizing TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays (Applied Biosystems™ by Thermo Fisher, Halethorpe, MD) with probes specific for GAPDH (expression control), IFNα4, IFNα2/11, H2-ab1, H2-k1, and FasL. qRT-PCR ran and analyzed with StepOnePlus™ machine and software (Applied Biosystems™ by Thermo Fisher, Halethorpe, MD).

Statistics

Tumor size, immunologic, RT-PCR, and flow cytometric analyses were statistically tested by one-way anova with Tukey's multiple comparisons test if multiple groups and by Student's t-test if two groups. Mouse survival studies were statistically tested by the log-rank test. Tumor time course regressions were analyzed by mixed effect linear regression models. STATA v11.2 (StataCorp, College Station, TX) and Prism 6 (GraphPad Software, Inc. San Diego, CA) were utilized for statistical analyses and figure creation. Significance level of $\alpha \leq 0.05$ was set for all experiments.

Results

Effect of αIL-10 on Therapeutic Vaccination Model

Intratumoral administration of neutralizing αIL-10 antibodies were given alone and in conjunction with a chemokine-fusion DNA vaccine (MIP3α-gp100) with known anti-tumor efficacy in the mouse B16F10 melanoma model. The top panel of figure 3.1 shows that αIL-10 as a monotherapy provides significant anti-tumor efficacy with a 43% reduction in average tumor size at day 14 post challenge ($p<0.001$). However, the tumor burden benefit of the monotherapy disappears at day 17 (Figure. 3.1 bottom panel), and this late, accelerated growth results in loss of a significant effect on the overall tumor growth rate when considered over the entire tumor growth cycle (Figure 3.2). The monotherapy did provide enough efficacy to have significantly enhanced survival (Figure 3.3; $p<0.01$), with an increase in median survival of 11%. These data support the hypothesis that neutralization of IL-10 provides clinical benefit in the melanoma system.

Figures 3.1-3 confirm results from our previous study that vaccination with MIP3α-gp100 provides consistent clinical benefit in the B16F10 mouse model, as measured by tumor size, tumor growth rate, and mouse survival. When combining the vaccine regimen and αIL-10 therapy, clinical benefit at early time-points was equivalent to both monotherapies, as seen in the top panel of figure 3.1. Over time, the combination therapy steadily provided anti-tumor efficacy whereas the two monotherapies began to lose efficacy. As seen in the bottom panel of figure 3.1, by day 17, tumors of the combination therapy group were on average 46% smaller than αIL-10 monotherapy ($p<0.001$) and 30% smaller than vaccination ($p<0.05$).

Further, figure 3.2 shows that the combination therapy group contains tumors that grow significantly more slowly, with a 31% reduction in growth rate compared to vaccine alone ($p<0.01$) and a 51% reduction compared to αIL-10 monotherapy ($p<0.001$). Finally, combination therapy provides significant enhancement in mouse survival compared to vaccine ($p<0.05$) and antibody ($p<0.001$) monotherapies (Figure. 3.3), with median survival enhancements of 10% and 21%, respectively. These results provide strong evidence that neutralization of IL-10 in the B16F10 melanoma microenvironment provides a benefit that is additive with the efficacy of a DNA chemokine-fusion therapeutic vaccine.

Analysis of Infiltrating Lymphocytes

The potential mechanism for the enhanced efficacy of αIL-10 therapy seen in figures 3.1-3 was initially hypothesized to be that IL-10 was inhibiting influx of effector T-cells from blood vessels into the tumor microenvironment [188, 189]. In this model, anti-IL10 therapy should increase the infiltration of lymphocytes. However, while both vaccination alone and vaccination combined with αIL-10 dramatically increased the number of infiltrating lymphocytes compared to the negative control ($p<0.001$), the two regimens were indistinguishable by this parameter (Figure 3.4). Similarly, the number (Figure 3.4) and percentage (Figure 3.5) of CD8+ infiltrating cells that produced IFN-γ upon *ex vivo* stimulation with $gp100_{25\text{-}33}$ peptide did not differ significantly between these two treatment groups.

The second hypothesis for the enhanced efficacy observed with addition of αIL-10 was that IL-10 was negatively affecting T-cell effector function by inhibiting

granzyme expression or by enhancing regulatory T-cell numbers in the environment, as seen in a similar model system[201]. Vaccine alone and combined with αIL-10 did not differ significantly in the number of CD4+Granzyme+ or CD8+Granzyme+ cells infiltrating the tumor (Figure 3.4). Similarly, the difference in percentage of CD4+ and CD8+ infiltrating T-cells with surface expression of the activation/exhaustion marker Programmed Death ligand-1 (PD-1) was not significant between the vaccine alone and vaccine plus αIL-10 groups (Figure 3.5). Also, in contrast to Kalli, et al. [201], no difference was seen in the percentage of infiltrating CD4+ T-cells that had a regulatory phenotype (Figure 3.5). The ratio between infiltrating CD8+ T-cells and regulatory CD4+ cells, a parameter generally correlating with T-cell based anti-tumor efficacy [136, 204], was also not significantly different between groups receiving vaccine alone and groups receiving both vaccine and αIL-10 (Figure 3.6). Therefore, the hypothesis that αIL-10 is providing anti-tumor efficacy by affecting influx or activity of specific T cell subsets is not supported by the data.

Notably, these parameters provide new insights into the therapeutic vaccination system. Data from Chapter 2 showed that influx of CD4+, CD8+, and gp100-reactive CD8+ tumor infiltrating lymphocytes was enhanced compared to mock vaccination (Figures 2.12-13). Figures 3.4-6 confirm and expand on this phenotype. The vaccine enhanced levels of infiltrating CD4+Granzyme+ cells ($p<0.05$) and CD8+Granzyme+ cells ($p<0.001$) over the negative control (Figure 3.4). The vaccine also enhanced the percentages of infiltrating CD8+ and CD4+ cells that contain the activation marker PD-1 (Figure 3.5; $p<0.001$). Although vaccination did not result in the reduction of regulatory T-cells, the CD8/Treg ratio was significantly higher than the negative control (Figure 3.6;

$p<0.01$). After vaccination, these data suggest that the tumor microenvironment contains not only higher levels of T-cells, but also T-cells that are mature effector cells with cytotoxic capabilities.

Expression Levels of Known IL-10 Responsive Genes

Transcriptional levels of *H2-k1* (MHC-1), *H2-ab1* (MHC-II), *Ifnα2/11,* and *Ifnα4* were analyzed in tumor lysates one day after administration of αIL-10 by real-time quantitative reverse-transcription PCR (qRT-PCR). Relative fold-expression values were calculated as noted in the methods and figure legend. Expression levels of *H2-k1, H2-ab1*, and *Ifnα2/11* remained relatively unchanged in vaccine alone versus with αIL-10 (Figure 3.7). Expression levels of *Ifnα4* were upregulated 2.8-fold ($p<0.05$) when adding αIL-10 to the vaccine (Figure 3.7). In addition, *Ifnα4* in the vaccine only group was upregulated 6.7-fold compared to negative control, so the combination group was upregulated 18.7-fold compared to negative control.

IFNαr1$^{-/-}$ Knockout Mice

Our data indicate that IFNα4 correlates with anti-tumor efficacy of αIL10 treatment, however the results do not differentiate between IFNα4 being associated with vs. its directly causing the enhanced anti-tumor efficacy. To address this issue, the standard therapeutic protocol was assessed utilizing mice with IFNα-receptor 1 knocked out (*IFNαr1$^{-/-}$*). Mice deficient in the receptor are unable to react to the IFNα family of cytokines [205].

In figure 3.8, tumor sizes at day 14 post challenge of wild type vaccine+αIL-10 treated mice were 61.5% smaller compared to wild type vaccine only ($p<0.05$), consistent with previous results. Vaccinated *IFNαr1*$^{-/-}$ mice have tumor sizes similar to vaccinated wild type mice, and the wild type combination treatment mice were 72% smaller (Figure 3.8; $p<0.01$). Importantly, the tumor sizes of the combination treatment in wild type mice were 73% smaller than *IFNαr1*$^{-/-}$ mice given the combination treatment (Figure 3.8; $p<0.001$).

Tumor growth rates followed a similar course (Figure 3.9). Wild type combination treatment mice had significantly lower tumor growth rates than wild type vaccine-only (52% less, $p<0.05$), *IFNαr1*$^{-/-}$ vaccine-only (54% less, $p<0.001$), and *IFNαr1*$^{-/-}$ combination treatment groups (63% less, $p<0.001$). Similarly, survival analysis (Figure 3.10), demonstrated that wild type mice receiving both treatments survived significantly longer than wild type vaccine-only mice ($p<0.05$), *IFNar1*$^{-/-}$ vaccine-only mice ($p<0.01$), and *IFNar1*$^{-/-}$ mice receiving both treatments ($p<0.01$). The group of wild type mice with both treatments showed an increase in median survival of 16.7% compared to the wild type vaccine-only group, 36.6% compared to the *IFNar1*$^{-/-}$ vaccine-only group, and 27.3% compared to the *IFNar1*$^{-/-}$ group with both treatments.

Discussion

Our data demonstrate that intratumoral neutralization of IL-10 significantly enhances the anti-melanoma efficacy of MIP3α-gp100 therapeutic DNA vaccination. Blockade of IL-10 has been shown to enhance T-cell responses to an otherwise ineffective DNA vaccine in a lymphocytic choriomeningitis virus model [206]. IL-10 neutralization also enhanced a dendritic cell-based melanoma vaccine and correlated with enhanced CD4+ granzyme+ tumor infiltrating lymphocytes (TILs) and reduction of regulatory CD4+ Foxp3+ TILs [201]. However, in the present model, levels of CD8+ TILs that produced IFN-γ upon vaccine peptide stimulation did not significantly change when adding αIL-10 treatments to vaccinated mice, nor did levels of CD4+ granzyme+ TILs or regulatory CD4+ Foxp3+ TILs. Overall levels of TILs did not change either, suggesting that αIL-10 was not altering T-cell migration or endothelial cell permeability.

Since αIL-10 did not appear to be affecting T-cells directly, intratumoral expression levels were assessed for some of the genes that are both known to be regulated by IL-10 and could indirectly enhance infiltrating T-cell responses. Genes such as *H2-K1* (MHC-I) [92, 196, 207], *H2-ab1* (MHC-II) [179], and *Ifnα* (Interferon-alpha) [94, 95] are known to be negatively regulated by IL-10 under certain conditions. An increase in either MHC molecule could easily enhance vaccine efficacy known to be mediated by both CD4+ and CD8+ effector T-cells (Figures 2.9-11). An increase in IFNα could provide a more general benefit by enhancing lymphocyte cytotoxicity[208], dendritic cell activity[209], expression of anti-angiogenic factors[210], and/or increasing apoptosis or cell cycle arrest[211]. IFNα4 and 11 are highly active in mice[212], and so levels of both were analyzed. While *Ifnα2/11, H2-k1, and H2-ab1* saw no increase in expression, *Ifnα4*

expression was increased almost 3-fold in combination treatment compared to vaccine-only and approximately 18-fold compared to the negative control.

This correlation was then shown to be the primary driver responsible for the efficacy of αIL-10 treatment. Knocking out a chain of the common receptor for interferon alpha cytokines (*Ifnαr1*$^{-/-}$) caused a disappearance of the enhanced vaccine efficacy phenotype associated with αIL-10 therapy. *Ifnαr1*$^{-/-}$ mice receiving vaccination and αIL-10 treatment did not show significant differences compared to *Ifnαr1*$^{-/-}$ vaccinated mice or wild type vaccinated mice, but did have larger tumors, a faster tumor growth rate, and reduced survival compared to wild type mice given both vaccine and αIL-10. Clearly, αIL-10 efficacy is primarily mediated by type-1 interferons and the responses they induce.

From our data, it is unlikely that the αIL-10 is having a substantial direct effect on T-cells in this system. The tumor time course data support this hypothesis. It is not until day 17 post challenge that the combination group significantly separates from the monotherapies. If αIL-10 were directly enhancing T-cell efficacy, one would expect to see earlier separation. However, it is telling that αIL-10 monotherapy loses efficacy by day 17 whereas the combination treatment is just then positively differentiating itself. There are several potential explanations for this phenotype. IFNα is known to have direct anti-tumor effects [211]. It is possible that the slight reduction in tumor size at early time-points in the combined treatments group allowed the vaccine response to achieve better efficacy over time due to the reduced burden. Alternatively, the IFNα could be enhancing dendritic cell activity and presentation [209]. Early responses in the αIL-10 monotherapy are most likely due to direct tumor effects by IFNα. The efficacy seen in the combination

therapy group starting at day 17 could be due to immune responses induced by early dendritic cell activity. While there seems to be no difference in number of TILs at day 17, the breadth and overall efficacy of the response could be enhanced in the combination therapy as opposed to the vaccine itself.

The next step in this line of research will be to elucidate the origin and relevant downstream function of IFNα. Activated plasmacytoid dendritic cells (pDCs) are considered primary sources of IFNα cytokines and are known to be regulated by IL-10 [96, 213, 214]. Therefore, detailed studies analyzing intratumoral pDCs are warranted in this system. qRT-PCR expression panels over time of all murine IFNα proteins would provide insight into the mechanism. IFNα4, along with IFNβ, is an immediate-early IFN depending only on phosphorylation of IRF-3 for expression [212]. Expression of other type-1 IFNs requires IRF-7, which needs priming of the cell by exogenous IFNα/β [212]. Understanding the timing of αIL-10-induced type-1 interferon expression could inform further treatment optimizations. These studies would then provide the framework for testable hypotheses of specific type-I IFN anti-melanoma mechanisms.

IL-10 is a complex cytokine with pleiotropic effects. Several studies provide evidence that IL-10 also plays a pro-inflammatory role by enhancing B-cell antibody production and cytotoxicity and longevity of CD8+ effector T-cells [91, 93, 215, 216]. IL-10 immunostimulatory activity on CD8+ T-cells could potentially explain the slight reduction seen here of gp100-reactive CD8+ TILs when αIL-10 was given with vaccine. Surprisingly, a recent study showed that treatment with pegylated-IL-10 provides anti-tumor efficacy [217]. However, the results of this study conclusively show that intratumoral neutralization of IL-10 provides therapeutic efficacy against B16 melanoma

alone and in concert with a DNA vaccine. This study further shows that *Ifnα4* expression is upregulated in the tumor after αIL-10 doses and that action of type-1 IFNs is necessary for αIL-10 treatment efficacy. Further study could guide investigators into understanding the specific contexts that provide the best anti-tumor efficacy from IL-10 neutralization.

Chapter 3: Figures

Figure 3.1: Effect of αIL-10 on tumor burden in therapeutic vaccine model. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of six doses. Top panel shows tumor burden at day 14 post challenge where all treatments looked equivalent. Bottom panel shows tumor burden at day 17 when treatments showed separation. The data are representative of 3-8 experiments of 5-9 mice per experiment, with range of sample sizes noted in the graph. αIL-10 itself provides significant therapeutic benefit at day 14 but not at day 17. The vaccine with αIL-10 treatment group by day 17 provided significant therapeutic benefit compared to all other treatments. Significance determined by Anova with Tukey's multiple comparison test. Boxplot whiskers delineate the range, and the box represents the middle 50% with median line. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Figure 3.1:

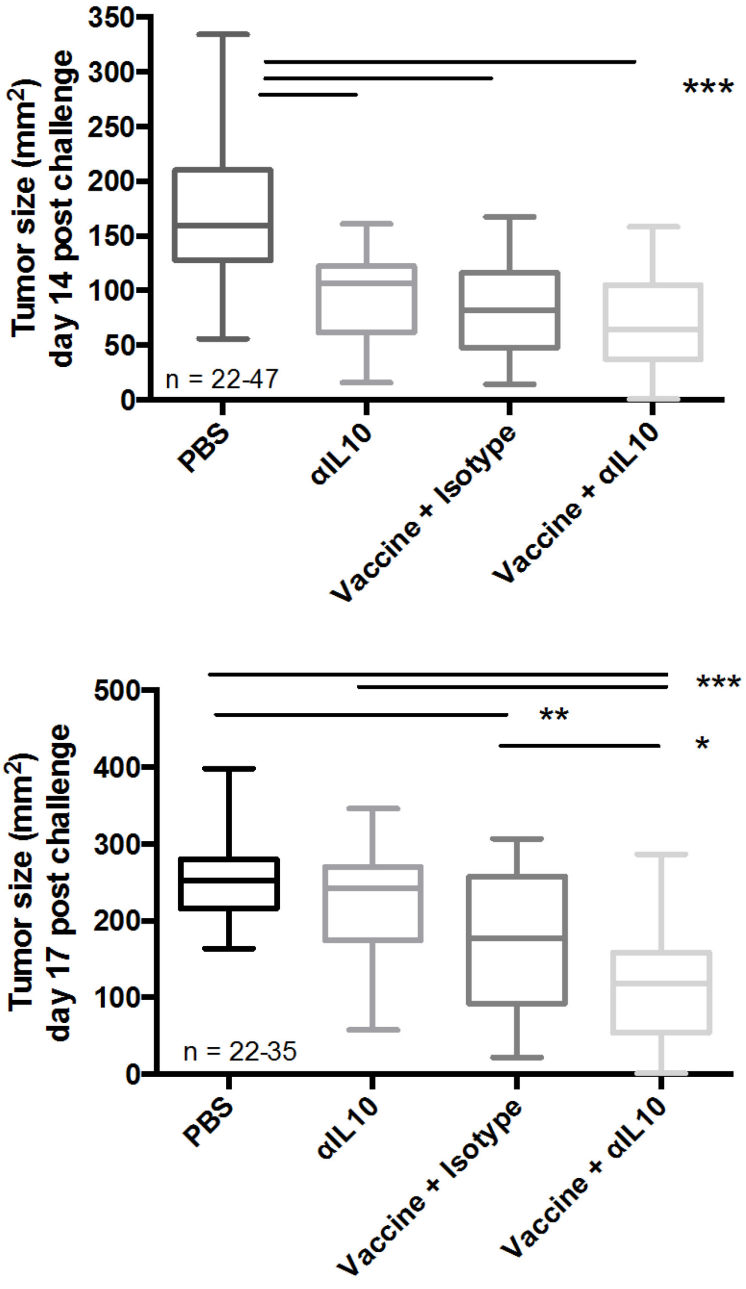

Tumor size (mm²) day 14 post challenge
350
300
250
200
150
100
50
0
n = 22-47
***
PBS
αIL10
Vaccine + Isotype
Vaccine + αIL10
Tumor size (mm²) day 17 post challenge
500
400
300
200
100
0
n = 22-35
***
**
*
PBS
αIL10
Vaccine + Isotype
Vaccine + αIL10

Figure 3.2: Effect of αIL-10 on tumor growth rate. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of six doses. Tumor growth rate was assessed between days 10 and 17, from when the tumor growth of the negative control group began accelerating to the time when mice began to be censored due to endpoints being reached. Graph shows data points with standard errors and linear regression lines. Numerical slopes are indicated adjacent to their respective regression lines. The data are representative of 4-9 experiments with 5-9 mice per experiment for sample sizes ranging from 28-53 per group. The group combining vaccination with αIL-10 therapy shows significantly slower tumor growth than all other treatments as evaluated by a statistical mixed effects regression model. *p<0.05, **p<0.01, ***p<0.001

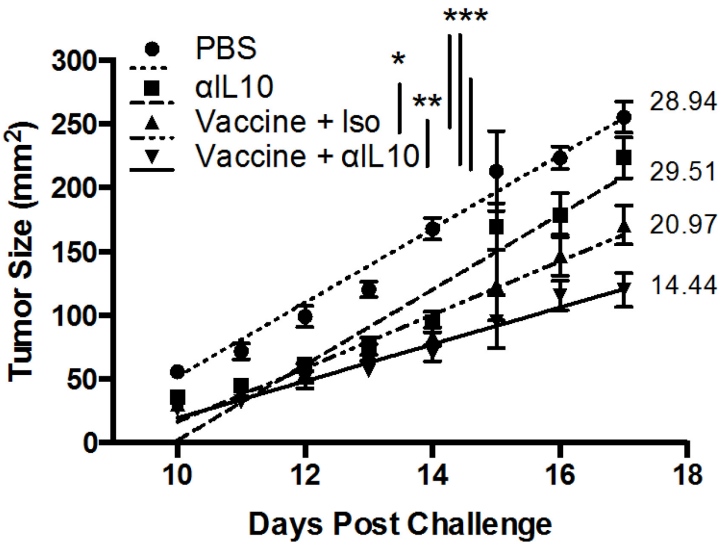


Figure 3.3**:** Effect of αIL-10 on mouse survival. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of six doses. Mice were removed from the study at the following endpoints: death, tumor size surpassing 2cm in any dimension, or excessive tumor bleeding and ulceration. Data are representative of three to four experiments comprising a total of 22-29 mice per group. The vaccine with αIL-10 treatment group provides significant survival enhancement compared to all other treatments, as evaluated by log-rank test. *p<0.05, **p<0.01, ***p<0.001

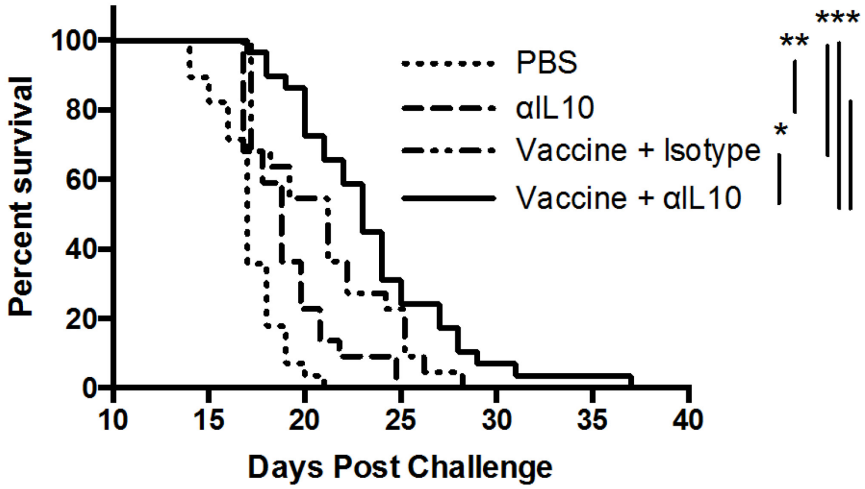


Figure 3.4**:** Flow cytometric analysis of tumor infiltrating lymphocytes by normalized numbers. Vaccinations occurred on days 3 and 10 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of four doses. Mice were euthanized on day 17, and tumor tissue was extracted for analysis by flow cytometry as outlined in the methods. Both treatment groups had significantly elevated levels of all cell types shown, but the vaccine + αIL-10 group showed no difference compared to vaccine-only in all cell types tested. Data are from 2-3 independent experiments of 3-8 mice each. Groups were evaluated by Anova with Tukey's multiple comparisons test. Error bars represent the standard error. *p<0.05; ***p<0.001.

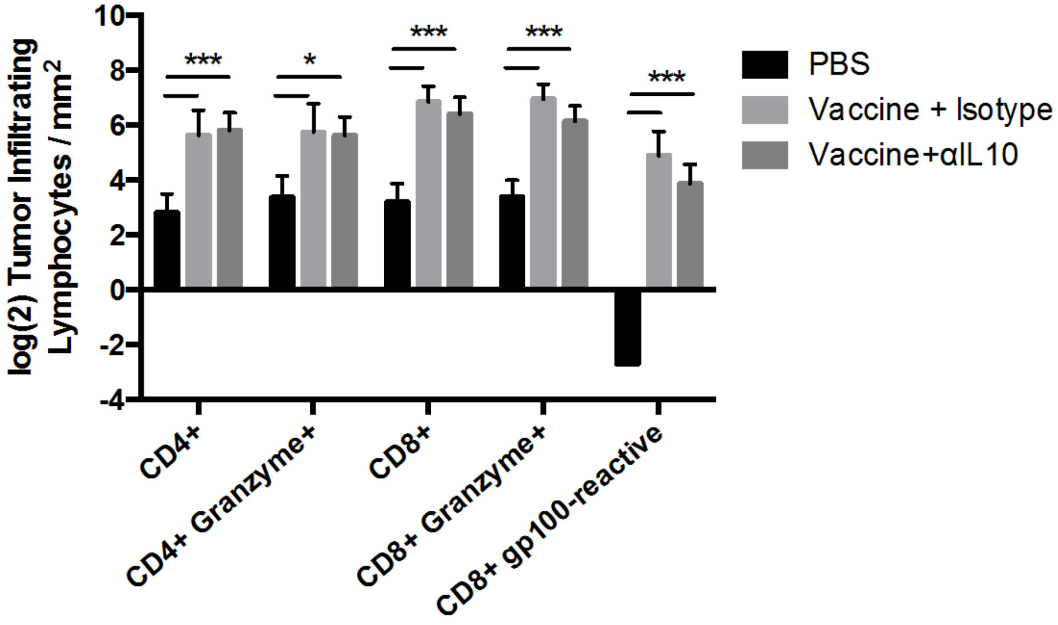


Figure 3.5: Flow cytometric analysis of tumor infiltrating lymphocytes by percentage of CD4+ or CD8+ T-cells exhibiting the analyzed characteristic. Vaccinations occurred on days 3 and 10 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of four doses. Mice were euthanized on day 17, and tumor tissue was extracted for analysis by flow cytometry as outlined in the methods. Both treatment groups had significantly elevated levels of all cell types except for the percentage of CD4+ cells that are T-regulatory cells. However, the vaccine + αIL-10 group showed no difference compared to vaccine only in all cell types tested. Data are from 2-3 independent experiments of 3-8 mice each. Groups were evaluated by Anova with Tukey's multiple comparisons test. Error bars represent the standard error. **p<0.01; ***p<0.001; n.s. p>0.05.

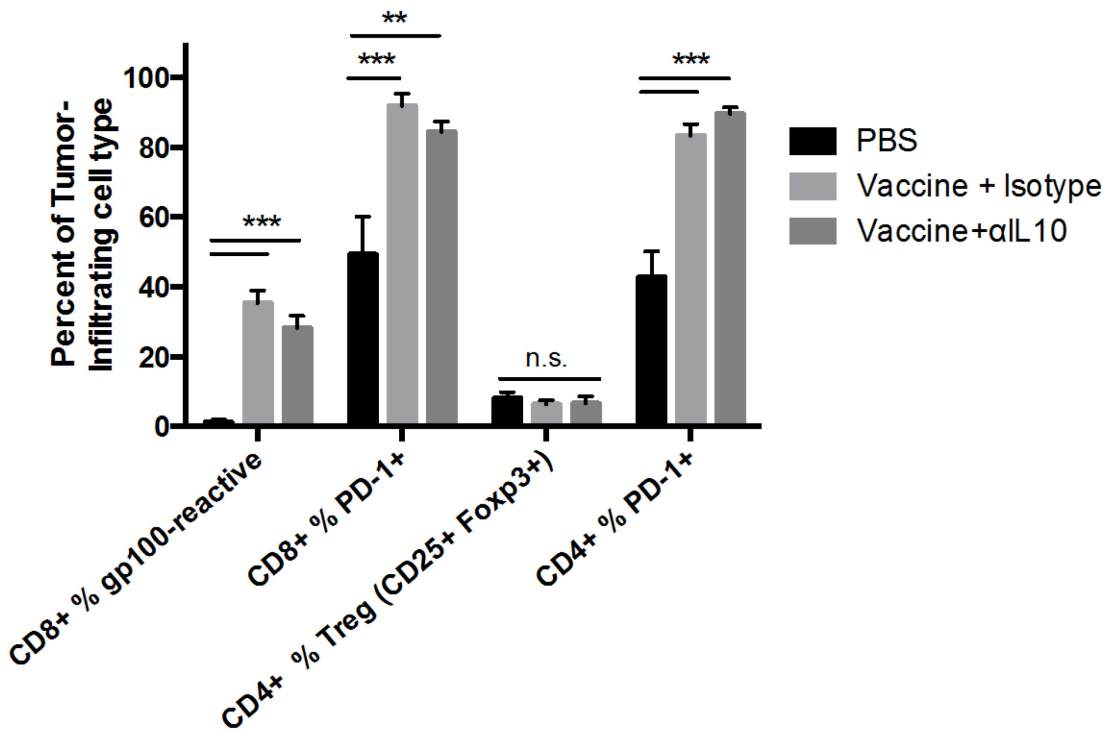


Figure 3.6: Calculating the ratio of tumor-infiltrating CD8+ T-cells to regulatory CD4+ T-cells. Vaccinations occurred on days 3 and 10 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of four doses. Mice were euthanized on day 17, and tumor tissue was extracted for analysis by flow cytometry as outlined in the methods. Both treatment groups had a significantly higher ratio than the negative control, but were no different from each other. Groups were evaluated by Anova with Tukey's multiple comparisons test. Data are from 2-3 independent experiments of 3-8 mice each. Error bars represent the standard error. **p<0.01; ***p<0.001.

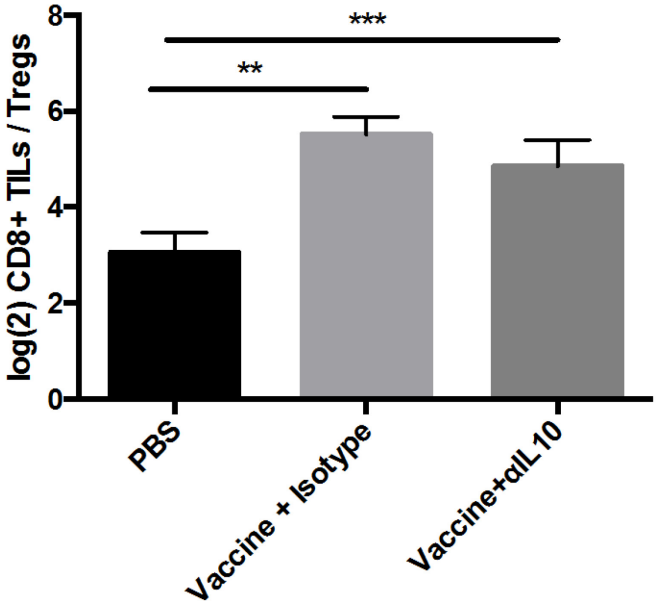


Figure 3.7: Real-time quantitative reverse-transcription PCR (qRT-PCR) analysis of loci potentially affected by αIL-10 treatment. Vaccinations occurred on days 3 and 10 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days until harvest. Mice were euthanized and tumors harvested one day after the αIL-10 dose where >80% of tumors were above minimum cutoff of 30 mm^2. qRT-PCR data was analyzed by the ΔΔCt method. Samples were normalized to GAPDH housekeeping gene control to form ΔCt values and then subtracted from a PBS vaccinated mouse to create the ΔΔCt values. Data shown as fold expression difference as compared to PBS vaccinated mouse by the formula $2^{(-\Delta\Delta Ct)}$. IFNα4 locus saw significantly enhanced transcriptional levels in vaccine + αIL-10 group as compared to vaccine only. No other locus provided significant results. Outliers more than two standard deviations greater than group mean were removed from the dataset. Data are representative of two independent experiments with 3-4 mice per group and were evaluated by Anova with Tukey's multiple comparisons test. Error bars represent the standard error. *p<0.05.

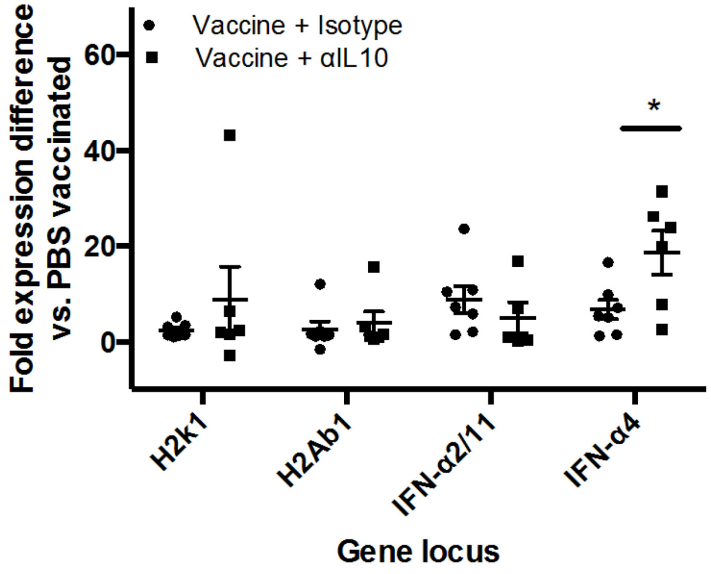


Figure 3.8: Tumor burden effects of Interferon alpha receptor knockout (*IFNαr1*$^{-/-}$) mice on vaccination and αIL-10 treatment. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of five doses. Data shown at day 14 post challenge – the final time-point including all mice. The group combining vaccine with αIL-10 treatment in wild type mice harbored a significantly reduced tumor burden compared to wild type vaccinated mice, *IFNαr1*$^{-/-}$ vaccinated mice, and, importantly, to *IFNαr1*$^{-/-}$ mice given vaccine and αIL-10 treatment. No other comparisons were significantly different. Data represent one experiment with 4-6 mice per group. Groups were evaluated by Anova with Tukey's multiple comparisons test. Error bars represent the standard error. *p<0.05; **p<0.01; ***p<0.001.

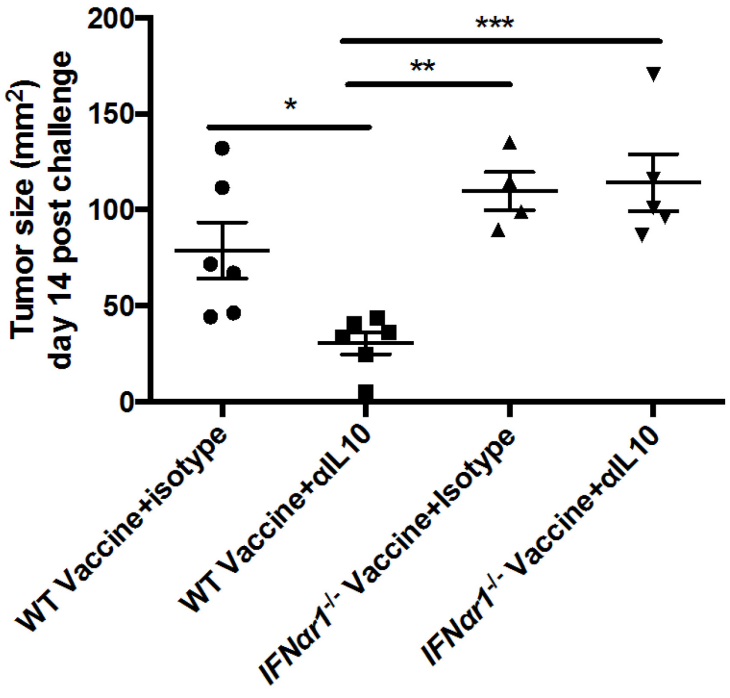


Figure 3.9: Tumor growth effects of *IFNαr1*$^{-/-}$ mice on vaccination and αIL-10 treatment. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of five doses. Tumor growth shown between day 8, when tumors began growing steadily, and 17, after which >10% of mice had been removed from the study. The group combining vaccine with αIL-10 treatment in wild type mice had tumors that grew significantly slower than wild type vaccinated mice, *IFNαr1*$^{-/-}$ vaccinated mice, and, importantly, to *IFNαr1*$^{-/-}$ mice given vaccine and αIL-10 treatment. No other comparisons were significantly different. Data represent one experiment with 4-6 mice per group. Groups were evaluated by mixed effects linear regression. Error bars represent the standard error, linear regression lines are included, and numerical slopes are indicated adjacent to their respective regression line. *p<0.05; ***p<0.001.

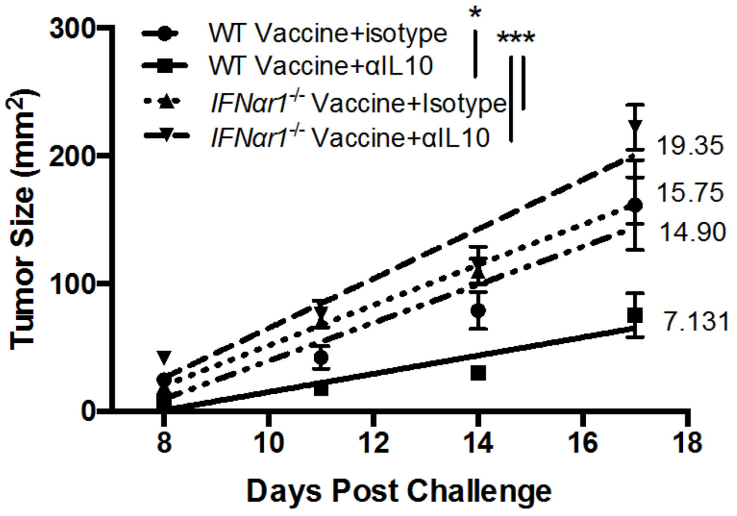
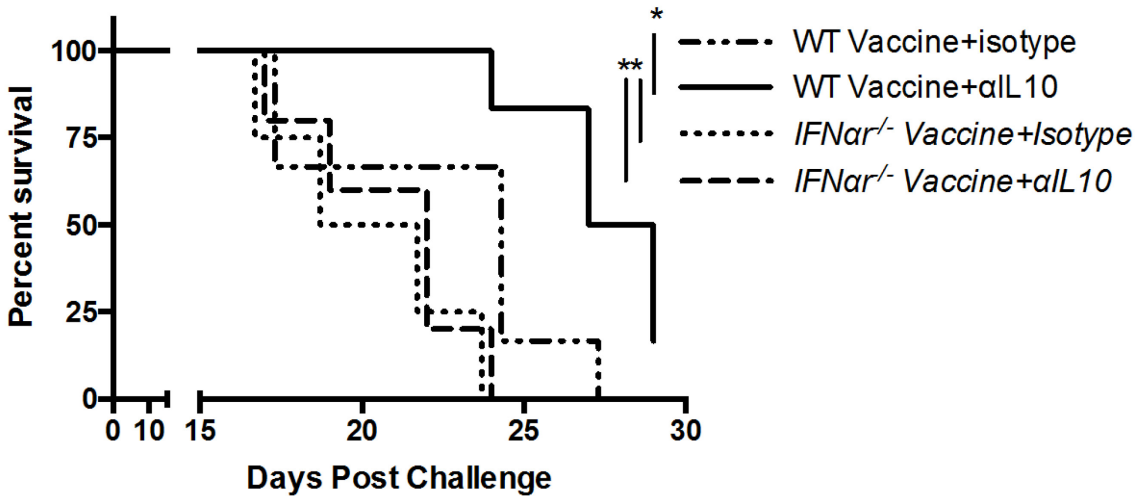


Figure 3.10: Survival effects of *IFNαr1*$^{-/-}$ mice on vaccination and αIL-10 treatment. Vaccinations occurred on days 3, 10, and 17 post challenge. Doses of αIL-10 began on day 5 post challenge and continued every three days for a total of five doses. Mice were removed from the study at the following endpoints: death, tumor size surpassing 2cm in any dimension, or excessive tumor bleeding and ulceration. The group combining vaccine with αIL-10 treatment in wild type mice exhibited enhanced survival compared to wild type vaccinated mice, *IFNαr1*$^{-/-}$ vaccinated mice, and, importantly, to *IFNαr1*$^{-/-}$ mice given vaccine and αIL-10 treatment. No other comparisons were significantly different. Data represent one experiment with 4-6 mice per group. Groups were evaluated by log-rank test. *p<0.05; **p<0.01.



CHAPTER 4

FUTURE DIRECTIONS

Section 1: Mechanism Elucidation

Role of CD4+ effectors in vaccine response

One intriguing finding from this work is that CD4+ T-cell effectors provide vaccine efficacy in the event of depleting CD8+ effector T-cells (Figs 2.10 and 2.11). This was unexpected, as studies with this vaccine platform in the lymphoma mouse model showed that CD4+ effector T-cells were unnecessary for the protection phenotype and that CD8+ effector T-cells fully carried the phenotype themselves [128]. Solid tumors are much different than liquid tumors, however. There is evidence discussed in Chapter 1 that, for melanoma, both CD8+ and CD4+ T-cells are important for effective anti-tumor immune responses [48, 51]. Beyond noting this phenotype, no further work was done to analyze the differences between the CD4+ T-cell compartments of MIP3α-gp100 and antigen-only vaccinated mice.

First, are the CD4+ T-cells directly cytotoxic by MHC-II restricted killing? There is evidence of melanoma expressing MHC-II [49]. MHC-II surface expression has not been assayed in the laboratory's B16F10 cell line. Initially one would utilize immunohistochemistry to detect the surface levels of MHC-II in cultured B16 cells and in B16 cells excised from growing tumor. If there is no MHC-II surface expression, then CD4+ T-cells cannot be directly cytotoxic to the tumor. If there is tumor MHC-II expression, then the CD4+ T-cells can be analyzed for killing ability. Figure 3.4 provided evidence that granzyme+CD4+ T-cells in vaccinated mice are infiltrating the tumor in significant numbers, with 53 such cells per mm^2 of tumor compared to 10 in the negative control. While this is less than the number of infiltrating CD8+Granzyme+ cells ($124/mm^2$), it is enough to provide efficacy in the absence of CD8+ cells. CD4+ T-cell

cytotoxic ability can be analyzed directly by an *in vitro* B16 cell killing assay. The CD4+ TIL population can be purified utilizing a flow sorter or anti-CD4+ coated beads and then assayed for inducing cell death of cultured B16F10 cells.

Second, are the CD4+ T-cells helping an immune cell population that are not CD8+ T-cells? From the results that depleting both CD4+ and CD8+ T-cells shows no protection (Figs 2.10 and 2.11), one can assume that antibodies do not play a significant role. The depletion experiment utilized a prophylactic vaccine approach, and figure 2.4 illustrates that a significant amount of antibody would be present in the circulation by the time of depletion. CD4+ T-cells can also mediate immune responses through production of IFN-γ that stimulates innate cells to produce a more inflammatory environment, with macrophages, neutrophils, and NK cells able to reduce tumor burden [53, 54]. The CD4+ TILs would need to be assayed for IFN-γ production. This could be accomplished in a few different ways. Initially one could assay for IFN-γ by intracellular cytokine staining flow cytometry in both unstimulated and nonspecifically stimulated scenarios. Secondly, one could analyze intratumoral levels of IFN-γ by ELISA assays and/or levels of IFN-γ transcript by qRT-PCR in mice depleted of CD8+ effector cells. Finally, one could perform an ELISPOT assay on mouse splenocytes after either purifying the CD4+ T-cell population or depleting the CD8+ T-cell population.

If the CD4+ T-cells are not being directly cytotoxic and a significant increase in IFN-γ is seen, additional depletions could be utilized to tease out the cell type being activated by the CD4+ T-cells. For instance, in a *Listeria monocytogenes* model, CD4+ T cell-activated macrophages were essential for clearance of the intracellular bacteria [218]. If in a CD8+ depleted mouse, other cell types are depleted or rendered inactive –

macrophages (clodronate liposomes), neutrophils (αLy6G), dendritic cells (αCD11c), NK cells (αNK1.1) – the results could show the cell type directly responsible for the CD4+ T-cell based vaccine efficacy.

Analyzing tumor evolution to vaccine response

An interesting question that arises directly from this work is why doesn't the vaccine work better? What allows the tumors to escape the induced immune response? It is possible that the primary method of immune evasion is attributable to a tolerogenic tumor microenvironment and not directly to the tumor. Chapter 3 showed that depleting the anti-inflammatory cytokine IL-10 enhanced vaccine efficacy, so the state of the microenvironment is clearly important. T-cells, although specific, may become exhausted by mechanisms of immune checkpoints. Combining vaccine with immune checkpoint inhibitors is discussed in a section below.

Another possibility is the effect of the regulatory CD4+ T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Figure 3.5 shows that the percentage of tumor-infiltrating CD4+ T-cells that are Tregs are not significantly different between MIP3α-gp100 vaccinated and mock vaccinated groups. Therefore, peripherally induced Tregs are not likely to explain the tolerance to the vaccine. A pilot study with *Aire*$^{-/-}$ knockout mice analyzed vaccine efficacy in a mouse lacking thymic natural Tregs (nTregs, discussed in Chapter 1). Figure 4.1 shows that efficacy of vaccinated knockouts was not enhanced over vaccinated wild type mice, and therefore nTregs likely do not play a major role in tumor escape from vaccine efficacy. Interestingly, mock vaccinated *Aire*$^{-/-}$

knockout mice were equally protected as vaccinated, so nTregs play a role in melanoma tolerance, but not in tolerance to this vaccine.

I hypothesize that this is due to utilization of the human gene for gp100 in the construct. It has been documented that responses in mice to the human gene product provide better protection than responses to the mouse gene product [219]. This is hypothesized to be because the T-cell clone specific for $gp100_{25\text{-}33}$, an immunodominant epitope, has differences in three MHC-anchoring amino acids that increase the avidity by two logs[219]. This particular T-cell clone escaped central tolerance, and so relief of central tolerance had no measurable effect on the vaccine. However, central tolerance has been shown to play a role in other melanoma antigens in the mouse, such as TRP-2 [220]. It should be noted that, in humans, there are clones for gp100 that escape central tolerance [221, 222], but it is not known if abrogation of central tolerance mechanisms would have an effect on MIP3α-gp100 vaccine response in the human system.

A second pilot study found a trend of higher concentrations of tumor infiltrating MDSCs in mice given MIP3α-gp100 vaccine with αIL-10 treatment compared to mice given mock vaccine (figure 4.2). This shows that MDSCs may play a role in tolerance to the vaccine response. Elevated tumor-infiltrating MDSC levels need to be confirmed with a larger sample size and in an experiment without the αIL-10 therapy. MDSCs play a pivotal role in melanoma T-cell tolerance [223, 224]. I hypothesize that MDSCs are being recruited to the area by non-IL-10 inflammatory mediators such as prostaglandin-E2 and GM-CSF as a response to vaccine-induced inflammation [225], and that affecting the regulatory functionalities of MDSCs would significantly increase the anti-tumor efficacy of the vaccine.

The tumor may also be directly evading immunity. It has been widely reported that melanoma in patients and in mice can readily lose or greatly reduce surface expression of MHC-I upon interaction with an inflammatory environment, especially IFN-γ [85, 86]. Tumors excised at different time points can be analyzed for MHC-I surface expression and transcript levels by immunohistochemistry and RT-PCR, respectively. It has also been reported that expression of non-essential antigens such as gp100 can be selected against by immunity-driven evolution [226]. Protein could be assayed by intracellular immunohistochemistry and expression by RT-PCR of tumor samples collected at progressing time points. If later stage tumors show a difference in MHC-I or gp100, that could be a direct mechanism by which the tumor evades the immune response.

Characterization of anti-tumor efficacy mediated by αIL-10-induced IFNα

As discovered in Chapter 3, blocking IL-10 additively enhances vaccine efficacy. This efficacy enhancement was not mediated by increased numbers of infiltrating T-cells, increased percentage of vaccine-specific CD8+ TILs, reduction of Tregs, or increased numbers or percentages of activated effector T-cells (Figures 3.4-6). The phenotype was, however, correlated with an increase in IFN-α4 expression in the tumor (Figure 3.7). Furthermore, *IFNαr1*$^{-/-}$ mice did not respond to αIL-10 treatment (Figures 3.8-10). As discussed in Chapter 1 and the introduction of Chapter 3, IFN-α is given regularly as an adjuvant therapy in melanoma patients [2, 107, 113], with myriad anti-tumor effects.

Plasmacytoid dendritic cells (pDCs) when activated are known as the primary cell type secreting IFNα, and their activation has been shown to be regulated by IL-10 [96,

185, 213, 214, 227]. By flow cytometry, it was seen in a pilot study that MIP3α-gp100 vaccine plus blocking IL-10 resulted in a dramatic increase of pDCs infiltrating the tumor over mock vaccine (Figure 4.3). This experiment needs to be expanded to compare vaccine alone to vaccine with αIL-10 to see if this influx of pDCs is due to blocking IL-10 or the vaccine response. An increase of activated pDCs would make sense when taken in context with the data in Chapter 3, with significantly increased levels of IFNα4 transcript in the tumor (Fig 3.7). If αIL-10 is increasing the level of pDCs, experiments could be performed to analyze the activation state of these pDCs, specifically their ability to produce type-I interferons, especially IFNα. This line of experimentation could confirm the origin of the IFNα4, providing more evidence for the proposed model.

IFN-α has been shown to enhance vaccine-induced CTL numbers, effector function, and anti-tumor activity[208]. The data in figure 3.4 indicate that the numbers of vaccine-induced CD8+ T-cells were actually decreased (not significantly) with αIL-10 treatment. By surface marker analysis, blocking IL-10 had no significant effect on numbers of granzyme+ T-cells or PD-1+ T-cells (Figures 3.4-5), suggesting that there is no difference in numbers of activated infiltrating effector cells. However, the functionality of the cells was not directly assessed. It was noted that in the PMA/ionomycin control groups of TIL intracellular cytokine staining assays, blocking IL-10 led to more general activation of CTLs (85% to 62%, n=1). This should be repeated as an experiment on its own to assess overall potential activation and not just those effectors induced by gp100 stimulation. If CTLs respond more robustly to nonspecific activation, *in vitro* killing assays could confirm potential cytotoxic

differences in the overall pools of CD8+ T-cells between the groups. Stimulation of IFN-γ production by effector CD4+ T-cells should also be investigated.

Section 2: Therapy Enhancements and Applications

Vaccine Optimization

The current vaccination administration system works with significant efficacy and immunogenicity in a therapeutic melanoma model, as shown in detail in Chapter 2. However, there is still room for improvement. The vaccine construct itself will remain constant, but total formulation and method of administration can be altered. First, when comparing results between i.m. electroporation and gene gun (figures 2.1 versus [130]), it appears that the protection seen from the gene gun is better. It has been reported that electroporation is superior to gene gun in a cervical cancer model [228], but the two techniques provide similar immune responses in an vaccine against β-amyloid [229].

It is known that the two methods elicit different immune responses [230]. Vaccinating against influenza hemagglutinin, gene gun and electroporation (EP) developed CD8+ effector responses of similar magnitude. However, Gene gun elicited Th2 skewed antibody responses whereas EP elicited a Th1 response, indicating that the CD4+ effector cells elicited are fundamentally different between the two techniques [230]. This may also help explain why that the gene-gun administered MIP3α-OFA vaccine elicited an anti-lymphoma response contingent solely on CD8+ effector T-cells [128], whereas the model presented here shows that both CD4+ and CD8+ effector T-cells play direct roles in efficacy (Figures 2.9-11). It would be interesting to directly test

gene gun and EP immunization strategies to see which provides better efficacy in melanoma and how the responses differ immunologically.

Adjuvants could also help to increase vaccine potency. This vaccine has been administered by direct i.m. injection of plasmid incorporated with Vaxfectin® (Vical, San Diego, CA), a lipid-based adjuvant that has been shown in some models to be an effective adjuvant for DNA vaccines [231–233], including a MIP3α-CSP anti-malaria vaccine [165]. No protection with a MIP3α-gp100 vaccine was seen, however (figure 4.4). Thus, it is unlikely that any lipid-based adjuvant would enhance efficacy of this vaccine. It was determined in the malaria model that the MIP3α vaccine component targets antigen to DCs but does not recruit DCs to the area [165]. GM-CSF stimulates dendritic cell maturation, development, and proliferation, and it has been shown to enhance T-cell responses to a DNA vaccine [155]. I hypothesize that adding a second plasmid to the vaccine expressing GM-CSF would significantly enhance vaccine potency.

Checkpoint Inhibitors

As described in Chapter 1, the checkpoint inhibitors αCTLA-4 and αPD-1 are revolutionizing cancer treatment by negating the effects of T-cell negative regulators, 'releasing the brakes' of the immune system, so to speak [133, 234]. These treatments only work if a T-cell immune response exists in the first place. It is hypothesized that the combination of a vaccine (to induce anti-tumor T-cells) and checkpoint inhibitors (to maintain T-cell potency) can lead to synergistic anti-tumor efficacy [136].

A pilot study examined whether blocking PD-1 in addition to blocking IL-10 would lead to further enhancement of mouse survival. However, figure 4.5 shows that

blocking PD-1 did not enhance therapeutic efficacy. Although not tested directly in this experiment, comparing figure 4.5 to figures 3.1-2 leads to the inference that αPD-1 did not add efficacy beyond what is regularly seen by αIL-10 itself. However, in other vaccine contexts, αCTLA-4 shows significant enhanced efficacy in mouse models [235, 236], and αPD-1 synergizes well with αCTLA-4 [204]. Therefore, I hypothesize that the efficacy of the MIP3α-gp100 vaccine would be significantly enhanced by αCTLA-4 treatment, which would be further enhanced by the addition of αPD-1. Other immunostimulatory antibodies such as agonists of 4-1BB [237] would be intriguing to test with the current vaccine platform as well.

Other Cancer Models

This vaccine platform has so far been tested only in one solid tumor model: B16 melanoma. One important question for this vaccine is the extent of its universal application to other tumor systems. A logical next system to analyze would be the 4T1 mouse breast tumor model, as it shares many important traits with B16. Like B16, 4T1 is a transplantable tumor system able to be grown in cell culture that originated spontaneously from the natural anatomical site without aid from mutagens [238]. In 4T1's case, the tumor originated from a mammary gland in a murine mammary tumor virus + (MMTV+) BALB/c mouse[239]. The transplantable syngeneic cell line is potently tumorigenic and invasive. Also like B16, cells are easily transplanted into the relevant tissue type so that tumor grows in the anatomically correct site [238]. Therapeutic potential against a 4T1 primary tumor would further show the utility of this vaccine platform.

A major strength of the 4T1 system over B16 is the ability to analyze metastatic potential in its natural context. In B16, a metastatic model is generated by an artificial system of tail-vein injection of B16 cells and analysis of tumor spots found on the lungs at later time points[104]. With 4T1, the primary tumor metastasizes progressively in a manner that mimics the clinical course of human metastatic breast cancer[238]. This would allow the vaccine to be utilized in a manner analogous to that used in the clinical setting. First tumors would be induced in the mice, and then the mice would be treated with vaccine. The tumor will likely shrink or at the very least slow its growth. At this point the tumor will be surgically removed, and the course of vaccinations will continue until complete. In control groups, metastases should develop after surgical removal of the primary tumor. The experimental groups will examine whether the vaccine can inhibit metastases from forming new tumors. This scenario is likely where a therapeutic vaccine would fit into current treatment plans: after the surgical removal of the primary tumor in hopes of inhibiting growth of metastases and preventing recurrence of the primary tumor. I hypothesize that this vaccine platform will exhibit significant efficacy in the 4T1 system, slowing primary tumor growth and inhibiting metastases.

Multiple antigens

An important aspect of this platform yet to be studied is its ability to provide immunogenicity against multiple targets. With a cancer system, it is preferable to create immunity against more than one protein to minimize the capability of tumor cells to undergo specific antigen immunoselection that leads to vaccine escape by cells no longer expressing vaccine antigen [226]. In the B16 melanoma model, there are several antigens

that can be utilized in vaccines in addition to gp100, including overexpressed antigens tyrosinase, tyrosinase-related proteins (TRP)-1 and TRP-2, and MART-1 (Table 1.2).

First, it has been shown that including long peptides in a cancer vaccine is preferable to the practice of only including the known CD8+ CTL epitopes that fit into MHC-1 [240]. Therefore, all constructs will be of the long peptide format. First, MIP3α-antigen constructs will be made with all antigens listed above, and all of them will be tested individually utilizing the standard therapeutic protocol. The two antigens that show the best protection will be analyzed further. Utilizing new cloning strategies, vaccines will be devised with the two MIP3α-gp100-antigen1/2 groups and a MIP3α-gp100-antigen1-antigen2 construct, all compared to the original MIP3α-gp100 construct to test for enhanced efficacy. Immunogenicity to gp100 and the new antigens will also be assessed to ensure all antigens included are stimulating immune responses. I hypothesize that targeting multiple antigens will lead to enhanced vaccine efficacy, as long as issues of antigenic competition do not minimize immune activation with multiple antigens.

Neoantigens

Finally, the most exciting extension of this research is the promise seen with neoantigens. As briefly introduced in Chapter 1, neoantigens are the result of mutations seen in proteins of the cancer. Neoantigens can be a part of driver mutations that share partial responsibility for the cancerous phenotype or can be bystander mutations that arise from the progressive genetic instability of cancer cells. In either case, the mutations create regions of proteins that no longer look like "self" to T-cells. These peptides, if immunogenic, can stimulate T-cells that are not restricted by mechanisms of central

tolerance, creating potentially strong immune responses against the "foreign" cancer neoantigens [134, 241]. It is hypothesized that many successful long-term anti-tumor immune responses generated by current therapies are actually due to the development of potent immune responses to tumor neoantigens [242, 243]. In addition, the neoantigen mutations are only found in the tumor, so specific off-target immune reactions are unlikely.

The issue with utilizing neoantigens in a vaccine is identifying them. Not only does one need to find the mutated sequences, but through *in silico* screens followed by *in vitro* verifications, the immunogenicity of the neoantigens must be analyzed. This includes analyzing the inherent cleavage sites by proteosomal machinery and the ability to bind MHC-1, MHC-II, and T-cell receptors. Tools to analyze these characteristics currently exist, and they are continuously being refined. However, as of right now, their functionality is that of a screen, as *in vitro* confirmation is necessary [134, 241, 244, 245]. The field is moving very quickly now, and protocols are already proposed for neoantigen-based therapies in human cancers [72].

A seminal paper has fully analyzed the B16F10 melanoma genome for immunogenic neoantigens [174]. This study found 563 nonsynonymous somatic point mutations in expressed genes, including both cancer driver genes and passenger genes. They selected 50 for immunogenicity analyses, and found one third of the peptides to be immunogenic, with 60% of those eliciting an immune response against their respective mutated neoantigens. Two of the most immunogenic were analyzed by peptide vaccination, with significant protection shown for both [174].

The groundwork has been laid for testing neoantigens in the B16 model. B16F10, like all cancers and immortalized cell lines, is prone to mutations over time. Therefore, the first step needed is to validate the mutations in the laboratory strain of B16 cells. This can easily be done by harvesting B16 cellular DNA and utilizing PCR with Taq polymerase to generate specific fragments of the regions notated in the Castle, et al. paper. Those fragments can be inserted into a TOPO® TA vector (ThermoFisher Scientific, Halethorpe, MD) and then sequenced. Direct comparison of the sequences here versus in the literature will show if the neoantigen mutations exist in the laboratory line. If not, cells will need to be requested from Castle, et al.

Once neoantigen sequences are confirmed, the fragments can be cloned and inserted into the vaccine MIP3α-antigen plasmid. Immunogenicity and efficacy will need to be determined using the protocols present in this dissertation along with any modifications that have arisen due to the above referenced optimizations. These assays will be performed in concert with the MIP3α-gp100 vaccine to determine relative efficacy of neoantigen vaccination. I hypothesize that the neoantigen vaccines will have enhanced immunogenicity and efficacy over the gp100 vaccine.

Some of the primary strengths of the MIP3α-antigen vaccine platform beyond enhanced efficacy are flexibility and ease of alteration. This modular DNA-vaccine can be successfully altered to any desired antigen and can be ready for immunization within a few weeks. The celerity of construction lends itself very well to clinical neoantigen discovery. It takes several weeks to identify and screen potential neoantigens. However, once the antigens are known, the personalized vaccine could be made quickly and in enough time to provide therapeutic efficacy for the majority of patients. Therefore, I

believe that neoantigen vaccination will be the primary translational focus for this vaccine platform in the melanoma system and will be a major avenue of future research.

Chapter 4: Figures

Figure 4.1: Analysis of *Aire* $^{-/-}$ vaccinated mice. In this experiment, the standard therapeutic protocol was utilized (see methods of Chapters 2 and 3). This figure represents one experiment with four mice per group. No comparisons were significant by Anova.

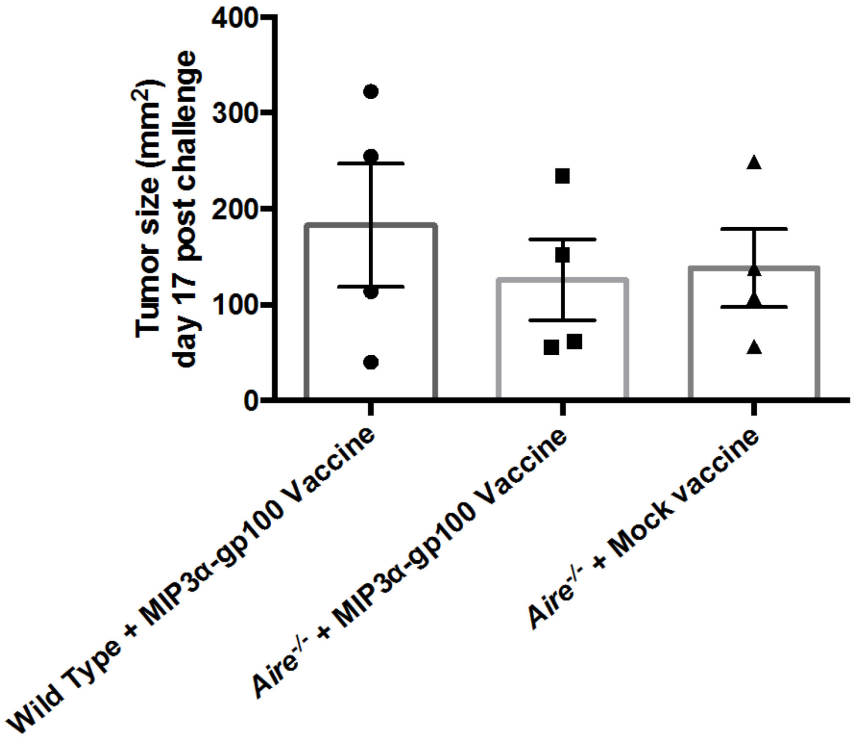


Figure 4.2: Tumor infiltrating MDSC analysis. The standard therapeutic protocol was utilized as defined in Chapter 3, except mice were sacrificed on day 13 post tumor challenge. Tumor infiltrating immune cells were enriched as described by methods of Chapters 2 and 3. MDSCs were analyzed by flow cytometry by being a Gr-1+, CD11b+, CD11c- cell population. Flow cytometry analyzed as described in Chapter 2, utilizing Gr-1-FITC, CD11b-APC, and CD11c-PE stains from eBioscience (San Diego, CA). Graph represents one experiment of n=3, and was analyzed by t-test ($p = 0.0515$).

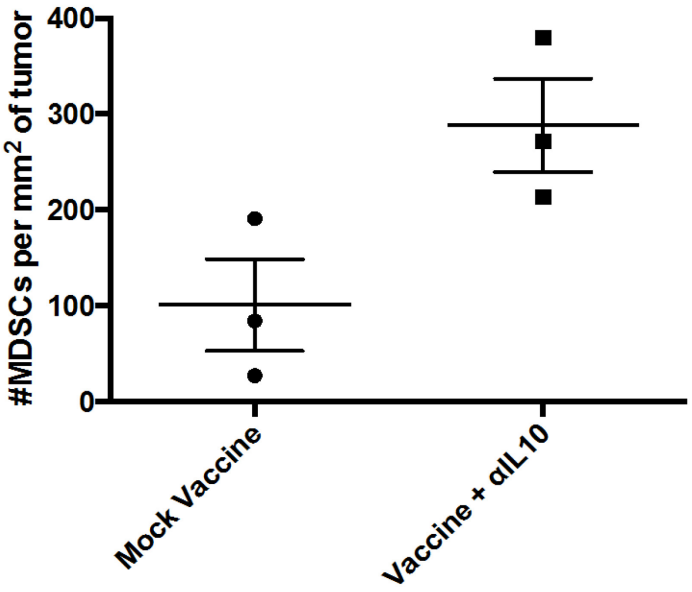


Figure 4.3: Tumor-infiltrating plasmacytoid dendritic cell analysis. The standard therapeutic protocol was utilized as defined in Chapter 3, except mice were sacrificed on day 13 post tumor challenge. Tumor infiltrating immune cells were enriched as described by methods of Chapters 2 and 3. pDCs were analyzed by flow cytometry and were defined as Gr-1 low, CD11b-, CD11c low, Ly6C+ cell population. Flow cytometry analyzed as described in Chapter 2, utilizing Gr-1-FITC, Ly6C-Percp-Cy5.5, CD11b-APC, and CD11c-PE stains from eBioscience (San Diego, CA). Graph represents one experiment of n=3, and was found to be significant by t-test ($p<0.05$).

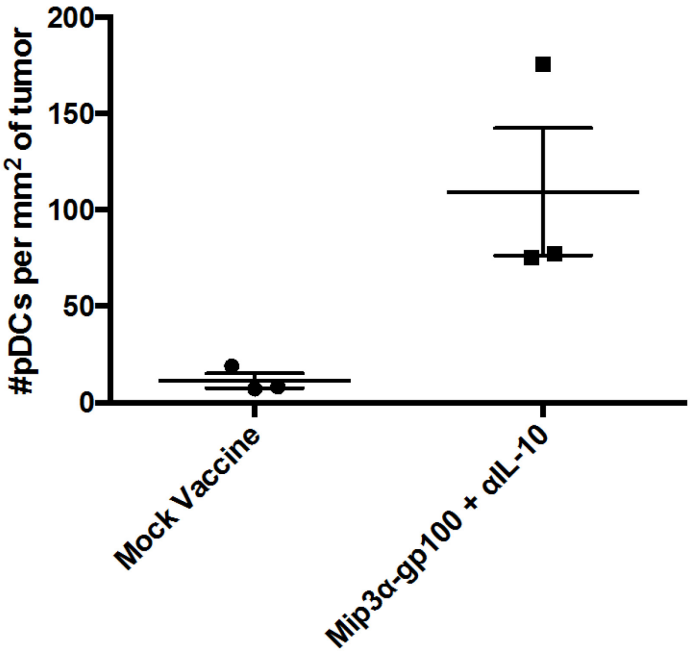


Figure 4.4: MIP3α-gp100 vaccine with Vaxfectin® (Vical, San Diego, CA) adjuvant formulation. Vaccine was administered i.m. with formulation of Vaxfectin® in a prophylactic protocol. Vaccine given every two weeks, and two weeks post third immunization the mice were challenged with $5x10^4$ B16 cells. This figure represents one experiment with 4-5 mice per group.

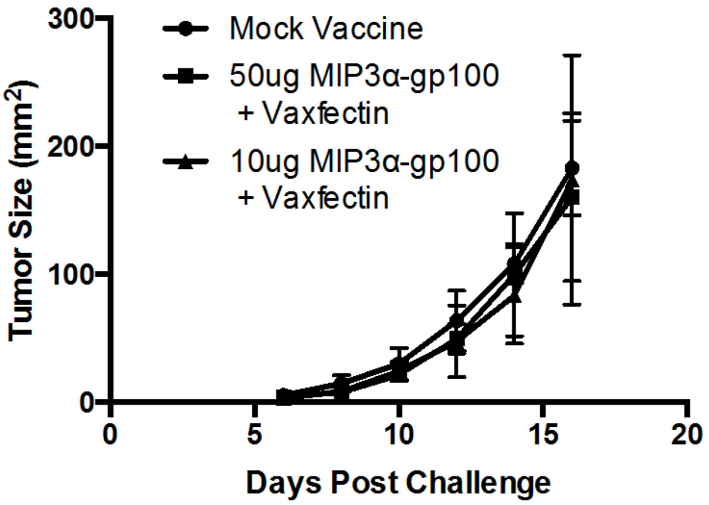
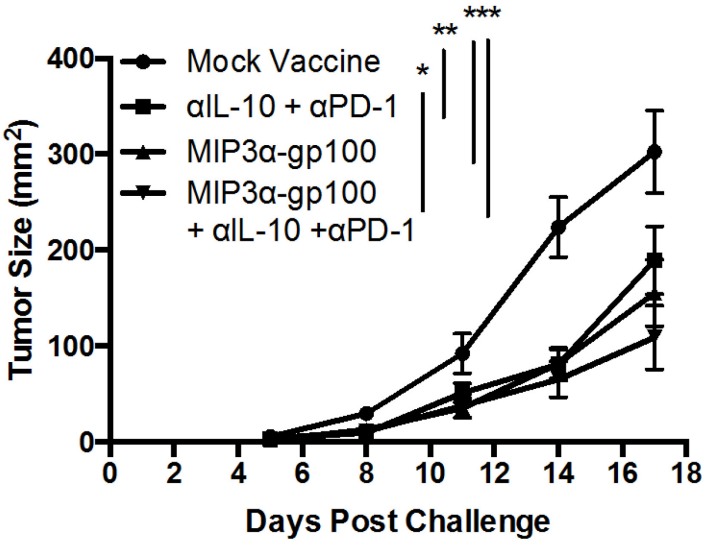


Figure 4.5: Analyzing effect of adding αPD-1 to MIP3α-gp100 + αIL-10 therapy. This experiment utilized the standard therapeutic protocol as defined in Chapter 3, with the addition of αPD-1 antibody (clone RMP1-14, BioXcell, West Lebanon, NH) administered intraperitoneally with doses of 250μg given on the same days as αIL-10. The graph represents one experiment with 7-8 mice per group. Data were evaluated by a statistical mixed effects regression model. *p<0.05, **p<0.01, ***p<0.001



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Gordy, J.T**.**, Jones, C.A., Rue, J., Cynda Crawford, P., Levy, J.K., Stallknecht, D.E., Tripp, R.A. and Tompkins, S.M. (2012), Surveillance of feral cats for influenza A virus in North Central Florida. *Influenza and Other Respiratory Viruses*, 6: 341-347.

- *Role* – Project leader: designed and performed experiments, analyzed data, and wrote and submitted paper

Driskell, E.A., Pickens, J.A., Humberd-Smith, J., Gordy, J.T**.**, Bradley, K.C., Steinhauer, D.A., Berghaus, R.D., Stallknecht, D.E., Howerth, E.W., Tompkins, S.M. (2012), Low Pathogenic Avian Influenza Isolates from Wild Birds Replicate and Transmit via Contact in Ferrets without Prior Adaptation. *PLOS One.* 7(6): e38067.

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Gordy, J.T., Luo, K., Zhang, H., Biragyn, A, and R.B. Markham. Fusion of the Dendritic Cell-Targeting Chemokine MIP3α to Melanoma Antigen Gp100 in a Therapeutic DNA Vaccine Significantly Enhances Immunogenicity and Survival in a Mouse Melanoma Model. Under review in *Journal for ImmunoTherapy of Cancer*, submitted 5/20/16

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- Poster, May 24, 2016, *Sidney Kimmel Cancer Center Fellow Research Day*, JHU School of Medicine (SoM), Baltimore, Md
- Poster, April 18-19, 2016, *19th Annual Conference on Vaccine Research*, Hyatt Regency, Baltimore, Md
- Oral, April 11th, 2016 and December 2nd, 2014, *Tumor Immunology Research in Progress*, JHU SoM, Baltimore, Md
- Poster, April 20th, 2015, *American Association for Cancer Research (AACR) National Conference*, Pennsylvania Convention Center, Philadelphia, Pa

"Surveillance of Feral Cats for Influenza A Infection"

- Oral, October 14th, 2010, *Science of Veterinary Medicine Research Day*, UGA CVM, Athens, Ga
- Poster, April 18-20, 2010, *Swine Origin H1N1 Virus: The First Pandemic of the 21st Century,* Emory University, Atlanta, Ga

- Poster, July 26-28, 2009, *Immunobiology of Influenza Virus Infection Conference*, UGA, Athens, Ga

"Developing New Methodologies for the Study of Small Open Reading Frames and Small Proteins in the Hyperthermophilic Archaeon *Pyrococcus furiosus*"

- Oral, April 10-12, 2008, *National Conference for Undergrad. Research (NCUR)*, Salisbury University, Salisbury, Md
- Oral, March 31, 2008, *Center for Undergrad. Research Opportunities (CURO) Symposium*, The Classic Center, Athens, Ga

Pedagogy:

Courses Taught

"Immunity and Cancer: How the Recent Paradigm Shift in Treatment Affects Public Health" (Spring 2016)*

- Proposed and accepted for Gordis Teaching Fellowship at Johns Hopkins University (JHU), Baltimore, Md
- Creator, organizer, and primary instructor of course, undergraduate level, class size 12

"Techniques in Molecular Biology", JHU (Summer 2015* and Summer 2016)

- College-level lecture/laboratory course during JHU Summer Institute for 17 students
- Fully responsible for all aspects of course: design, preparation, curriculum, evaluation, lectures, and laboratory

Lectures Given

"Introduction to Cancer Immunology," Introduction to Immunology, Towson University, Towson, Md (Fall 2015)

"Evolution of Antibiotics and Resistance," Evolution of Infectious Diseases, JHSPH (Fall 2014 and Fall 2015)

"Nucleotide Structure and Function," Introduction to Biological Molecules, JHU (Summer 2014)

Preparing Future Faculty Teaching Academy, 2013-14, JHU

Phases I and II: Foundation and Immersion. Completed via TA Certification and Teaching at the University Level course

Phase III: Capstone. Completed by preparing and teaching two labs and a double lecture of the Johns Hopkins University undergraduate summer course Introduction to Biological Molecules under tutelage of Dr. Richard Shingles (July 2014)

Teaching Assistant (TA)

"Evolution of Infectious Diseases", JHSPH (Fall 2012 – 2015)*

- Actively participated in Graduate-level classroom pedagogy, administration, online presence, and evaluation
- Organized and implemented office hours and group review sessions, and acted as liaison b/w students and faculty

"Introduction to Molecular Biology Techniques", JHSPH (January 2015)*

- Leader of course organization, pedagogy, and implementation
- Created syllabus, course description, course objectives, and course online components

Tutoring

TutoringZone (2008-2010); Athens, Ga

- Developed lesson plans for Q&A sessions (2-6 ppl), weekly group sessions (5-25), and large exam reviews (10-50)

UGA Div. of Academic Enhancement (2005-2006); and UGA Athletic Assoc, (2006-2007); Athens, Ga

- Facilitated drop-in sessions, computer lab help, and private sessions in individual and small-group formats

*Student evaluations available upon request

Honors, Awards, and Activities:

Fellowships and Grants

Gordis Teaching Fellowship (2015-2016): Supports teaching course of my design to

Delta Omega Scholarship (2015): Merit-based research grant judged by expert ΔΩ panel

Student Assembly Conference Fund (2015): Funded AACR National Meeting reg. dues

Excellence in Undergraduate Research Award (2008): Funded travel and expenses to

CURO Summer Fellowship (2007): One of 27. Granted $2500 scholarship for summer

Activities and Services

Faculty Liaison (2013-2015): Acted as intermediary between faculty and graduate

Recruitment Committee (2013-2015): Assisted with recruitment process of incoming

Social Media Chair (2014-2015): Responsible for updating and fostering growth of

Alumni Day Moderator (2014): Moderated for alumni day panel comprising of MMI

Article Review Assistant (2010-2011): Assisted reviewing manuscripts in *Influenza and*

CURO Political Liaison (2008): Member of group touting benefits of undergraduate

Journal of Undergraduate Research Opportunities (2006-2008): Edited research papers

Eagle Scout, (2004): Troop 99. Eagle Project: cleaning and renovating church property

Honors and Awards

Bliss Fellowship (2013): Recognizes an outstanding student in molecular microbiology

Dr. Harry J Lawler Award Fund (2012): Recognizes an outstanding student in molecular

CURO Scholar (2008): Submitted undergraduate thesis, performed 12 research credit

Phi-Beta-Kappa (2008): Inducted into academic honors society