

The Development of Cancer Vaccines Targeting Neoantigens for the Treatment
of Malignant Astrocytomas

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults. Conventional therapies for GBM typically fail to provide lasting antitumor benefits, owing to their inability to specifically eliminate all malignant cells.

Immunotherapy is currently being pursued as a strategy to address this unmet need, in light of the cell-specific cytotoxicity an immune response can afford. Of the various immunotherapeutic modalities, cancer vaccines are currently being evaluated as a means to direct the adaptive immune system to target residual GBM cells that remain following standard-of-care treatment. To date, no cancer vaccines have been proven effective against GBM; however, only a few have reached phase III clinical testing. Clinical immunological monitoring data suggests that GBM vaccines are capable of stimulating immune responses reactive to GBM antigens, but whether these responses have an appreciable antitumor effect on GBM is still uncertain. Nevertheless, there have been several promising outcomes in early phase clinical trials, which lend encouragement to this area of study.

In this dissertation, we explore the therapeutic potential of cancer vaccines targeting malignant astrocytoma-specific somatic missense mutations – or neoantigens. This pursuit was inspired by recent data from a phase III clinical trial with a protein vaccine targeting the GBM-specific antigen EGFRvIII, revealing that most recurrent

tumors were composed of EGFRvIII-deficient or -suppressed tumor cell variants. This outcome, known as antigen escape, is likely a consequence of the profound heterogeneity of GBM tumors and, altogether, suggests that monovalent immunotherapeutic strategies targeting subclonal GBM antigens are likely insufficient to treat this disease. Conversely, personalized cancer vaccines targeting patient-specific missense mutations have the potential to elicit a multivalent, tumor-specific immune response that may target a broader repertoire of GBM cells.

Chapters 1-4 offer a comprehensive review of GBM, an overview of immunotherapy for malignant brain tumors, and promising vaccines that are currently being explored for the treatment of GBM. In chapter 5, we present a novel method that we have developed for evaluating neoantigen-specific lymphocytes from miniscule amounts of solid tumor tissue, which we believe can aid in immunological monitoring of neoantigen-specific immune responses in the clinic. In chapter 6, we elucidate the mechanism of an efficacious neoantigen vaccine, which led to the development of rationally-designed, neoantigen-targeting, synthetic long peptide vaccines with enhanced immunogenicity and efficacy using a universal helper epitope. In chapter 7, we explore the utility of minigene-transfected dendritic cell (DC) vaccines for targeting neoantigens, in which we reveal several significant limitations of traditional GM-CSF + IL-4-generated DCs. Finally, chapter 8 discusses future prospects for enhancing the therapeutic response by cancer vaccines. Together, this original work provides several

encouraging insights for the development and evaluation of personalized cancer vaccines for GBM.

Dedication

This dissertation is dedicated to my loving wife, Hanqian, my father, Steve, my mother, Leesa, my sister, Angela, my grandparents, Mary, Madeline, Ronald, and Elwood, and to the rest of my family who unconditionally encourage and support me in whatever path I choose.

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1. An Introduction to Malignant Gliomas

1.1 Permissions and collaborations

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1.2 A primer on gliomas

Gliomas arise from glial cells. The three types of glial cells that are known to produce tumors are the astrocytes, oligodendrocytes, and ependymal cells. Astrocytes, which make up 20–40% of all glial cells, are regarded as the supportive cells of the CNS and have a wide range of functions including, but not limited to, metabolic and biochemical regulation, structural support, and maintenance of the blood–brain barrier. Oligodendrocytes also play a supportive role, albeit not as extensive as astrocytes, by providing electrical insulation by encasing neuronal axons within the CNS with a lipid-rich myelin sheath. Finally, ependymal cells line the ventricular system and central canal and are involved in the production of cerebrospinal fluid. The exact etiological mechanisms that cause these cells to transform into cancerous cells with uncontrolled

growth have not been fully clarified, though several intrinsic and environmental factors have been proposed.

Gliomas represent the second most common primary CNS-resident tumor (~27%), behind only meningiomas (~36%). However, given that most meningiomas are benign, gliomas hold the title of the most common primary malignant CNS-resident tumor by a wide margin (~80%). Although they are not staged, gliomas are classified by a World Health Organization grading system that reflects their organization and structure relative to normal tissue (i.e., differentiation), growth potential, and aggressiveness. Grade I and II gliomas generally have a more differentiated phenotype, are slower-growing, and less aggressive. These low-grade gliomas make up the majority of brain tumors that afflict children and adolescents. Alternatively, grade III and IV gliomas are poorly differentiated, grow rapidly, and are highly aggressive. These gliomas are designated as malignant or high-grade gliomas, occur more frequently in adults, and make up >90% of all diagnosed gliomas (Ostrom et al., 2017).

There are several types of gliomas that occur in both children and adults, which generally include oligodendrogliomas, ependymomas, astrocytomas, optic nerve gliomas, and mixed gliomas. Gliomas can develop in the brain, spinal cord, and brain stem—the latter more commonly occurring in children and young adults. Grade III gliomas includes anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic

oligoastrocytoma, and anaplastic ependymoma. The brain stem glioma known as diffuse intrinsic pontine glioma is also classified as a high-grade glioma and is exceptionally difficult to treat due to its location. Grade IV includes only the astrocytoma known as glioblastoma (GBM), which typically develops *de novo* (i.e., primary GBM) but can also derive from lower-grade gliomas (i.e., secondary GBM). GBM is the most common and aggressive primary malignant brain tumor, representing ~55% of all gliomas (Figure 1), and has an incidence of ~2–3 in 100,000 people in the United States and Europe (Ostrom et al., 2017).

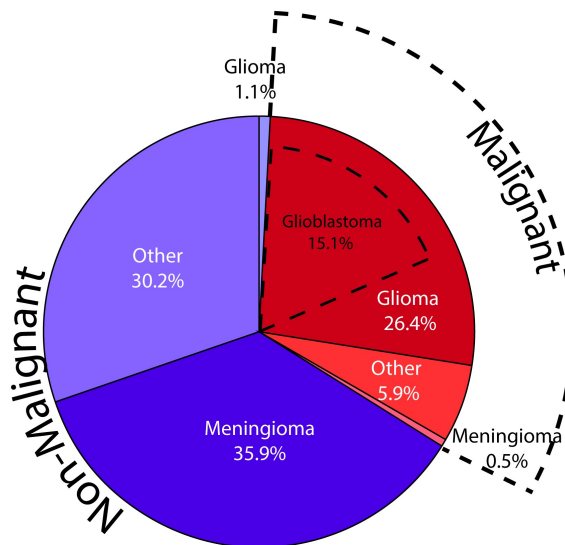


Figure 1: Distribution of malignant and nonmalignant brain and CNS-resident tumors.

Data derived from CBTRUS statistical report on primary brain and other central nervous system tumors diagnosed in the United States (Ostrom et al., 2017), and figure reproduced with permission (A.M. Swartz et al., 2017).

Multidimensional genomic analyses have shown that GBM represents a very diverse class of tumors and can be divided into several subtypes, each with their own unique signature. The classical subtype is frequently characterized by aberrations in the EGFR gene. The mesenchymal subtype typically exhibits deletions in the NF1 gene. The proneural subtype is typified by alterations in the PDGFRA gene, as well as point mutations in IDH1; this group has been shown to make up a large fraction of secondary GBMs. Finally, the neural subtype is distinguished by the expression of a number of neuronal markers (Verhaak et al., 2010); however, it has recently been proposed that this ‘subtype’ is actually just neural cell contamination (Q. Wang et al., 2018).

1.3 Current management of newly-diagnosed GBM

The current standard-of-care therapy for GBM begins with gross tumor resection. In many cases, complete tumor resection is not practical as it may potentially lead to impairment of neurological function, leading to a poorer quality of life in patients; however, advancements in image-guided surgical modalities have enhanced tumor debridement, while mitigating the damage to surrounding tissues. These techniques include intraoperative and functional magnetic resonance imaging (MRI), cortical

mapping, tractography, and fluorescence-guided tumor resection. Surgery alone is generally insufficient as a treatment considering the highly infiltrative nature of GBM, as evidenced by tumor recurrence in patients who underwent a hemispherectomy (Dandy, 1928). Though GBM is unique from most other cancers in that these malignancies rarely metastasize, in ~95% of cases, recurrent tumors are found within 2 cm of the original tumor (Hou et al., 2006), highlighting the infiltrative nature of this disease.

Resection is followed by fractionated focal radiotherapy administered at 2 Gy per fraction once daily five days per week over a 6-week period, for an accumulated dose of 60 Gy. Concomitant with radiotherapy, temozolomide (TMZ) chemotherapy is given daily at a dose of 75 mg/m². After a 4-week break, patients then receive maintenance TMZ on days 1-5 every 28 days for 6 cycles at a dose of 150-200 mg/m² (Stupp et al., 2005).

TMZ is an alkylating agent whose therapeutic benefit is derived from its ability to methylate N-7 or O-6 positions of guanine residues, ultimately leading to cell death. The enzyme encoded by O⁶-methylguanine-DNA methyltransferase (MGMT), O⁶-alkylguanine DNA alkyltransferase, diminishes the efficacy of TMZ by removing alkyl groups from DNA. Consequently, it has been demonstrated that the silencing of MGMT expression via methylation of the MGMT promoter contributes to a better prognosis in GBM patients (Hegi et al., 2005; Stupp et al., 2005).

Using this strategy, median progression-free survival (PFS) is approximately 6.9 months and median overall survival (OS) is roughly 14.6 months (Stupp et al., 2009; Stupp et al., 2005). While this protocol has been shown to extend survival to a greater extent than radiotherapy alone (Stupp et al., 2005), tumors almost invariably recur.

1.4 Alternative investigational therapies for GBM

Nitrosoureas have demonstrated the ability to kill glioma cells *in vitro*; however, the short half-life (~20 minutes) and systemic toxicity of these chemicals limit their clinical use. Alternatively, implantation of carmustine (3-bis (2-chloroethyl 1)-1-nitrosourea)-impregnated polymer wafers (Gliadel® wafers) within the surgical cavity have been used to slowly release the chemotherapeutic agent locally over a 2-3 week period (Brem et al., 1995). Phase III trials have shown that Gliadel® wafer implantation, post-resection is a safe and effective method of treating newly diagnosed and recurrent GBMs (Westphal et al., 2003).

Approved by the FDA in 2009, the humanized monoclonal IgG1 antibody bevacizumab (Avastin®) has proven safe for the treatment of GBM (Chamberlain, 2011; H. S. Friedman et al., 2009). Bevacizumab targets the angiogenic vascular endothelial growth factor (VEGF), which is secreted by highly vascularized GBM (Gil-Gil et al., 2013; Kreisl et al., 2011). The recent results of the two Phase III randomized clinical trials RTOG 0825 and AVAGlio, which investigated the use of bevacizumab along with the

current standard of care therapy in newly diagnosed GBM patients, demonstrated mixed results, however. These studies showed that, although bevacizumab was well tolerated, median overall survival was not significantly increased (Weller et al., 2013).

Several alternative therapeutics are currently being investigated for the treatment of GBM, though the list is much too long to address here. Additional targets include receptor tyrosine kinases (e.g. VEGFR, EGFR, HER2, and PDGFR), signal transduction molecules (e.g. mTOR, PKC, and AKT), and post-translational modifiers (e.g. HDAC and farnesyl transferase) (Agnihotri et al., 2013). Conventionally, the therapeutic targeting of biological molecules employs synthetic small molecule ligands or antibodies. Though these modalities have their advantages, small molecule drugs often have undesirable off-target effects, and there is debate as to whether antibodies can readily cross into the brain parenchyma of GBM patients.

1.5 Therapies for recurrent GBM and other malignant gliomas

Although there is no established standard of care for alternative (non-GBM) malignant gliomas or recurrent GBM, treatment is generally similar. As with newly-diagnosed GBM, therapy only slightly prolongs survival, in most cases. Not only are the conventional therapies used to treat malignant gliomas incapacitating and damaging to healthy tissues, they ultimately suffer from a lack of tumor selectivity. A hallmark feature of malignant gliomas is their ability to infiltrate and diffuse into normal, healthy

CNS tissues. Consequently, any residual tumor cells with stem cell-like attributes that remain following therapy have the potential to repopulate a new tumor. To overcome these limitations, a novel modality that can selectively target malignant glioma cells is likely required.

2. Immunotherapy for Malignant Gliomas

2.1 Introduction to immunotherapy for malignant gliomas

Malignant gliomas are an especially aggressive group of diseases that are largely recalcitrant to conventional therapies. Even with modern advancements in treatment, the median overall survival for patients afflicted with these diseases is only roughly 15 months. The inability of standard therapies to safely and specifically eliminate all cancerous cells leaves patients vulnerable to tumor relapse and underscores the demand for alternative treatment modalities. In response, researchers across the globe are now attempting to exploit the immune system's natural cytotoxic capacity to mount an aggressive immunological siege upon cancer—a field known as immunotherapy. From this therapeutic paradigm, a variety of novel treatment strategies were born, several of which are showing promise against multiple cancers.

Although great progress is being made in this area of research, the use of immunotherapy for malignant gliomas is still a relatively new concept. Furthermore, these CNS-resident neoplasms are proving to be particularly resilient in the face of potent immunological intervention. Despite these obstacles, studies have elucidated the undeniable connection between the immune system and CNS-resident tumors. With each immunotherapeutic attempt that is made, the evasive mechanisms of these tumors

become clearer, providing hope that one day immunotherapy will be at least a component of the successful eradication of malignant gliomas.

2.2 A brief history of cancer immunotherapy

The notion that the immune system can detect and eliminate cancerous cells has been around since 1909, when Paul Ehrlich made this proposition (Strebhardt et al., 2008). In the 1950s, Sir Frank MacFarlane Burnet and Lewis Thomas elaborated upon this theory by claiming that malignant cells arise within a host quite regularly but are quickly eliminated by the immune system—a concept known as immunosurveillance (Burnet, 1957). This theory was dealt a significant blow in the 1970s when it was realized that athymic nude mice, which at that time were considered to be devoid of a functioning immune system, did not develop cancer at an increased rate (Stutman, 1974). However, it was later determined that these mice were not so immunodeficient after all (Ikehara et al., 1984; Maleckar et al., 1987), and studies with mice lacking immunological effector molecules (e.g., IFN- γ) did, in fact, exhibit a higher incidence of cancer (Street et al., 2001; Street et al., 2002). More recently, in 2004, Gavin Dunn, Lloyd Old, and Robert Schreiber put forth a theory known as immunoediting (Dunn et al., 2004), positing that tumors are sculpted by the immune system, selecting for tumor cells with a low-immunogenic phenotype. Thus, it would appear that cancer has managed to exploit an evolutionary weak point in the immune system, leaving many to question

whether the immune system truly has the capacity to eradicate all malignant cells within a host. Fortunately, however, there seems to be more to the story.

One of the major considerations of cancer immunotherapy regards the sufficiency of the host immune system to target malignant cells; in other words, the immune system must possess immune cells that can detect tumor cells in order for immunological rejection to occur. From an immunotherapeutic standpoint, cancer truly is a wolf in sheep's clothing, inasmuch as its components are more or less normal cellular material. Most tumor antigens are identical to normal antigens to such an extent that tumor cells easily evade detection by innate immune cells, which scout for pathogen-associated motifs. Fortunately, evolution has provided us with an adaptive immune system that can be educated *de novo* to target specific molecules. But again, given the similarity between most tumor and normal antigens, most adaptive immune cells that recognize these homologous antigens are thought to be destroyed or rendered nonresponsive (i.e., anergic) to avoid the consequence of autoimmunity. Nevertheless, several decades worth of preclinical studies have repeatedly demonstrated that adaptive immune cells do indeed possess the capacity to target tumor cells, proofed by the abrogation of antitumor effects or exacerbation of tumor growth in the context of adaptive immune cell depletion.

Early attempts to target tumor antigens took a brute force approach by using vaccines consisting of autologous, typically irradiated, cancer cells. Despite an exhaustive variety of approaches, these therapies induced only moderate and short-lived antitumor benefits, at best. Then, in the early 1990s, in the wake of cytokine-gene cloning, researchers began evaluating the antitumor effects of tumor cells transfected with genes encoding various immunological signaling molecules. Though results often varied among studies depending on the cancer type and site of tumor challenge, striking antitumor effects were frequently seen in preclinical studies using the immunomodulator granulocyte-macrophage colony stimulating factor (Dranoff et al., 1993; Sampson et al., 1996). As an alternative to the brute force approach, investigators at that time also began identifying specific tumor antigens that could be recognized by adaptive immune cells (Anichini et al., 1993; Brichard et al., 1993; Gaugler et al., 1994). Notably, several melanocyte-specific proteins (e.g., MART-1) were found to stimulate tumor infiltrating lymphocytes (TILs) isolated from melanoma patients (Anichini et al., 1993), and vaccines consisting of these melanocytic proteins were able to produce significant antitumor responses in preclinical models of melanoma. This was a rather striking observation considering that clonal deletion of self-reactive lymphocytes was demonstrated a few years earlier (Kappler et al., 1987; Nemazee et al., 1989). We now know these processes are incomplete, and self-reactive immune cells do indeed exist in

the periphery (Yu et al., 2015). However, activation of these cells does pose the risk of autoimmunity, which is a critical concern in the field of immunotherapy. The readministration of *ex vivo*-expanded TILs into melanoma patients, while effective in several cases, frequently induced autoimmune effects (e.g., vitiligo) (Dudley et al., 2002; Yeh et al., 2009)—a not too surprising outcome considering the nature of the recognized antigens. Though the treatment of one disease with another is not unprecedented, autoimmunity can have just as debilitating effects as malignant cancer. Consequently, researchers are beginning to focus their attention on safer tumor-specific antigens, which only became possible in recent years.

Upon the advent of next generation sequencing (NGS) in the early 21st century, large-scale tumor sequencing efforts quickly ensued. Using this technology, it became possible to peer inside tumor cells at the molecular level to determine their exact genetic and transcriptomic compositions. Although it was largely known that cancer was a disease caused by genetic mutations, the comprehensive landscape of these alterations was unknown prior to the availability of NGS. Much of the findings of these studies were not all too surprising: (1) genetic mutations were found in coding and noncoding DNA, (2) oncogenic and tumor-suppressor genes were frequently perturbed, and (3) tumors thought to develop from environmental stressors (e.g., melanoma and lung cancer) had a far higher number of mutations. However, it became evident that it may

be possible to target the expressed mutations, or neoantigens, within tumors. The benefit of using neoantigens as immunotherapeutic targets is two-fold: they are inherently tumor-specific and neoantigen-cognate adaptive immune cells are less likely to be subject to deletion or quiescence. Consequently, efforts are now being made to target the repertoire of tumor-specific neoantigens within tumors.

Numerous studies have demonstrated the immunogenicity of tumor-specific and tumor-associated antigens. How, then, are endogenous immune responses to these antigens prevented? The answer lies, in part, in the developmental process of a tumor. From incipience, cancers evolve an immunosuppressive phenotype that develops gradually, balancing on the cusp of immunological rejection with constant signals relayed to the immune system that it is simply a normal, healthy cluster of cells. Paracrine-acting suppressive molecules secreted by the tumor subdue local effector functions, whereas endocrine signaling molecules diminish activation of new antitumor immune cells in the secondary lymphoid organs, as well as promote systemic immunosuppression. In those unlucky few, the balance between immunological rejection and immunosuppression skews in the favor of tumor development. By the time of diagnosis, the arsenal of immunosuppressive mechanisms exhibited by the tumor is frequently evidenced by the infiltration of immunosuppressive immune cells (e.g., regulatory T-cells [Tregs]), the reeducation of the stroma into an immunosuppressive

phenotype, and the elaboration of immunosuppressive signaling molecules from the tumor cells themselves.

Overturning the effects of tumor-mediated immunosuppression is now a primary focus of immunotherapy. The history of this strategy dates back to the late 1800s, when a bone surgeon by the name of William B. Coley noticed that a patient suffering from a recurrent neck sarcoma experienced tumor regression following a case of erysipelas, or *Streptococcus pyogenes*, infection (Coley, 1910). In response to this observation, Dr. Coley crafted his own treatment cocktail consisting of killed *Streptococcus pyogenes* and *Serratia marcescens*— eponymously termed Coley's toxin. Although the antitumor effects of Coley's toxin are mixed, several cases of tumor regression following treatment have been documented. Several explanations of the antitumor effects of these bacterial components have been proposed, including the activation of innate toll-like receptors; however, the exact mechanisms are unknown. Suffice it to say that the stimulation of the host's immune system by Coley's toxin seems to be sufficient to reverse immunological suppression, thereby promoting antitumor responses, in some instances. As the field of immunology progressed, the suppressive pathways of the immune system began to develop understanding. In the late 1980s, a molecule known as cytotoxic T lymphocyte associated antigen 4 (CTLA-4) was discovered (Brunet et al., 1987), and shortly thereafter, the receptor programmed death 1

(PD-1) (Ishida et al., 1992) and its ligands (PD-L1 and PD-L2) (Freeman et al., 2000; Latchman et al., 2001) were identified. These molecules are collectively known as checkpoints, due to their role in keeping the immune system in check by promoting immunosuppression. Checkpoint inhibition via antibody-mediated blockade is gaining considerable attention in the field of immunotherapy in light of the potent antitumor responses it engenders against several tumors (e.g., melanoma and lung cancer) (Brahmer et al., 2010; Hodi et al., 2010). These effects highlight the role of immunological suppression in subduing endogenous antitumor responses. Unfortunately, these therapies do not seem to be equally efficacious in all cases or against all tumors; however, further optimization of clinical protocols using checkpoint inhibitors is required before their true effectiveness can be confirmed. It is unlikely, however, that checkpoint blockade, alone, will be the “magic bullet” envisioned by Dr. Ehrlich in the 1900s. The list of immunosuppressive mechanisms utilized by cancer is an extensive one, and therapeutic remediation of alternative pathways is showing great promise against certain cancers.

The immune system truly is a remarkably complex, and at times seemingly inextricable but equally astonishing, network of cells and cellular processes that staggers the imagination when its ontogeny is contemplated. From a therapeutic perspective, the immune system boasts potent cytotoxic potential with the ability to resolve structures at

the nanometer level, giving it a distinct advantage over conventional cancer treatment strategies. Leveraging these capabilities to produce safe, selective, and durable antitumor responses is the foremost goal of immunotherapy, and while this field has been mired with hurdles, great progress has been made and intriguing discoveries unearthed along the way. Studies are continuously showing that the body does indeed have a defense network in place that can eradicate malignant cells, but cancer—masquerading as a normal cell—is a formidable expert at attrition warfare and has decisively co-opted the immune system for its own benefit. However, there have been several strong indications that cancer’s immunological evasive mechanisms are reversible, as will be illustrated throughout this book. We truly are in the eve of the “Golden Age” of cancer immunotherapy, and these next few years will be critical in establishing immunotherapy as a respected modality in the armamentarium of cancer therapeutics.

2.4 Major challenges of brain tumor immunotherapy

From the serendipitous discovery of bacteria-mediated tumor suppression in the 19th century to the striking response rates promoted by modern molecular-guided immunotherapies, immunotherapy is proving to be a powerful modality for the treatment of cancer. Nevertheless, hopefulness is met with reasonable skepticism, particularly with regard to brain tumor immunotherapy. Although some types of cancer

do seem to be amenable to immunotherapeutic intervention, there are several aspects of brain tumors that make the use of immunotherapy a controversial proposition.

CNS tissues represent a very unique immunological environment compared to peripheral tissues (Figure 2). Seminal studies in the early 20th century demonstrated that grafts implanted within the CNS are rejected much slower than grafts placed in the periphery (Medawar, 1948), leading to the notion that the CNS was, in essence, an immunologically privileged site. This concept was further supported by the apparent lack of draining lymphatics in the brain and CNS tissues (Louveau, Harris, et al., 2015) and the presence of a highly restrictive blood–brain barrier (Saunders et al., 2014). From an evolutionary standpoint, it is reasonable to assume that the CNS has developed means to safeguard itself from the destructive effects of inflammation, given the indispensability of these tissues; however, we now know that the immune privilege of these tissues is not absolute. Studies have shown that immune cells do indeed have the potential to access these compartments, as evidenced by disease states such as multiple sclerosis and cancer. The ability of immune cells to access the CNS-resident tumors can further be aided by the disruption of the blood–brain barrier by invading glioma cells (Watkins et al., 2014). Additionally, a lymphatic system was recently elucidated in the dural sinuses of mice (Louveau, Smirnov, et al., 2015), which carry immune cells and antigen to the deep cervical lymph nodes (Harris et al., 2014). Thus, it is currently well-

established that there exists an appreciable degree of immunological surveillance of CNS tissues and CNS-derived antigen, albeit not as extensive as that which occurs in peripheral tissues. Nevertheless, it is also clear that these mechanisms are not sufficient to promote complete endogenous rejection of CNS-resident tumors.

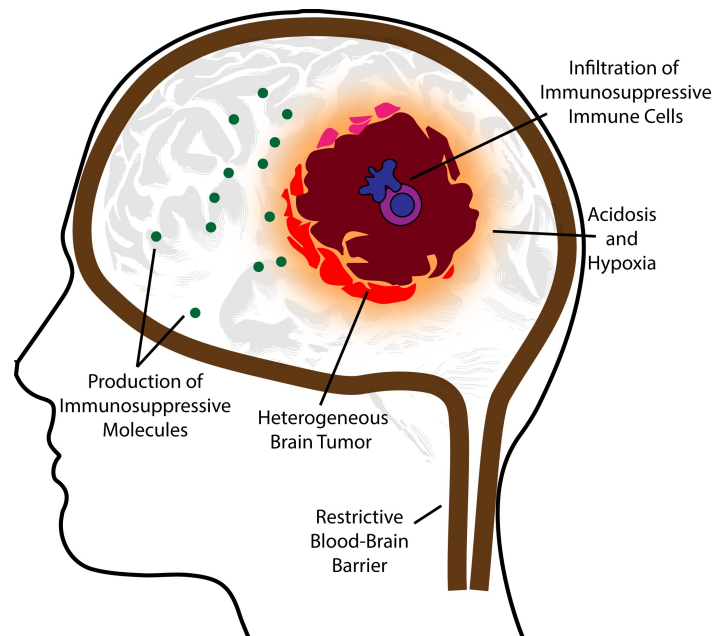


Figure 2: The immunological barriers to CNS-resident tumors.

Figure reproduced with permission (A.M. Swartz et al., 2017).

A second aspect that can limit the effectiveness of brain tumor immunotherapy has less to do with the anatomy of the CNS, but rather the architecture and behavior of gliomas (Figure 2). Immunological evasion is one of the hallmarks of cancer (Hanahan et

al., 2011), and malignant gliomas are true experts. Much like viruses that frequently mutate to avoid immunological detection, malignant gliomas exhibit profound heterogeneity (Kumar et al., 2014; Soeda et al., 2015; Sottoriva et al., 2013), which likely enables their escape from monovalent immunotherapeutic intervention (Sampson et al., 2010). Even upon the activation of high numbers of diverse tumor-reactive immune cells, malignant gliomas may still be protected by additional lines of defense. For example, the malignant glioma milieu is often associated with acidosis and hypoxia. This environment is extremely detrimental to effector immune cell function but is able to sustain immunosuppressive immune cells (e.g., Tregs), as well as stimulate angiogenesis (Fukumura et al., 2001). Additionally, malignant gliomas are known to produce a host of immunosuppressive molecules, such as transforming growth factor (TGF)- β , interleukin (IL)-10, and indoleamine-2,3-dioxygenase (IDO), that dramatically inhibit the immune response. These factors are also thought to contribute to the profound immunosuppression identified in malignant glioma patients, characterized by lymphopenia and T-cell dysfunction.

2.5 Hopefulness of brain tumor immunotherapy

The aforementioned characteristics of brain tumors arguably represent the worst-case scenario for immunotherapy. Few other cancers are situated behind such a vital organ that also maintains partial immunological seclusion. Nevertheless, the need

for safer and more selective therapies for brain tumors is undeniable, and the evaluation of brain tumor immunotherapy has, thus far, not been a fruitless endeavor. The studies carried out in this pursuit have provided great knowledge into the immunobiology of the CNS and CNS-resident tumors. It is now clear the immunotherapy has the potential to stimulate immunological changes within patients afflicted with brain tumors and, in several cases, impart molecular changes within the tumor itself (Sampson et al., 2010).

The finding that the CNS could indeed reject xenografts (Mason et al., 1986), suggested that “immune privilege” is not absolute (Carson et al., 2006). This was corroborated by molecular characterization of cells within the brain that revealed expression of proteins associated with the innate immune system. The most notable of these cells are the microglia – the CNS-resident macrophages – which express pattern recognition receptors (PRRs): Toll-like receptors (TLR), Nod-like receptors (NOD), and RIG1-like receptors (RLR). Upon activation of microglia, they transform from a ramified morphology into an amoeboid morphology, enabling them to perform various functions including phagocytosis, neuroprotection, and cytotoxicity (Blaylock, 2013). Interestingly, PRRs have also been found on endothelial cells, astrocytes, oligodendrocytes, and even neurons (Lafon et al., 2006; Lampron et al., 2013), profoundly implicating the role of a formidable innate immune system within the CNS.

Even more intriguing are studies demonstrating adaptive immune activity within the CNS. In general, studies suggest that localization of both naïve and effector lymphocytes to the CNS requires the expression of CNS-tropic adhesion molecules. These molecules include PSGL-1, $\alpha4\beta1$ integrin, and LFA-1, which bind to endothelial P-selectin, VCAM-1, and ICAM-1, respectively (Calzascia et al., 2005; Engelhardt et al., 2012). Once localized within CNS microvessels, lymphocytes extravasate into the perivascular spaces by migrating through endothelial cells, rather than between them, in a process known as transcellular diapedesis (Carrithers et al., 2000; Engelhardt et al., 2004). The perivascular sites act as drainage zones for choroid plexus-derived CSF fluid, as well as CNS parenchyma interstitial fluid, and are home to various antigen presenting cells (APCs), including microglia. It is here that T cells sample the CNS environment through their interaction with resident APCs. Furthermore, the recent identification of a draining lymphatic system (Louveau, Smirnov, et al., 2015) suggests a means by which adaptive immune cells can become activated upon exposure to CNS-derived antigens, such as those derived from brain tumors. Only upon activation are lymphocytes able to infiltrate the CNS parenchymal basement membrane and glia limitans (Bartholomaeus et al., 2009; Calzascia et al., 2005; Hickey, 1991; Vajkoczy et al., 2001), a process that has been shown to depend on the focal activity of various matrix metalloproteinases – namely MMP-2 and MMP-9 (Agrawal et al., 2006).

Despite our incomplete understanding of neuroimmunology, several CNS-targeted immunotherapies have been developed that demonstrate efficacy for the treatment of high-grade gliomas in preclinical models. The immune system is unquestionably active within the CNS, albeit to a much lesser extent compared to most other areas of the body. Thus, in order to enhance the potential of these truly powerful therapeutics, further examination of neurobiological and immunological processes is required.

3. Promising Cancer Vaccines for the Treatment of GBM

Conventional therapies for glioblastoma (GBM) typically fail to provide lasting antitumor benefits, owing to their inability to specifically eliminate all malignant cells. Cancer vaccines are currently being evaluated as a means to direct the adaptive immune system to target residual GBM cells that remain following standard-of-care treatment. A vaccine is a type of active immunotherapy that provokes the immune system into acquiring long-term immunity against an antigen of interest. Fundamentally, this activity is prompted by the administration of an immunogen in conjunction with an adjuvant – an immunological stimulator – thereby directing the activation of antigen-specific lymphocytes. Vaccines have traditionally been used in a prophylactic capacity; however, their ability to therapeutically mediate antitumor immunity is now being appreciated. Investigative cancer vaccines take many forms, including autologous/allogeneic tumor cells, tumor lysates, synthetic peptides, proteins, antigen-loaded dendritic cells, nucleic acid-based, and viral vectors (Evel-Kabler et al., 2006; Rosenberg et al., 2004; L. W. Xu et al., 2014). Ultimately, the goal of a cancer vaccine is to promote the presentation of tumor-specific antigens by dendritic cells, leading to the subsequent activation of tumor-specific adaptive immune cells (Figure 3).

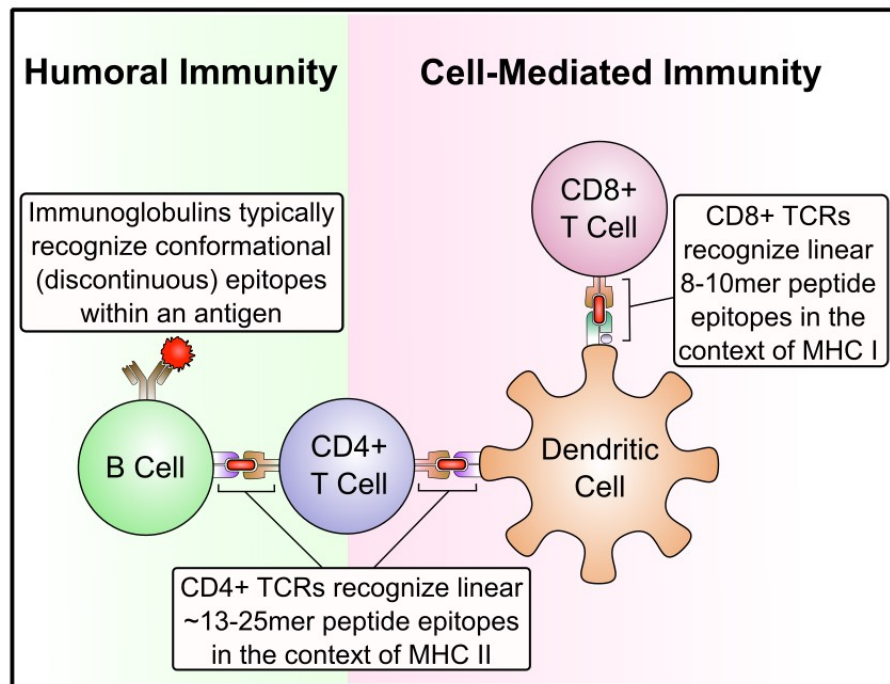


Figure 3: Epitope presentation and recognition by cells of the adaptive immune system.

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3.1 Cancer vaccine platforms

3.1.1 Peptide vaccines

Peptide vaccines contain peptides of about 8-30 amino acids in length and are, therefore, among the simplest forms of cancer vaccines. They are designed to encompass tumor-associated or tumor-specific epitopes that can be recognized by immunoglobulins or, when bound to MHC molecules, by TCRs. The identification of MHC-restricted tumor epitopes has been greatly facilitated by the use of *in silico* MHC

binding algorithms which predict peptide binding to class I or class II molecules (Castle et al., 2012). However, due to the highly polymorphic nature of human MHC genes, not all patients will react similarly to a given epitope (Purcell et al., 2007). To enhance their immunogenicity, these short peptides are often conjugated to a carrier protein, such as keyhole limpet hemocyanin (KLH) and tetanus toxoid. Peptide vaccines are typically administered with an immunostimulatory adjuvant capable of eliciting local inflammation. These include inflammatory cytokines, and pathogen-derived molecules called pathogen-associated molecular patterns (PAMPs). While cytokines can mediate inflammation directly, PAMPs have to be recognized by pattern recognition receptors (PRRs), which in turn lead to the induction of inflammatory cytokines. One of the most studied and promising PRR family is the Toll-like receptors (TLRs) (Warshakoon et al., 2009). The ease by which GMP-grade peptides and TLRs ligands can be synthesized, their relative stability and low cost makes adjuvant peptide vaccines very amenable to the clinical setting.

3.1.2 Dendritic cell vaccines

In vitro-derived DCs are used quite extensively as vaccines for GBM immunotherapy. Their appeal stems from the unique capacity of DCs to transform naïve T cells into potent cytotoxic effector T cells. Traditionally, DCs used for vaccines are derived from autologous peripheral blood monocytes. These cells are then

differentiated into DCs using various cytokines, including but not limited to granulocyte macrophage colony stimulating factor (GM-CSF), Interleukin 4 (IL-4), and FMS-like tyrosine kinase 3 ligand (FLT-3L). Prior to immunization, DCs are often matured using various cocktails that convert them from antigen-capturing cells into antigen-presenting cells. The “gold standard method” of DC maturation involves incubation in the endogenous pyrogens IL-1 β , tumor necrosis factor- α (TNF- α), and IL-6 (Jonuleit et al., 1997). Recently, a novel cocktail formulation including TNF α , IL-1 β , interferon- α (IFN α), interferon- γ (IFN γ), and polyinosinic:polycytidylic acid has been shown to generate highly mature DCs with enhanced migratory capacity as well as elevated production of IL-12 and improved T cell priming activity in comparison to the “gold standard method” (Mailliard et al., 2004).

A major advantage of DC vaccines is their amenability to various forms of antigen loading. DC vaccines have been tested in many forms, including pulsation with tumor lysate, proteins, or MHC-restricted epitopes. Alternatively, DCs can be transfected – usually through electroporation – with mRNA encoding tumor antigens, including total tumor mRNA. An advantage of this approach is that it does not require knowledge of dominant tumor antigens, as DCs inherently possess endogenous antigen processing and presentation mechanisms. Additionally, transgene-derived antigen processing can be augmented through the inclusion of signal sequences that shuttle the

encoded protein to subcellular compartments specialized in antigen processing (e.g., lysosomal-associated membrane protein 2 (LAMP2) for late endosomes and lysosomes and ubiquitin for the proteasome).

Increasing the effectiveness of DC vaccines is a subject of ongoing research. It was recently demonstrated that preconditioning the vaccine site with the tetanus/diphtheria toxoid enhanced DC migration to the lymphoid organs, which was associated with enhanced antitumor efficacy against GBM in humans (D. A. Mitchell et al., 2015). While the full mechanism of these effects has not been completely elucidated, these findings are encouraging for the future use of DC vaccines for GBM. DC vaccines may also be used to augment the effectiveness of other immunotherapeutic modalities, as demonstrated in a recent study showing that a CMV pp65-loaded DC vaccine could enhance the polyfunctionality of adoptively transferred CMV pp65-cognate T cells (Reap et al., 2018).

3.1.3 Nucleic acid vaccines

Nucleic acid vaccines can be divided into DNA and RNA vaccines. DNA vaccines are designed to encode one or more tumor antigens driven by a strong promoter. These vaccines are typically administered intramuscularly or intradermally, where resident APCs take up the DNA vector, migrate to the lymphoid organs, and present encoded antigens to immune cells. Interestingly, non-lymphoid cells (e.g.,

myocytes, keratinocytes, and fibroblasts) have also been implicated in DNA vaccine-mediated immune responses (Shirota et al., 2007). DNA vaccines generated from bacteria will possess unmethylated CpG motifs, which act as PAMPs recognized by TLR9. Thus, DNA vaccines can act as both an antigen source as well as an adjuvant for a simplified vaccine. These features, combined with a low manufacturing costs, make DNA vaccines an appealing choice for immunotherapy.

RNA vaccines have gained considerable attention in recent years for cancer immunotherapy. RNA vaccines are thought to be safer than DNA vaccines since RNA cannot integrate into the genome. Similar to DNA vaccines, RNA can be used to encode multiple tumor antigens as well as act as its own adjuvant. Immunological RNA PRRs include TLR3, TLR7, TLR8, Rig 1 like receptor I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), NLR family pyrin domain containing 3 (NLRP3), and nucleotide-binding oligomerization domain-containing protein 2 (NOD2). A major limitation to RNA-based vaccines, however, is their labile nature when exposed to extracellular and ubiquitous host RNA nucleases. For this reason, RNA vaccines are typically administered as nanoparticles or lipoplexes, which can provide protection from nucleases as well as facilitate their uptake by professional APCs (Kranz et al., 2016).

3.1.4 Tumor cell vaccines

Cancer cells can express a large number of antigens that are capable of being recognized by the immune system. However, successfully identifying the comprehensive set of these tumor antigens remains a major challenge. For this reason, vaccines that contain tumor cells as a major component represent an efficient means of capturing the entirety of immune-reactive tumor antigens within a singular vaccine. Several implementations of this approach have been evaluated for gliomas. For example, glioma cells may be lysed and administered directly into hosts (Belmans et al., 2017) or pulsed onto DC vaccines (Prins et al., 2013). Glioma cells ectopically expressing cytokines or other immunostimulatory factors have also been shown to elicit profound antitumor benefits in preclinical studies (Sampson et al., 1996).

3.1.5 Oncolytic viruses

The first laboratory-adapted virus used for oncolytic purposes was based on a Herpes simplex virus mutant and was reported in 1991 (Martuza et al., 1991). The development of these viruses paved the way for the adaptation and utilization of novel viruses with the sole purpose of mediating tumor lysis, therefore, creating the field of Oncolytic Viral Therapy (Chiocca, 2002). While the original studies focused on the capacity of these viruses to mediate tumor lysis *in vitro* and *in vivo*, these studies did not incorporate the immune system as a mechanism of tumor rejection, since most of these

studies involved immunodeficient mouse models and human xenografts. It was not until the late 1990s-early 2000s, that the field examined the role of an intact immune system on immunocompetent mouse models (Guo et al., 2017). To their surprise, the studies utilizing immunocompetent mouse models demonstrated that the inoculation of oncolytic viruses, led to what is currently referred to as “immunogenic tumor cell death” leading to the initiation of tumor specific immune responses and T cell mediated immunity capable of mediating long-term immunity against distal metastasis and tumor re-challenge (De Munck et al., 2017; Prestwich, Harrington, et al., 2008). These observations demonstrated that these oncolytic viruses serve as an “in situ” vaccine, and led to the novel design of oncolytic viruses aimed at potentiating these immune responses.

3.2 Promising vaccines for GBM

3.2.1 Vaccines targeting high-frequency GBM antigens

The lack of conserved tumor antigens that are shared among patients also presents a significant challenge to the development of universal vaccines for GBM. Nevertheless, GBM has been shown to express several antigens that seem to occur at a reasonably high-frequency, making the idea of off-the-shelf vaccines possible. Likely the most well-studied of these is the neoantigen EGFRvIII (Nishikawa et al., 1994) – expressed in ~25-65% of all primary GBMs (Heimberger et al., 2005). EGFRvIII arises

from a splicing mutation within the epidermal growth factor receptor (EGFR) gene that results in the deletion of exons 2-7 and the introduction of a novel glycine residue (Huang et al., 1997). This results in the formation of a constitutively active form of the EGF receptor that bestows tumorigenic function (Ekstrand et al., 1994). Initial attempts to target EGFRvIII used a vaccine composed of a 14mer peptide spanning the EGFRvIII mutation conjugated to the carrier protein KLH, known as rindopepimut, which induces a prominent humoral response to EGFRvIII (Heimberger et al., 2003). Early phase I and II clinical trials for patients with newly diagnosed GBM demonstrated a significant antitumor benefit with this vaccine compared to historical controls (Sampson et al., 2011; Sampson et al., 2009). Furthermore, a phase II trial evaluating rindopepimut in combination with bevacizumab (anti-VEGF) in patients with recurrent GBM also demonstrated a survival benefit (D. A. Reardon et al., 2015). However, a more recent multicenter phase III clinical trial, ACT IV, showed no survival benefit compared to the KLH-alone vaccine cohort (Weller et al., 2017). At this time, the cause of treatment failure in ACT IV remains unclear. It may be likely that optimization of standard-of-care over time has simply yielded better survival outcomes, and historical controls may not be suitable for clinical trial design. Furthermore, this study challenges the notion that EGFRvIII-targeting immunotherapies are responsible for the outgrowth of EGFRvIII-deficient GBM cells (Sampson et al., 2011; Sampson et al., 2010), considering that

EGFRvIII loss was observed in the control arm to a similar degree as that of the treatment arm. Nevertheless, these studies suggest that monovalent immunotherapeutic strategies targeting subclonal, heterogeneously-expressed tumor antigens are likely insufficient to promote durable antitumor responses against this devastating disease.

A high frequency (80-90%) of low grade gliomas (LGGs) possess mutations in isocitrate dehydrogenase 1 (IDH1) with more than 90% of containing an arginine to histidine switch at position 132 (IDH1^{R132H}) (Yan et al., 2009). This high-frequency neoantigen exhibits higher clonal expression than EGFRvIII (Platten et al., 2018) and is expressed in over 70% of GBMs that arise from LGG (i.e. secondary GBM) (Nobusawa et al., 2009). This mutation results in the production of the oncometabolite 2-hydroxyglutarate and can affect gene expression through epigenetic alterations (Turcan et al., 2012). Preclinical studies have suggested that IDH1^{R132H} is presented by gliomas (Bunse et al., 2015) and peptide vaccines spanning this mutation may elicit IDH1^{R132H}-reactive CD4⁺ and CD8⁺ T-cell responses, leading to antitumor benefits (Schumacher et al., 2014). A phase I trial evaluating the safety of an IDH1^{R132H} peptide vaccine for high grade gliomas has recently been initiated and expected to be completed in 2019 (NCT02454634).

EGFRvIII and IDH1^{R132H} are regarded as ideal immunotherapeutic targets given their exclusive expression within GBM cells, thereby mitigating the risk of

autoimmunity or immunosuppressive mechanisms. However, GBM also expresses several non-mutated tumor associated antigens (TAAs) that are overexpressed within tumors and, therefore, are thought to embody suitable immunotherapeutic targets. Included in this group is survivin, a protein involved in the inhibition of apoptosis by blocking caspase activity. Correspondingly, survivin's expression in astrocytomas is associated with a poorer prognosis (Kajiwara et al., 2003). The expression of survivin can be found in all GBM subtypes (Verhaak et al., 2010), making it a broadly applicable target. The survivin vaccine, known as SVN53-67/M57-KLH or SurVaxM, was well-tolerated in a phase I clinical trial (Fenstermaker et al., 2016). As such, SurVaxM in combination with the alkylating chemotherapeutic TMZ is set to be tested in a phase II trial for patients with newly diagnosed GBM (NCT02455557).

3.2.2 Dendritic Cell Vaccines

Two of the most well-studied DC vaccines for GBM are DCVax®-L and ICT-107. DCVax®-L consists of autologous GBM-lysate pulsed DCs. When evaluated in a phase I/II clinical trial for patients with newly diagnosed GBM, the observed median PFS was over 2 years, which is more than 3 times greater than that seen with standard-of-care treatment (Polyzoidis et al., 2014). Additionally, median OS was greater than 3 years, and 27% of treated patients survival greater than 6 years with 2 patients currently surviving beyond the 10-year mark (Polyzoidis et al., 2014). Recent results from a

randomized, placebo-controlled phase III clinical trial for DCVax®-L (NCT00045968) are encouraging (Liau et al., 2018). The median OS of the intent to treat group (n=331) was noted at 23.1 months. Furthermore, an increased OS was observed in a population (n=100) with no previously known prognostic factors associated with this outcome. Therefore, it is tempting to speculate that a patient subpopulation may be highly responsive to this therapy (Liau et al., 2018). The investigators concluded that DCVax®-L is safe and may be efficacious in patients with glioblastoma when combined with standard-of-care.

ICT-107 is comprised of autologous DCs pulsed with peptides derived from TAAs that are expressed at a high-frequency within GBM stem-like cells: melanoma-associated antigen 1 (MAGE1), human epidermal growth factor receptor 2 (HER2)/neu, tyrosinase-related protein 2 (TRP-2), glycoprotein 100 (gp100), absent in melanoma 2 (AIM-2), and interleukin 13 receptor α IL-13R α -2 (Phuphanich et al., 2013). In a phase I trial with HLA-A1⁺ and/or HLA-A2⁺ patients with newly diagnosed or recurrent GBM, treated patients exhibited a median PFS of 16.9 months and a median OS of 38.4 months (Phuphanich et al., 2013). These effects were reportedly associated with the expression of at least 4 TAAs within tumor samples, though all patient tumors expressed at least 3 and 75% expressed all 6. In a double-blind, placebo-controlled phase II trial, a significant enhancement in OS was not witnessed in patients with newly diagnosed

GBM; however, post-hoc analysis revealed stronger effects from ICT-107 in the HLA-A2⁺ cohort (Wen et al., 2014) and, as such, are the subjects in an ongoing phase III trial named STING (NCT02546102).

The association between GBM and cytomegalovirus (CMV) remains a controversial topic, given that some researchers have detected CMV antigens in GBM (Cobbs et al., 2002; D. A. Mitchell et al., 2008) while others have not (Baumgarten et al., 2014). Nevertheless, CMV antigens are being actively pursued as vaccine targets for GBM, and some clinical studies have yielded striking results. In a small, randomized phase I clinical trial for patients with newly diagnosed GBM, immunization with DCs transfected with mRNA encoding pp65 (a dominant antigen of CMV) within a vaccine site preconditioned with tetanus/diphtheria toxoid (TenivacTM) significantly improved clinical outcomes compared to preconditioning with unpulsed DCs (D. A. Mitchell et al., 2015). In another phase I trial, pp65-transfected DCs were administered in the context of adjuvant GM-CSF and dose-intensified TMZ, resulting in prolonged PFS and OS compared to historical controls (Batich et al., 2017). Two follow-up phase II trials, ATTAC II (NCT02465268) and ELEVATE (NCT02366728), have commenced to further assess efficacy and enhanced DC vaccine migration upon TenivacTM preconditioning with and without GM-CSF, respectively.

3.2.3 Multivalent Peptide Vaccines

GBM tumors are profoundly heterogeneous. Different regions of a tumor can present to the immune system differing antigens (Patel et al., 2014; Sottoriva et al., 2013), and no singular antigen has been found that is ubiquitously expressed throughout all GBM cells. The clinical implication of this has been demonstrated in clinical trials with the EGFRvIII vaccine rindopepimut. Histological evaluation of EGFRvIII expression following immunization suggested that tumor recurrence was largely a result of the outgrowth of GBM cells that do not express EGFRvIII (Sampson et al., 2010). These data support the notion that multivalent vaccine strategies are likely needed to more comprehensively target an individual GBM tumor.

One such vaccine that attempts to address this limitation is IMA950. This vaccine is comprised of 9 HLA-A*02-restricted epitopes derived from “self” antigens that are highly expressed in GBM, 2 HLA class II-binding peptides that may also elicit cytotoxic CD8⁺ T cell responses, and a Hepatitis B virus core antigen epitope that serves as a positive vaccine control (Rampling et al., 2016). The results of a phase I trial testing the safety of IMA950 with the TLR3 ligand polyinosinic-polycytidylic acid, poly-L-lysine and carboxymethylcellulose (poly-ICLC) in combination with chemo-radiotherapy in newly diagnosed, HLA-A*02-positive GBM patients have recently been described. PFS was reported as 74% at 6 months and 31% at 9 months and median OS at 15.3

months. Interestingly, stratification of survival data based on the occurrence of an injection site reaction (ISR) revealed that patients who experienced an ISR (n = 25) had a median OS of 26.7 months compared to 13.2 months for patients that did not exhibit an ISR. A phase I/II trial evaluating IMA950 with TMZ and poly-ICLC for newly diagnosed, HLA-A*02-positive GBM patients (NCT01920191) has recently finalized, though results have not yet been reported.

Patient-specific neoantigens that arise from missense or splicing mutations have garnered significant interest as vaccine targets due to their inherent tumor-specificity. As such, they are thought to bestow an excellent safety profile. Furthermore, because neoantigens expressed in adult tumors are generally not presented during the negative selection phase of T cell development, neoantigen-reactive lymphocytes are less likely to be eliminated or rendered functionally unresponsive compared to T cells reactive to “self” antigens. A phase I trial evaluating personalized neoantigen vaccines for newly diagnosed, MGMT-unmethylated GBM has been initiated and is estimated to complete in 2019 (NCT02287428).

Immatics biotechnologies, who developed IMA950, has partnered with other biotechnology companies (e.g., BioNTech), universities, and medical centers in order to coordinate a project known as GAPVAC, or Glioma Actively Personalized Vaccine Consortium. The primary objective of GAPVAC is to develop personalized peptide

vaccines targeting tumor-associated antigens highly represented within gliomas (i.e. TUMAPs) and patient-specific neoantigens. GAPVAC-101 (NCT02149225), a phase I trial for patients with newly diagnosed GBM, commenced in 2014. In this study, patients were first immunized with 5-10 TUMAPs, poly-ICLC, and GM-CSF 15 days following the first maintenance TMZ cycle, receiving 11 vaccines over 22 weeks. Patients were also immunized with 1-2 mutant peptides with poly-ICLC and GM-CSF starting 15 days after the fourth maintenance TMZ cycle, receiving 8 vaccines over 10 weeks. GAPVAC-101 is expected to complete in July 2018.

3.2.4 Oncolytic viruses

Over the years many oncolytic viruses have been evaluated against GBM in both the pre-clinical and clinical setting. Viruses derived from: Herpes Simplex Viruses-1 (HSV-1), Retroviruses, Adenovirus (Ad), Reoviruses, and Polio Viruses (PVS) are amongst the most commonly used viruses (Foreman et al., 2017; Wollmann et al., 2012). Most of these viruses have been tested in phase III clinical trials and have demonstrated to be safe and promising.

HSV-1 is one of the most commonly used oncolytic viruses. HSV-1 is a double-stranded DNA enveloped virus with tropism for the CNS (Conrady et al., 2010). Several gene modified HSV-1 viruses have been developed that selectively infect and lyse CNS tumors while sparing neuron infection (Grandi et al., 2009). Viruses such as G207,

HSV1716, M032, and G47 Δ have been demonstrated in preclinical studies to mediate immune activation and elicit tumor specific immune responses (Foreman et al., 2017). Several trials in patients with brain tumors including GBM, have been completed (NCT00157703, NCT00028158) and have demonstrated safety, while other trials with more recent vector designs are on their way (NCT02457845, NCT02062827). However, despite the great safety profile of HSV-1 based viruses, limited antitumor efficacy has been observed in patients with GBM. The recent approval of IMLYGIC[®], an oncolytic HSV-1 virus expressing GM-CSF for melanoma patients highlights the promising potential of such HSV-1 based oncolytic viruses for the treatment of cancer (Andtbacka et al., 2015).

TOCA-511 is a replication competent retrovirus that encodes the suicide gene Cytosine Deaminase, which converts the pro-drug call 5-fluorocytosine (5-FC) into the active anticancer drug, 5-Fluorouracil, a chemotherapeutic agent which causes “immunogenic cell death” (Perez et al., 2012). Pre-clinical studies of this oncolytic viruses have shown that the main mechanism of tumor rejection here requires the presence of an intact immune system and systemic tumor-specific T cell immunity (L. A. Mitchell et al., 2017). Two recent clinical studies demonstrated promising results with an increased OS in patients with recurrent GBM (Cloughesy et al., 2016; Cloughesy et al., 2018). One study of 45 patients showed an increased OS of 13.6 months (Cloughesy et

al., 2016), and in the other study of 56 patients there was a durable response rate of 21.7% (Cloughesy et al., 2018). A phase II/III study has since been initiated (NCT02414165).

A combination of two replication incompetent adenoviruses has recently been developed with very promising results. Ad-HSVtk + Ad-FLT-3L are human adenoviruses serotype 5, deleted in E1A and E3 genes (Castro et al., 2014). Ad-HSVtk encodes the suicide gene Herpes Simplex Virus Thymidine Kinase (HSVtk), which converts the pro-drug ganciclovir or valganciclovir into a nucleoside analog which gets incorporated into the DNA leading to the termination of DNA replication, and induction of “immunogenic cell death” (King et al., 2011). The other virus, Ad-FLT-3L, encodes the immunomodulatory molecule FLT-3L, which enhances the recruitment, differentiation and proliferation of APCs (King et al., 2011). When these agents are combined the “immunogenic cell death” elicited by Ad-HSVtk results in the release of HMGB1 protein which interacts with TLR-2 present on the APCs recruited by Ad-FLT-3L, leading to tumor antigen uptake, maturation and activation of the endogenous T cells (Curtin et al., 2009). This T cell response then is capable of eradicating multifocal glioma (King et al., 2008). These encouraging results have led to the initiation of a phase I/II study (NCT01811992).

Reoviruses are nonenveloped, double-stranded RNA viruses which upon internalization into host cells triggers Type I IFN production due to the activation of RIG-I, in response to the presence of the viral genome in the cytosol (Holm et al., 2007). Normal cells limit the replication and infectivity of Reovirus, however, most tumors, including GBM, have increased Ras activity which allows the virus to replicate selectively in GBM tumors to mediate oncolysis (Wilcox et al., 2001). Reovirus infection of immune cells present within the tumor triggers the induction of DC maturation and activation of tumor specific T cells leading to the desired “in situ” vaccine effect (Prestwich, Errington, et al., 2008). A recent phase I study conducted by Europe (EudraCT - 2011-005635-10) has demonstrated promising results in patients with brain tumors. Intravenous infusion of Reovirus was shown to infect brain tumors and to increase T cell infiltration (Samson et al., 2018). These studies have led to the initiation of a recent phase I study (NCT02444546) of Reovirus in combination with GM-CSF with the expectation of inducing more robust tumor-specific immunity.

Delta-24-RGD also known as DNX-2401 is an oncolytic replication competent adenovirus which has been genetically modified to increase selectivity for tumor cells (Jiang et al., 2009). Deletion of a 24bp segment within the E1A gene allows for viral replication within tumors with an active retinoblastoma (Rb) pathway (Fueyo et al., 2000), and inclusion of an arginine-glycine-aspartate (RGD) motif in the adenovirus fiber

enhances selectivity for tumors (Fueyo et al., 2003). Delta-24-RGD has been demonstrated to prolong survival in mice with established brain tumors (Jiang et al., 2014). Virus injection in glioma bearing mice resulted in the induction of T cell immunity (Jiang et al., 2014). These studies led to the initiation of a phase I study (NCT00805376) in recurrent glioma. The results were extremely encouraging, 20% of patients survived more than 3 years from treatment, and detectable T cell responses were seen within the tumor (Lang et al., 2018). New trials are currently accruing in which the Delta-24-RGD oncolytic virus will be evaluated with other immunomodulatory agents (NCT02798406).

PVSRIPO is a recombinant live attenuated poliovirus type 1 derived from the Sabin strain, in which the Internal Ribosome Entry Site (IRES) has been replaced with that of human rhinovirus type 2 (Gromeier et al., 1996). PVSRIPO was adapted to infect glioma cell cells, therefore, displaying tropism against malignant tumors such as GBM (Ochiai et al., 2006). PVSRIPO leads to a rapid productive infection resulting in tumor lysis and the induction of an inflammatory response characterized by type I IFN expression, recruitment of myeloid cells, and activation of DCs and tumor antigen-specific CD8⁺ T cells (Brown et al., 2017). These novel findings have resulted in the initiation of a phase Ib in pediatric glioblastoma and a phase II clinical study in patients with brain tumors (NCT03043391 and NCT02986178, respectively). PVSRIPO has

received a breakthrough therapy designation from the FDA as a potential treatment for patients with recurrent GBM due to the encouraging results obtained in the recently published phase I clinical study (NCT01491893) (Desjardins et al., 2018).

As these agents progress through clinical development (Table 1), inclusion of immunomodulatory agents to enhance their clinical efficacy without compromising their safety profile has become of paramount importance.

Table 1: Promising vaccine clinical trials for GBM

Figure reproduced with permission (Adam M. Swartz et al., 2018)

Trial name and ClinicalTrials.gov Code	Sample Size	Phase	Treatment Arm(s)	Primary Outcome	Sponsor
NOA-16 NCT02454634	39	I	IDH1 peptide vaccine	Safety, tolerability, and immunogenicity of vaccine	National Center for Tumor Diseases, Heidelberg
SurVaxM NCT02455557	64	II	SurVaxM	Progression-free survival	Roswell Park Cancer Institute
DCVax [®] -L NCT00045968	331	III	DCVax [®] -L	Progression-free survival and Overall Survival	Northwest Biotherapeutics
STING NCT02546102	414	III	ICT-107	Overall survival	ImmunoCellular Therapeutics, Ltd.
IMA950 NCT01920191	19	I/II	IMA 950 + Poly-ICLC	Safety and tolerability	Geneva University Hospitals, Centre of Oncology
Personalized NeoAntigen Vaccine NCT02287428	16	I	Standard radiation therapy + personalized NeoVax	Safety, tolerability, and study feasibility	Dana Farber Cancer Institute
GAPVAC NCT02149225	16	I	APVAC1 and APVAC2 plus Poly-ICLC and GM-CSF concurrent to TMZ	Safety and biological activity of vaccine	Immatics Biotechnologies GmbH
Toca-511 + Toca-FC NCT02414165	380	II/III	Replicative Competent Retrovirus - Cytosine Deaminase + 5-FC	Overall survival	Tocagen Inc.
Ad-hCMV-TK and Ad-hCMV-Fh3L NCT01811992	18	I	Dose escalation of Ad-hCMV-TK and Ad-hCMV-Fh3L	Maximum tolerated dose	University of Michigan Cancer Center
CAPTIVE NCT02798406	48	II	DNX-2401 + pembrolizumab	Objective response rate	DNATRIX, Inc.
PVSRIP0 NCT01491893	61	I	PVSRIP0	Maximum tolerated dose or optimal dose	Duke University
Neoantigen Vaccine + Checkpoint Blockade NCT03422094	30	I	NeoVax + Nivolumab or NeoVax + Nivolumab + Ipilimumab	Safety, tolerability, and study feasibility	Washington University School of Medicine

4. A Closer Look at the First-in-Class GBM Vaccine, Rindopepimut

4.1 Introduction to EGFRvIII and rindopepimut

Driver mutations (i.e. those that promote a selective growth advantage) are notoriously difficult to identify due to the heterogeneity amongst tumor samples; that is, driver mutations are not ubiquitous in tumors of the same type. Using computational methods, one study estimated there are 49 missense driver mutations in GBM (Carter et al., 2009). Popular candidate proteins are those that contribute to the “hallmarks of cancer,” such as growth factor signaling. One such protein is the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase involved in transducing signals that modulate cell proliferation, inhibition of apoptosis, angiogenesis, and cell migration, adhesion, and invasion in response to the binding of ligands including extracellular epidermal growth factor (EGF) and transforming growth factor- α (TGF- α). This receptor is often overexpressed in GBM (Wong et al., 1987). Additionally, a class III deletion mutation, known as EGFRvIII, has been identified in ~25-65% of newly diagnosed primary GBM (Moscatello et al., 1995).

The gene that encodes EGFRvIII is characterized by the deletion of exons 2-7, resulting in a novel junction between exon 1 and exon 8. This removes amino acids 6-273 from the extracellular, ligand binding domain of EGFR and inserts a glycine not found in the original reading frame (Frederick et al., 2000; Wong et al., 1992). EGFRvIII has

been shown to enhance tumorigenicity via low but constitutive ligand-independent signaling (Batra et al., 1995), promote tumor cell migration (Boockvar et al., 2003), and confer protection from RT and TMZ-therapy (Lammering et al., 2004; Montgomery et al., 2000; Nagane et al., 1996). Cells expressing EGFRvIII can also induce tumorigenicity in neighboring EGFRvIII-negative cells by secreting EGFRvIII-bound oncosomes that incorporate into the plasma membrane of neighboring cells (Al-Nedawi et al., 2008). Furthermore, amplification of EGFRvIII is possible given its presence on extrachromosomal double-minute chromosomes (Vogt et al., 2004). In light of its oncogenic role and prevalence in GBM, EGFRvIII provides an ideal target for GBM immunotherapy (A. M. Swartz et al., 2014; Wikstrand et al., 1998).

Rindopepimut is a 14-mer peptide that spans the mutation site of the GBM-specific antigen EGFRvIII (PEPvIII: NH₂-Leu-Glu- Glu-Lys-Lys-Gly-Asn-Tyr-Val-Val-Thr-Asp-His-Cyt- COOH) conjugated to the immunogenic carrier protein keyhole limpet hemocyanin (KLH). The name rindopepimut was determined by the United States Adopted Name Council of the American Medical Association according to the naming convention for peptide immunotherapies, while the drug was licensed to Pfizer (NY, USA) in 2008. Since 2010, the license for rindopepimut has been owned by Celldex Therapeutics (MA, USA).

4.3 Pharmacodynamics of rindopepimut

Preclinical and clinical data indicates that vaccination with rindopepimut results in increased PEPvIII-specific antibodies, suggesting a B cell-mediated humoral response (Heimberger et al., 2002; Heimberger et al., 2003; Heimberger et al., 2011; Lai et al., 2011; Sampson et al., 2011). The data on rindopepimut's ability to elicit a cell-mediated response is less clear. Supporting the idea that cytotoxic T cells are involved in anti-EGFRvIII immunity is preclinical data showing that depletion of CD8+ T cells diminishes rindopepimut's antitumor efficacy (Heimberger et al., 2003) and positive clinical DTH tests showing anti-PEPvIII responses (Sampson et al., 2011; Sampson et al., 2009; Sampson et al., 2010). Further studies are needed to elucidate the pharmacodynamics of rindopepimut.

4.4 Safety of rindopepimut

Autoimmunity and intracranial inflammation are concerns with any CNS-targeted immunotherapy; however, these were not apparent complications associated with rindopepimut vaccination. All rindopepimut vaccines used in preclinical and clinical trials were generally well accepted and typically never exceeded grade 2 toxicity (NCI: Common Toxicity Criteria). The most common adverse effect witnessed in vaccinated patients was a low-grade reaction at the site of injection. In very rare cases, grade 3 toxicity was exhibited, thereby resulting in discontinuation of treatment.

4.5 Preclinical studies

Rindopepimut was initially used to generate monoclonal antibodies specific to EGFRvIII (Wikstrand et al., 1995). One such IgG2a antibody, Y10, demonstrated the ability to protect mice from tumor growth after subcutaneous challenge with melanoma cells (B16) stably expressing EGFRvIII (B16-EGFRvIII). Y10-mediated tumor protection was shown to be a result of autonomous, complement-mediated, and antibody-dependent cell-mediated cytotoxicity (ADCC). Moreover, protection was dependent on the Fc receptor and independent of complement, granulocytes, NK cells, and T lymphocytes, as indicated by depletion experiments. Although systemic delivery of Y10 was not able to ameliorate intracerebral (i.c.) challenge with B16-EGFRvIII, direct injection of the antibody into the tumor did increase median survival by 286% and increase long-term survival in 26% of mice (Sampson et al., 2000).

To determine whether an immunological memory against EGFRvIII could be established – a function not afforded by passive immunity – a novel method of tumor-antigen presentation using dendritic cells was adapted based on early studies demonstrating that dendritic cells, pulsed with B16 melanoma cell extract or RNA, were able to generate an adaptive immune response (Ashley et al., 1997; Porgador et al., 1996). To mediate EGFRvIII specificity, 1 µg rindopepimut was pulsed into dendritic cells (rindopepimut-DCs), and C3H mice were vaccinated intraperitoneally, followed by i.c.

challenge with syngeneic K1735-EGFRvIII melanoma cells one week later. Treated mice experienced a ~600% increase in median survival time compared to mice vaccinated with 1 µg rindopepimut alone or PBS. Rindopepimut-DC vaccinated mice that survived were rechallenged, and all survived. Interestingly, both rindopepimut-DCs and rindopepimut alone resulted in a similar IgG1 response; however, in the rindopepimut-DC vaccinated mice, there was a more dramatic IgG2a response – the same antibody class as Y10 (Heimberger et al., 2002).

To evaluate the efficacy of rindopepimut vaccination in the presence of an adjuvant, C3H mice with established i.c. K1735EGFRvIII tumors were vaccinated with 100 µg rindopepimut with co-administration of Freund's complete adjuvant. This resulted in a 26% increase in median survival with 40% of mice surviving long-term compared to KLH-vaccinated C3H mice. A clinical relevant modality was also assessed using 100 µg rindopepimut with co-injection of Freund's incomplete adjuvant plus GM-CSF, which is thought to enhance antigen presentation (Gilboa et al., 1994; Sampson et al., 1996), resulting in a ~60% increase in median survival. Sera from the surviving mice had increased concentrations of anti-PEPvIII IgG1 antibody, and passive transfer of sera into s.c. B16-EGFRvIII challenged mice resulted in tumor protection. Interestingly, depletion of CD8+ T cells in C57BL/6J mice diminished vaccine efficacy, although a cellular immune response was not detectable. This may partially explain the

effectiveness of the rindopepimut vaccine on established i.c. tumors.

Immunohistochemical analysis of relapsed tumors indicated that 80% exhibited an outgrowth of EGFRvIII-negative cells, suggesting a cause of treatment failure (Heimberger et al., 2003).

4.6 Clinical trials with rindopepimut

4.6.1 Phase I for newly-diagnosed GBM: VICTORI

Because of the success of rindopepimut in preclinical *in vivo* studies, clinical studies were performed to determine safety and efficacy in human subjects. The first of these was a small-scale phase I safety trial performed at Duke University Medical Center, known as VICTORI. Criteria for patient selection included newly diagnosed GBM patients, treated with current standard of care resection/RT/TMZ, over the age of 18, with a KPS of ≥ 80 . Eligibility was not dependent on EGFRvIII expression in tumors, however. For this study, 12 patients were vaccinated 3 times in the upper thigh, once every 2 weeks, with PBMC-derived autologous DCs pulsed with 500 μg rindopepimut and evaluated without therapy until progression was evident. The maximum administered dose of 1×10^8 rindopepimut-DCs was well accepted with minimal toxicity. The results indicated that T cells from vaccinated patients underwent antigen-specific proliferation *in vitro*, with T cells from 83.3% of patients responding to PEPvIII and 91.7% responding to KLH. Delayed type hypersensitivity (DTH) skin tests were

performed to evaluate the presence of a cellular immune response. In all cases, patients were positive before and after vaccination for tetanus toxoid. No patient was responsive to PEPvIII or KLH prior to vaccination; however, 56% and 100% of patients had a positive response to PEPvIII and KLH post-vaccination, respectively. The median progression free survival (PFS) was 10.2 months, and the median overall survival (OS) was 22.8 months after histological diagnosis (Sampson et al., 2009).

4.6.2 Phase II for newly-diagnosed GBM: ACTIVATE

A phase II trial was performed at Duke University Medical Center to evaluate the efficacy of rindopepimut, known as ACTIVATE. In this study, the use of DCs was abandoned due to their expense and difficulty to culture. Instead, 18 patients were vaccinated 3 times in the upper thigh, once every 2 weeks, with 500 µg rindopepimut with 150 µg GM-CSF as an adjuvant. Vaccinations were administered once a month, thereafter, until evidence of progression or death. Generally, rindopepimut exhibited low toxicity. Patients were again selected based on their status as a newly diagnosed GBM patient, treated with current standard of care (i.e. gross tumor resection and chemo-radiation therapy), over the age of 18, with a KPS of ≥ 80 ; however, EGFRvIII-expression was now an inclusion criterion. DTH skin tests indicated that, after vaccination, only 18% of patients had a positive response against PEPvIII. Additionally, humoral responses were evaluated, and 43% had positive responses post vaccination.

Although the sample size in this trial is too small to make any significant determinations, patients with positive DTH and humoral responses against PEPvIII did display an increased OS compared to those that were negative for these responses. Median PFS and OS from histological diagnosis for rindopepimut vaccinated patients was 14.2 and 26.0 months, respectively, compared to 6.4 and 15.2 months, respectively, for matched controls who were contemporaneously-treated according to standard of care at MD Anderson. Upon tumor progression, patients within both the control and experimental cohorts received additional anti-tumor therapies. These treatments include TMZ treatment, protein kinase inhibitors, angiogenesis inhibitors (i.e. anti-VEGF antibody and 2-methoxyestradiol), topoisomerase inhibitors, IL13 infusion, and alternative chemotherapeutic agents (other than TMZ). Among recurrent tumors where pathologic tissue could be obtained, 82% lost all EGFRvIII expression. One of the two recurrent tumors that expressed EGFRvIII exhibited < 1% of total cells staining positive for EGFRvIII. Four rindopepimut-vaccinated patients survived beyond the completion of this study (Sampson et al., 2010). At the time of this review, two of these patients are still alive, receiving only a monthly treatment with rindopepimut plus GM-CSF.

4.6.3 Phase II for newly-diagnosed GBM: ACTII

A counterintuitive result from recent studies suggested that enhanced TMZ-induced lymphopenia could improve antitumor immune responses (Sampson et al.,

2011; L. A. Sanchez-Perez et al., 2013). To determine whether rindopepimut efficacy could be enhanced by maintenance TMZ therapy, a Phase II trial, known as ACTII, was conducted to evaluate rindopepimut vaccination in the context of standard TMZ dosing (STD) and dose intensified (DI) TMZ treatment. Patient selection criteria were similar to that of ACTIVATE: recently diagnosed GBM patients, have undergone gross tumor resection, have received chemo-radiation therapy, over the age of 18, a KPS of ≥ 80 , and exhibit positive EGFRvIII expression. Twenty-two patients were selected for this trial. Rindopepimut was administered, with 150 μg GM-CSF, in the upper thigh on the 21st day of a 28 day TMZ cycle. STD resulted in mostly grade 2 lymphopenia (< 800 cells/ μL), and DI treatment resulted in predominantly grade 3 lymphopenia (< 500 cell/ μL) by the 6th cycle. Though TMZ treatment diminished both T cell and B cell counts, an unexpected statistical increase in CD4+CD25+FOXP3+ T regulatory cells was witnessed in DI-treated patients. Cell-specific and humoral responses were negative prior to vaccination but were almost all positive post vaccination. Interestingly, antibody titers and DTH responses were significantly increased in DI-treated patients. Because of the small sample size, distinctions could not be made concerning the effects of STD and DI treatment on median PFS and OS; however, the PFS and OS of all vaccinated patients from the time of histological diagnosis was 15.2 and 23.6 months, respectively, compared to 6.4 and 15.2 months, respectively, in matched historical controls from the

ACTIVATE trial (Sampson et al., 2011). Upon tumor progression, most patients received additional anti-tumor therapies including TMZ treatment, repeat resection, protein kinase inhibitors, rapamycin, topoisomerase inhibitors, angiogenesis inhibitors (i.e. anti-VEGF antibody), topoisomerase inhibitors, and alternative chemotherapeutics (other than TMZ).

4.6.4 Phase II for newly-diagnosed GBM: ACTIII

ACT III was a single arm, phase II trial performed at 31 centers in the United States. Sixty-five newly diagnosed GBM patients with EGFRvIII expression were selected for this trial, regardless of HLA subtype. Additional criteria included ≥ 18 years of age, no progression after gross tumor resection and chemo-radiation therapy, and a KPS score of ≥ 70 . Patients were vaccinated in the upper thigh with 500 μg rindopepimut with 150 μg of the adjuvant GM-CSF and treated with STD maintenance therapy. Vaccinations were administered bimonthly, for the first 3 doses, and then on the 21st day of a 28-day treatment cycle until intolerance, tumor progression, or death. Eighty-five percent of patients developed enhanced antibody titers against EGFRvIII, which increased over time. Cellular responses were also evaluated; however, the results were obfuscated by TMZ-induced lymphopenia. No correlation was found among the various HLA types. Median PFS and OS from histological diagnosis was 12.3 and 24.6 months, respectively, compared to 6.4 and 15.2 months, respectively, in matched historical

controls from the ACTIVATE trial (Lai et al., 2011). EGFRvIII was eliminated in 4/6 (67%) of tumor samples obtained after > 3 months of therapy (personal communication, Celldex). (Sampson et al., 2011). Upon tumor progression, most patients received additional anti-tumor therapies including TMZ treatment, repeat resection, angiogenesis inhibitors (i.e. anti-VEGF antibody), alternative chemotherapeutics (other than TMZ), radiation, and other investigational agents.

Table 2: Summary of phase I and phase II clinical trials with rindopepimut

Table reproduced with permission (A. M. Swartz et al., 2014)

Table 1. Summary of Phase I & II Rindopepimut Trials								
Authors	Trial Name	Sample Size	Patient Eligibility	Treatment Groups	Toxicity ¹	EGFRvIII-Expression Post-Vaccination	Progression-Free Survival	Overall Survival
Sampson et al. 2009 ref. 60	VICTORI (Phase I)	12	Newly diagnosed GBM, received standard of care treatment, ≥18 yrs. old, KPS ≥80, negative for pregnancy, immunosuppression, and/or infection	Up to 1 x 10 ⁸ autologous dendritic cells electroporated with 500 µg Rindopepimut, 3 doses, 2 weeks apart, injected i.d. in inguinal region	≤ grade 2		Median 10.2 mo. (C.I. ₉₅ , 5.7-12.6) after histologic diagnosis	Median 22.8 mo. (C.I. ₉₅ , 17.5-29.0) after histologic diagnosis
Sampson et al. 2010 ref. 61	ACTIVATE (Phase II)	18	Newly diagnosed EGFRvIII-positive GBM, gross tumor resection, received chemo-radiation, ≥18 yrs. old, KPS ≥80, negative for pregnancy, immunosuppression, and/or infection	500 µg Rindopepimut + 150 µg GM-CSF administered biweekly for first 3 doses, followed by monthly doses until tumor recurrence, injected i.d. in inguinal region	≤ grade 3 ²	11 recurrent patient tumors examined: 9 exhibited no EGFRvIII expression; of the 2 that were positive, 1 exhibited <1% EGFRvIII expression	Median 14.2 mo. (C.I. ₉₅ , 9.9-17.6) after histologic diagnosis*	Median 26 mo. (C.I. ₉₅ , 21.0-47.7) after histologic diagnosis***
Sampson et al. 2011 ref. 63	ACTII (Phase II)	22	Newly diagnosed EGFRvIII-positive GBM, gross tumor resection, received chemo-radiation, ≥18 yrs. old, KPS ≥80, negative for pregnancy, immunosuppression, and/or infection	STD ² and DI ² TMZ cohorts; 500 µg Rindopepimut + 150 µg GM-CSF administered biweekly for first 3 doses, followed by monthly doses until tumor recurrence, injected i.d. in inguinal region	≤ grade 3 ²	12 recurrent patient tumors examined: 11 exhibited no EGFRvIII expression	Median 15.2 mo. (C.I. ₉₅ , 11.0-18.5) after histologic diagnosis (all patients)**	Median 23.6 mo. (C.I. ₉₅ , 18.5-33.1) after histologic diagnosis (all patients)**
Lai et al. 2011 ref. 64	ACTIII (Phase II)	65	Newly diagnosed EGFRvIII-positive GBM, gross tumor resection, received chemo-radiation, ≥18 yrs. old, KPS ≥80, negative for pregnancy, immunosuppression, and/or infection	STD ² TMZ; 500 µg Rindopepimut + 150 µg GM-CSF administered biweekly for first 3 doses, followed by monthly doses until tumor recurrence, injected i.d. in inguinal region	≤ grade 3 ²	6 recurrent patients tumors examined: 4 exhibited no EGFRvIII expression	Median 12.3 mo. after histologic diagnosis**	Median 24.6 mo. after histologic diagnosis***

* p < 0.05; treatment vs. historical controls
** p < 0.01; treatment vs. historical controls
*** p < 0.001; treatment vs. historical controls
¹ National Cancer Institute: Common Toxicity Criteria
² STD (standard TMZ maintenance treatment): 200 mg/m² for 5 days of 28-day cycle
³ DI (dose-intensified TMZ maintenance treatment): 100 mg/m² for 21 days of 28-day cycle
⁴ One patient removed from study due to presumed allergic reaction
⁵ Allergic drug reactions concentrated in DI TMZ cohort
⁶ Two patients removed from study due to toxicity. Both conditions resolved after discontinuation of treatment.
KPS: Karnofsky performance status; GM-CSF: granulocyte macrophage colony stimulating factor; i.d.: intradermally; TMZ: temozolomide; C.I._{95%}: 95% confidence interval; mo.: months

Table 3: Summary of rindopepimut

Table reproduced with permission (A. M. Swartz et al., 2014)

Summary of rindopepimut^a
Background
<ul style="list-style-type: none">• Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant glioma.• The epidermal growth factor receptor deletion mutation EGFRvIII is found in ~30% GBM.
Current Therapy
<ul style="list-style-type: none">• The current standard of care for GBM is maximal tumor resection with adjuvant radiotherapy and temozolomide chemotherapy. Median overall survival with this treatment is ~15 months.• Additional therapies being investigated for GBM include a carmustine-impregnated wafer (Gliadel[®] wafers) and an anti-VEGF monoclonal antibody (Avastin[®]).
Overview of Rindopepimut
<ul style="list-style-type: none">• Rindopepimut is a 14-mer peptide spanning mutation site of EGFRvIII, conjugated to the carrier protein keyhole limpet hemocyanin.
Pharmacodynamics
<ul style="list-style-type: none">• Rindopepimut vaccination increases EGFRvIII-specific antibody titers in preclinical and clinical studies.• Some rindopepimut-vaccinated patients exhibited positive EGFRvIII skin test

responses, though data suggesting a cell-mediated response remains inconclusive.

- Vaccination with rindopepimut results in widespread elimination of EGFRvIII-expressing tumor cells.

Clinical Efficacy

- A Phase I trial (VICTORI) evaluated treatment of newly diagnosed GBM patients with autologous dendritic cells electroporated with 500 µg rindopepimut, demonstrating that Rindopepimut is a safe and immunogenic vaccine.
- Phase II trials (ACTIVATE, ACTII, and ACTIII) evaluated the safety, immunogenicity, and efficacy of 500 µg rindopepimut/150 µg GM-CSF in newly diagnosed, EGFRvIII-positive GBM patients. ACTII evaluated vaccine effects in the context of enhanced temozolomide-induced lymphopenia. These studies demonstrated a statistical increase in progression-free and overall survival compared to controls.

Safety and tolerability

- All evaluated rindopepimut vaccines were generally well-tolerated, and toxicity very rarely exceeded grade 2 based on NCI's Common Toxicity Criteria.

4.6.5 Phase II for recurrent GBM: ReACT

ReACT is a non-pivotal Phase II trial for patients with recurrent EGFRvIII-positive GBM. Criteria for selection were individuals who have relapsed on current standard of care treatment and were naïve to anti-VEGF neutralizing antibody, or bevacizumab, therapy. Seventy-two relapsed GBM patients were accrued for this study. Patients were vaccinated with 500 µg rindopepimut plus GM-CSF or KLH alone, each along with bevacizumab, in a double-blinded manner. Vaccines were administered until tumor progression ensued, at which point patients were treated with alternative therapeutics.

Rindopepimut was generally tolerated in this study, with grade I to II injection site reactions being the most common. Rindopepimut generated pronounced anti-EGFRvIII titers (1:12,800 to 1:6,553,600) in 80% of patients. Impressively, treatment with rindopepimut plus bevacizumab was associated with prolonged overall survival compared to the KLH plus bevacizumab control arm (hazard ratio = 0.47), and treatment effects were more favorable in patients having a high Karnofsky score (D. A. Reardon et al., 2015). These results, published in 2015, provided further evidence that rindopepimut was capable of mediating antitumor effects in GBM patients and extended the therapeutic benefit to patients with recurrent GBM.

4.6.6 Phase III for newly-diagnosed GBM: ACTIV

Given the favorable therapeutic responses observed with rindopepimut in previous clinical trials, a phase III trial, known as ACTIV, was initiated. Patient inclusion criteria for this randomized, double-blinded, two-arm registration trial included EGFRvIII-positive *de novo* GBM patients who have received current standard of care treatment and have not progressed following chemo-radiation therapy. This study was carried out at 165 locations in 22 countries and accrued 745 patients. Patients were randomly assigned to the treatment cohort or the control cohort. Patients in the treatment arm received vaccinations with 500 µg rindopepimut plus GM-CSF in combination with TMZ. The control arm was treated with KLH alone along with maintenance TMZ treatment. Vaccinations will be administered until tumor progression ensues.

Rindopepimut was mostly tolerated in this study. Serious adverse events were exhibited in a subset of patients, however. Seizure and brain edema were observed in 5% vs 6% and 2% vs 3% of patients within the treatment arm versus the control arm, respectively. Interestingly, anti-EGFRvIII humoral responses and EGFRvIII-loss in recurrent tumors were very similar among the rindopepimut- and KLH-treated groups. Unfortunately, this trial was discontinued for futility in light of results from interim analysis. This was due to the observation that there was no significant difference in

overall survival between the treatment arm (20.1 months) and the control arm (20.0 months).

4.7 Lessons learned from rindopepimut

While early phase clinical trials with rindopepimut in patients with newly-diagnosed GBM suggested a rindopepimut-mediated treatment benefit, the large-scale phase III clinical trial did not. At this time, the disparate results from these trials remains unclear; however, several different ideas have been proposed. Understanding the basis of the perceived treatment failure will be critical for improving brain tumor immunotherapy.

Of the 745 subjects in ACTIV, 445 patients were classified as having minimum residual disease (MRD; tumor volume $< 2\text{cm}^2$) and 338 were classified as having significant residual disease (SRD, tumor volume $> 2\text{cm}^2$). Interestingly, there appeared to be a survival benefit in the SRD cohort, suggesting the perhaps an appreciable tumor burden is required for rindopepimut-mediated antitumor effects; however, globally, this therapeutic response may be masked in the intent-to-treat population by the MRD cohort. This may explain the observed antitumor effects in the ReACT trial, given that MRD was not an inclusion criterion. However, bevacizumab on its own can have favorable effects on the immunotherapeutic response (Osada et al., 2008; Thomas et al., 2017), and its coadministration may enhance rindopepimut's therapeutic effects.

Additionally, it has been proposed that the enhanced overall survival observed in both treatment arms (i.e. rindopepimut and KLH alone) of ACTIV may be a result of improvements in the standard-of-care over the years. Thus, historical controls may not be appropriate for clinical studies for patients with GBM.

Another explanation to the apparent effects of KLH alone is that simply receiving a generic, but highly immunogenic, vaccine can have therapeutic effects against GBM tumors, and there are several lines of evidence that make this plausible. This begins with the early revelation that patients with tuberculosis had lower incidences of cancer (Pearl, 1929). Then Dr. William B. Coley noted that patients with aggressive cancers treated with his eponymously-named bacterial concoction experienced tumor regression. While many consider this early work to be controversial, generic bacteria-based vaccines have demonstrated utility for other cancers. As an example, *Bacillus Calmette–Guérin* (BCG) has been used to treat superficial bladder cancers for the last 40 years, and to date no other therapy has surpassed its treatment effects for this disease (Fuge et al., 2015). With respect to GBM, work from our lab has shown that GBM patients preconditioned with tetanus-diphtheria toxoid prior to DC vaccination experienced greater survival compared to patients who did not receive this treatment (D. A. Mitchell et al., 2015). This effect was associated with the CCL19 and CCL21-dependent enhancement of dendritic cell migration to the lymph nodes. Thus, generic

vaccines, such as KLH used in ACTIV, could prompt enhanced migration of tumor antigen-loaded DCs to the draining lymph node, which could consequently lead to an improved adaptive immune response. In fact, there was very little difference in the rate of anti-EGFRvIII humoral response between the rindopepimut-treated cohort and the KLH alone-treated cohort (HR = 1.10 for slow response, HR = 1.01 for rapid response) (Weller et al., 2017). Thus, the finding that EGFRvIII expression was lost in recurrent tumors from patients treated with KLH to a similar degree as that of those treated with rindopepimut may be explained by generic immunological stimulation.

Though the results from ACTIV are initially discouraging, there are many insights to be gained from this study. It is clear that antigenic heterogeneity and subclonal neoantigen expression represent significant challenges for GBM vaccines, given the observed outgrowth of EGFRvIII-deficient tumor cells. This may be remedied by activating the cell-mediated arm of the immune system, as opposed to the humoral arm, due to the ability of T-cell-mediated responses to induce epitope spreading. This is, in fact, a major objective of this thesis work. Nevertheless, further elucidation of these principles will undoubtedly be necessary for the improvement of cancer vaccines for GBM and other malignant gliomas.

5. The Development of a Novel Method for Detecting Neoepitope-Reactive Tumor Infiltrating Lymphocytes Using the ELISpot Assay

5.1 Introduction

The detection of antigen-specific lymphocytes is crucial for immune monitoring, as well as investigating the immunological basis of cancer, autoimmunity, and pathogenic disease. Among the most widely-used assays for this purpose are the ELISpot assay, multimer analysis, and intracellular staining (ICS) (Phetsouphanh et al., 2015). Of these methods, the ELISpot assay offers several clear advantages. The ELISpot is based on the principle that soluble molecules secreted by stimulated cells are captured onto antibody coated plates, allowing effector cells to be enumerated (Czerkinsky et al., 1988). In contrast to multimer analysis, the ELISpot is a functional assay that can test lymphocyte reactivity to a large number of antigens in a high throughput manner without an underlying knowledge of exact epitopes or MHC alleles (Calarota et al., 2013). Furthermore, the ELISpot is capable of detecting low avidity T cells, which includes many tumor-reactive or autoimmune T cells and most CD4⁺ T cells, while the use of multimers is typically reserved for the detection of high avidity T cells (Wooldridge et al., 2009). In comparison with ICS, both methods have the ability to sensitively detect antigen-specific lymphocytes that produce a variety of soluble molecules (e.g., cytokines, chemokines, and antibodies); however, the ELISpot is

superior for the detection of *bona fide* effector cells given that it measures only secreted, and therefore biologically-relevant, effector molecules (Calarota et al., 2013).

Tumors frequently contain an enriched population of tumor-reactive lymphocytes that are desirable for investigating tumor immunobiology. Unfortunately, isolating TILs from small mouse tumors has historically been a challenge due to their low frequencies and a limited supply of tissue (Prevost-Blondel et al., 1998). Furthermore, the oft-used method of dissociating tumors with enzymes, such as DNase and collagenase, are prone to inconsistencies and have been shown to alter surface T-cell coreceptor expression levels, which may affect T-cell function (Mulder et al., 1994). Here, we present a simple and enzyme-free protocol for processing TILs from small mouse intracerebral tumors for ELISpot analysis. Briefly, tumors are gently dissociated into a single cell suspension using a paddle blender. Cells are then plated for several hours in a tissue culture-treated flask to remove adherent cells (e.g., tumor cells, macrophages, stromal cells) that could otherwise sterically interfere with effector molecule capture onto the plate. Finally, a 2-day *in vitro* culture period in medium containing IL-2 allows lymphocytes to recover from a non-responsive state (Mognol et al., 2017). This methodology permits an enrichment of viable and responsive effector immune cells suitable for ELISpot analysis.

5.2 Materials and Methods

5.2.1 Tumor cell preparation and intracerebral injection

B16F10 mouse melanoma and a variant expressing chicken ovalbumin, B16OVA, were used for this study. Upon injection of B16OVA into syngeneic C57BL/6 mice, the host immune system spontaneously activates CD8⁺ T cells recognizing the epitope SIINFEKL bound to the MHC I allele K^b. B16 cells were cultured in DMEM containing 10% heat inactivated FBS and 1x penicillin streptomycin to 85% confluency at 37°C/ 5% CO₂. Cells were then passaged at a 1:8 dilution and harvested by trypsinization at approximately 60-80% confluency two days later. B16 melanoma cells were washed thrice in ice-cold 1x PBS, viable cells counted using trypan blue exclusion, and cells resuspended to 4 x 10⁶ cells mL⁻¹ in 1x PBS. Tumor cells were then mixed thoroughly in an equal volume of 10% methylcellulose in PBS using a sterile 1 mL syringe. 6 to 12-month-old C57BL/6 mice were premedicated with buprenorphine SR and anesthetized in an isoflurane chamber. Anesthetized mice were then placed into a stereotactic frame (Kopf), and a ~1 cm sagittal incision was made to reveal the skull. One drop of bupivacaine was added to the incision site. A 25-gauge injection needle was placed 2 mm right of the bregma and inserted to a depth of 4 mm below the surface of the skull. 1.0 x 10⁴ cells in a volume of 5 μL were delivered into the caudate nucleus. Following injection, the hole was sealed using bone wax and the incision closed with a surgical

staple. C57BL/6 mice inoculated intracerebrally with B16 melanoma cells become moribund in ~18-22 days. All animal experiments were performed in accordance with Duke University Institutional Animal Care and Use Committee-approved protocols.

5.2.2 Intracerebral tumor harvesting

Moribund mice were sacrificed in a CO₂ chamber followed by decapitation. In a laminar flow hood, using sterile forceps and scissors, the skull plate covering the tumor injection site was carefully removed. B16F10 and B16OVA tumors are distinguishable from normal brain tissue by their darker color. The tumor burden was removed using jeweler's forceps and placed in a non-tissue culture-treated dish containing 15 mL of T-cell medium (RPMI, 10% heat inactivated FBS, 1 mM glutamine, 1x NEAA, 1x sodium pyruvate, 1x penicillin streptomycin, 55 μ M β -Me, and 100U mL⁻¹ IL-2). The tumor was minced into small pieces using scissors, transferred into a paddle blender bag (standard size, 105 x 155 mm), and agitated in a paddle blender (Stomacher® 80 Biomaster, Seward) for 30-60 min at 37°C on medium speed. The dissociated tumor sample was then filtered through a 70 μ M strainer into a sterile 50 mL conical tube, and the single cell suspension was washed once with fresh T-cell medium. After pelleting cells at 350g for 10 min, cells were resuspended in fresh T-cell medium and transferred into a tissue culture-treated flask, maintaining a ratio of 2-3 cm² plating area for every 1 mg of isolated tumor mass. Following an 8-12 h incubation period at 37°C/ 5% CO₂, non-

adherent cells were pelleted, resuspended in 15 mL T-cell medium, and incubated in a T75 flask at 37°C for an additional 2.5 days. This additional resting period allows TILs to become responsive to stimulation and does not result in a significant expansion or diminished viability of TILs (Figure 4).

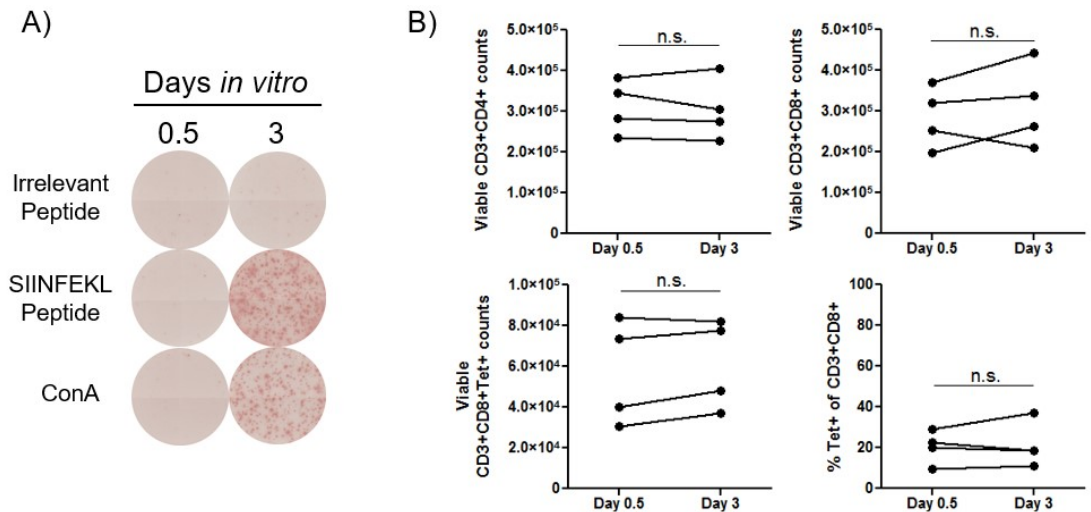


Figure 4: TIL resting period revives antigen-responsiveness without promoting significant proliferation or cell death.

Figure reproduced with permission (A. M. Swartz et al., 2018)

A) Day 20 intracerebral B16OVA tumor tissue was processed according to the described protocol, using a 12 h *in vitro* incubation period to remove adherent cells. The presence of SIINFEKL-cognate T cells in 25K viable TILs was compared between samples cultured *in vitro* for 0.5 days vs 3 days using the ELISpot assay. B) Viable CD3+CD4+, CD3+CD8+, and CD3+CD8+SIINFEKL/K^b tetramer+ T-cell counts, determined by flow cytometry, from day 20 intracerebral B16OVA tumors at days 0.5 and 3 of *in vitro* resting period (n=4). Difference in viable T-cell counts not significant by Mann-Whitney U test.

5.2.3 Tumor infiltrating lymphocyte processing for ELISpot analysis

Non-adherent cells were washed once in R10 (RPMI, 10% heat-inactivated FBS, 1x penicillin streptomycin, and 1x NEAA) and then carefully layered onto 5 mL cell-separation gradient (Lympholyte-M, Cedarlane Labs). Isopycnic centrifugation was performed at room temperature at 1,300g for 25 min with no brake. The buffy coat layer was carefully removed and washed twice in fresh R10 medium. Viable lymphocytes were quantified using a flow cytometer (Guava® easyCyte, Millipore Sigma); however, we have also found that judicious counting of viable lymphocyte-sized cells using trypan blue exclusion yields very similar results. Generally, >85% of lymphocytes are viable. Following one final wash in fresh R10, cells were resuspended to the desired concentration for the ELISpot assay. Typically, 25-100K TILs per well are sufficient for analysis. While these numbers are much lower than that of traditional ELISpots (> 150K cells), we have not found evidence of insufficient antigen presentation as a result of low cell density. It is probable that the non-lymphocytic cells that are not removed via plastic adherence assist in antigen presentation.

5.2.4 IFN γ ELISpot assay

96-well PVDF plates (Multiscreen HTS filter plates, Millipore Sigma) were treated with 15 μ L 70% ethanol for 30 seconds and then washed thrice with 1x PBS. 100 μ L 10 μ g mL⁻¹ IFN γ antibody (clone AN18 antibody, Mabtech) was added to each

treated well and ELISpot plates were incubated at 4°C overnight. The following day, ELISpot plates were washed once with 1x PBS and blocked for 2 h at 37°C with 150 µL R10 medium. Lymphocytes from the previous step were stimulated with R10 medium alone, 1 µM irrelevant peptide, 1 µM SIINFEKL peptide, or 4 µg mL⁻¹ concanavilin A (ConA), and 25K cells were added per well, performing each sample in duplicate. ELISpot plates were incubated for 18-24 h at 37°C. Following incubation, ELISpot plates were washed 6 times with wash buffer (1x PBS + 0.05% tween). After removing wash buffer, 1 µg mL⁻¹ biotin-conjugated IFN γ antibody (biotinylated clone R4-6A2 antibody, Mabtech) in 1x PBS plus 0.5% BSA was added to each well and incubated at 37°C for 2 h. ELISpot plates were then washed 4 times with wash buffer. 100 µL avidin-conjugated peroxidase (Vectastain ABC, Vector Laboratories) was added to each well and incubated at room temperature for 1 h. ELISpot plates were washed twice in wash buffer and then thrice in 1x PBS. ELISpot plates were developed in 100 µL AEC solution (AEC staining kit, Sigma) for 8 min and then rinsed with copious amounts of water. ELISpot plates were air dried and evaluated in blinded fashion (ZellNet Consulting, Inc., Fort Lee, NJ) using an ELISpot reader (KS ELISpot reader, Zeiss) with KS ELISpot software version 4.9.16. The plate evaluation process including the setup of optimal reading parameters followed the International guidelines on ELISpot plate evaluation (Janetzki et al., 2015).


- 
1. Harvest tumor tissue and cut into small pieces in 15 mL T-cell medium. Transfer tissue to a standard sized paddle blender bag.
 2. Agitate in a paddle blender for 30-60 min at 37°C on normal setting.
 3. Filter debris using 70 μm strainer then incubate single cell suspension in a T150 flask at 37°C for 8-12 h in medium containing 100U/mL IL-2. Next, transfer non-adherent cells to a new flask, and incubate for an additional 2.5 days.
 4. Enrich viable leukocytes using isopycnic centrifugation, and wash cells twice.
 5. Enumerate viable lymphocytes and perform ELISpot assay.

Figure 5: An enzyme-free method for processing tumor infiltrating lymphocytes from mouse tumors for ELISpot analysis.

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5.3 Results and Discussion

Using the protocol outlined above (Figure 5), we achieve reproducible detection of antigen-specific lymphocytes from mouse intracerebral tumor tissue (Figure 6). We have also found that this protocol preserves the versatility of the ELISpot assay. This includes antibody-mediated MHC blockade and bead-based cell subset depletions that facilitate the determination an epitope's MHC-restriction and effector subset, respectively (M. Wang et al., 2010). Additionally, titrating the dose of stimulating peptide enables an approximation of T-cell avidity (Hesse et al., 2001). This method may also be used to detect low avidity, antigen-specific CD4+ T cells that reside within the tumor environment, though for this purpose we add an additional 100K spleen-derived, non-irradiated leukocytes from an untreated mouse to provide a source of professional antigen presenting cells (APCs), as a precaution. We also advise using this approach when stimulating with elongated peptides that require processing prior to being presented on MHC molecules.

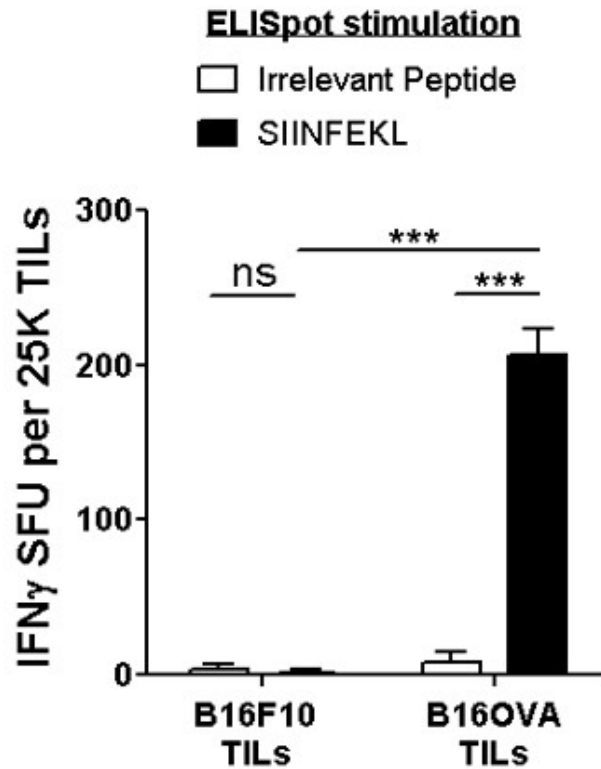


Figure 6: SIINFEKL-cognate lymphocytes from mouse intracerebral tumors are detectable by ELISpot

Figure reproduced with permission (A. M. Swartz et al., 2018)

Quantification of IFN γ spot forming units (SFU) from TILs enriched from B16F10 and B16OVA intracerebral tumors (n=3 per group). ***p < 0.001 by two-way ANOVA with Bonferroni post-hoc test. Data are representative of 3 independent experiments.

Here, we have developed an enzyme-free protocol for processing lymphocytes from mouse tumors for subsequent analysis using the ELISpot assay. While our data here are demonstrated using an intracerebral B16 melanoma tumors, we have successfully employed this strategy for the detection of antigen-specific lymphocytes

from subcutaneous tumors and non-melanoma tumors (e.g., SMA-560). Given the simplicity of this approach, this method may also be adapted to various source tissues wherein lymphocytes represent a minor population.

We believe that this methodology is highly-adaptable to the immunological monitoring of neoantigen-specific vaccine responses in the clinic. Because this procedure utilizes the host's own antigen presenting cells, this approach is not limited by patient-specific MHC polymorphisms or the pre-determination of defined epitopes, which are drawbacks of the oft-used tetramer analysis. Furthermore, this method can be reliably used to detect MHC I or MHC II-specific lymphocyte response. As we demonstrate in the next chapter, this approach allowed us to correlate antitumor responses by neoantigen-specific synthetic long peptide vaccines with the tumor infiltration of neoantigen-reactive lymphocytes, providing proof-of-concept that this methodology can help elucidate the mechanism of neoantigen-targeting vaccine responses.

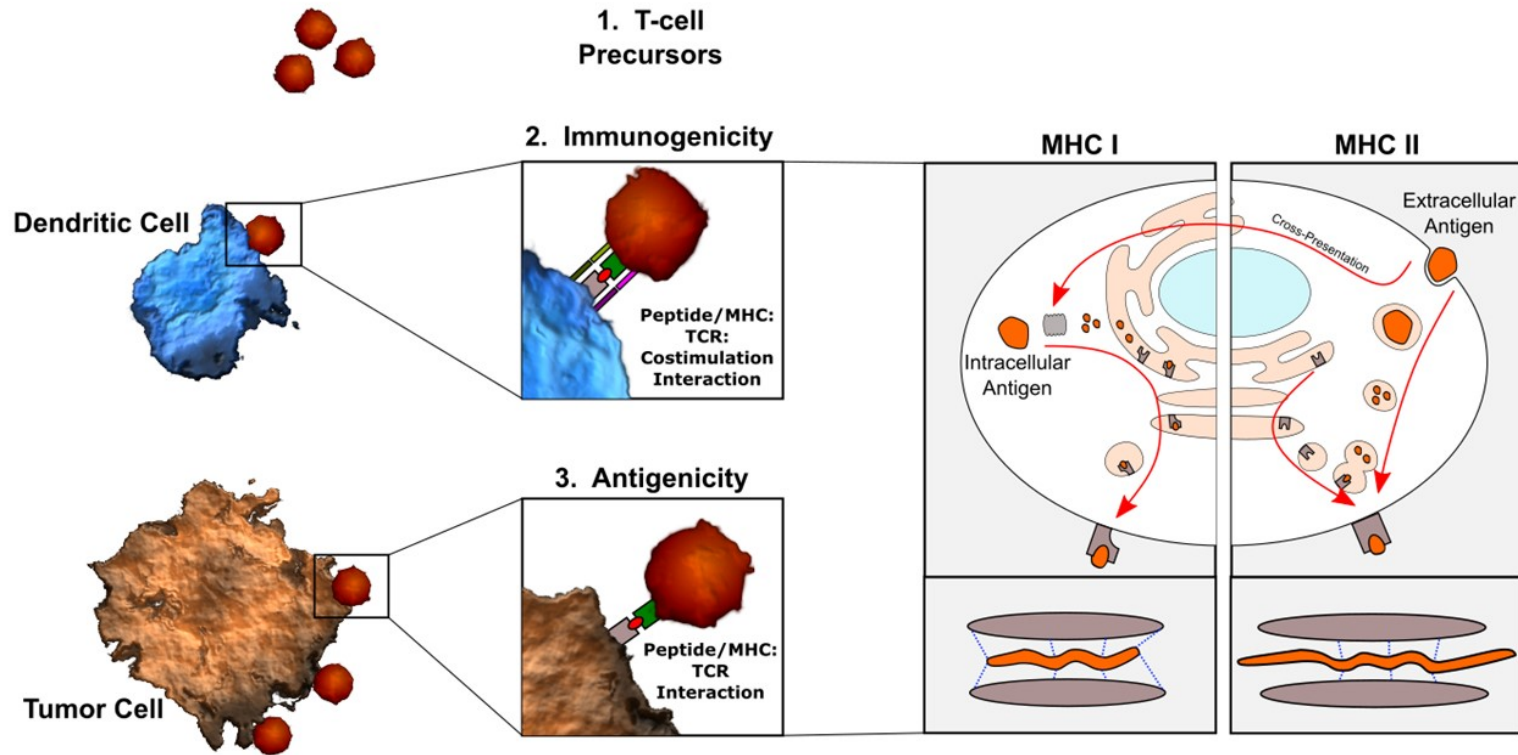


Figure 7: Immunological requirements for T-cell activating cancer vaccines

Original artwork by Swartz AM, designed using Inkscape v0.92

These conditions must be met for a cancer vaccine to afford antitumor benefits: 1) the host must harbor T-cell precursors that recognize the tumor-specific antigen; 2) the immunogen (e.g., synthetic long peptide) must contain a tumor-specific epitope, which must be adequately processed and loaded onto the MHC molecules of activated dendritic cells – the primary activators of T cells (immunogenicity); and 3) in order for activated T cells to react with the tumor, the tumor, or some neighboring cell, must process the tumor-specific epitope from the native tumor antigen and present it on MHC molecules (antigenicity). MHC I, recognized by CD8+ T cells, can present tumor epitopes from intracellular or extracellular (cross presented) antigens in a proteasome- and TAP-dependent manner. MHC I stabilizes peptides at their amine and carboxy termini, thus restricting epitope length to ~8-10 amino acids, as well as through amino acid R-group binding to hydrophobic pockets within MHC I molecules. MHC II, recognized by CD4+ T cells, generally presents epitopes from extracellular antigens, processed via the endolysosome. MHC II stabilizes peptides only through amino acid R-group binding; therefore, epitope length is not rigidly restricted.

6. The Rational Design of Synthetic Long Peptides Using a Universal Helper Epitope Can Improve the Therapeutic Potential of Neoantigen Vaccines

6.1 Introduction

Recurrence of glioblastoma following standard-of-care therapy with gross tumor resection, radiotherapy, and temozolomide chemotherapy is almost certain given the inability of these modalities to selectively eliminate all malignant cells that infiltrate into the brain parenchyma. Immunotherapy, which affords molecular-guided precision and high cytotoxic potential, is currently being explored to address this unmet need. GBM tumors exhibit a high degree of intertumoral heterogeneity (Brennan et al., 2013; Q. Wang et al., 2018); therefore, immunotherapies that are not attenuated by patient-specific variation are needed. Cancer vaccines offer an immunotherapeutic modality that can be tailored to an individual patient in an effort to maximize the response to their unique set of tumor antigens.

Results from a recent phase III trial with a proteinaceous vaccine targeting the GBM-specific antigen EGFRvIII, rindopepimut, revealed that the majority of recurrent tumors were largely composed of EGFRvIII-deficient tumor cells (Weller et al., 2017). These data suggest that monovalent vaccines targeting subclonal, heterogeneously-expressed GBM antigens are likely insufficient to treat this devastating disease. Cancer vaccines targeting neoantigens arising from somatic missense mutations, hereafter

referred to only as neoantigens, provide an opportunity to induce a multivalent, tumor-specific immune response with the potential to target a broader repertoire of GBM cells that, consequently, may impede or prevent relapse.

ND GBM expresses, on average, only 35 somatic mutations (Alexandrov et al., 2013; Vogelstein et al., 2013). Though it may be practical to immunize against all or most ND GBM neoantigens, ensuring their true immunotherapeutic potential is crucial given their relative low numbers. By examining the mechanism of an efficacious SLP vaccine targeting the SMA560-specific neoantigen Odc1, we discovered a reliance on a bipartite SLP vaccine that induces both a neoepitope-specific CD8⁺ T-cell response and a CD4⁺ T-helper response. Furthermore, provision of T-cell help using the universal helper epitope P30, from tetanus toxin, was able to sustain the therapeutic benefits of the Odc1 vaccine and, remarkably, enhance the immune and antitumor responses by other neoantigen vaccines. Together, these data demonstrate a clinically-amenable vaccine design strategy with the potential to broaden the therapeutic range of neoantigen-targeting SLP vaccines and, fundamentally, suggest that the native neoantigen sequence may not always be optimal for neoantigen-targeting vaccines. These data provide encouraging insights for clinical neoantigen-vaccine design, especially for ND GBM and other tumors exhibiting a low mutational burden.

6.2 Methods and Materials

6.2.1 Cell lines, mice, and media

All animal experiments were performed in accordance with Duke University's Institutional Animal Care and Use Committee-approved protocol. Wild-type VMDk mice were bred in-house under pathogen-free conditions at Duke University Medical Center. Male 129S6 mice were acquired from Taconic Farms. For immunogenicity and tumor studies, 6-12-week-old mice were used. SMA560 tumor cells were cultured in IMEM-Zinc Option medium (Gibco) containing 10% FBS (Gemini Bio-Products) and 1x penicillin-streptomycin (Gibco). R10 medium consisted of RPMI (Gibco), 10% FBS, 1x MEM-NEAA (Gibco), and 1x penicillin-streptomycin. T-cell medium consisted of R10 medium with 1 mM glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 50 μ M beta-mercaptoethanol (Gibco), and 100 U/mL IL-2. FACS buffer consisted of 1x PBS (Gibco) plus 2% FBS (Gemini Bio-Products).

6.2.2 Next-generation exome sequencing and ribosome profiling

Exome libraries were captured from SMA560 tumor cells and syngeneic healthy VMDk brain tissue using the SureSelect Mouse All Exon kit (Agilent). Sequencing was performed on an Illumina HiSeq2000 as 100bp paired end reads, and the resulting reads were aligned to the mm9 mouse reference genome. Variants were called using the Illumina CASAVA pipeline. Alterations occurring outside of the protein coding region

were removed from subsequent analyses. Ribosome profiling was performed as described previously (Reid et al., 2015). Briefly, SMA560 cells were lysed on ice in buffer containing 1% NP-40, and the clarified supernatant was subjected to micrococcal nuclease treatment for 30 min at 37°C to generate ribosome-protected mRNA fragments. Following Trizol (Invitrogen) enrichment and isopropanol precipitation, the purified mRNA was treated with PNK for 30 min at 37°C and then electrophoresed through a 15% TBE-urea polyacrylamide gel alongside a 35 nt oligo (IDT). The ~35 nt mRNA band was carefully removed, processed to remove the polyacrylamide, and ligated into a sequencing library (NEBNext® Small RNA Library, NEB). Sequencing was performed using Illumina MiSeq as 50bp single end reads. Reads were mapped to the GRCm38 reference assembly using HISAT2 and transcript abundance was determined using Cufflinks(Trapnell et al., 2010), as a measure of their FPKM (fragments per kilobase of transcript per million mapped reads) values. Cross-referencing the ribosome profiling results with the exome sequencing data served as an indicator of neoantigen expression within SMA560. Genes with an FPKM > 0.25 were regarded as “expressed” and included in downstream studies. For all identified immunogenic SLPs, the presence of the mutated transcript within *in vivo*-derived SMA560 tumors was confirmed using RT-PCR and Sanger sequencing.

6.2.3 Bioinformatic prioritization of mutations

The 225 expressed missense mutations were analyzed using Immune Epitope Database (IEDB) MHC I and II-binding prediction algorithms using a 29mer peptide sequence with the mutation in the center. IEDB percentile rank scores were reported for the top-ranked predicted epitope containing the mutation. Peptides were excluded if the highest-ranked predicted epitope (1) had a MHC II percentile rank of >50, in light of a prior study indicating their high unlikelihood of generating an immune response (Kreiter et al., 2015) or (2) had a predicted MHC I percentile rank scores of >10. Mutant peptides were ranked by their best MHC I and MHC II percentile rank score and the top 39 from MHC II predictions and the top 41 from MHC I predictions were selected for empirical determination of immunogenicity.

6.2.4 RMA-S MHC I stabilization assay

RMA-S cells were cultured in R10 medium for 48 h at 26°C/ 5% CO₂. Cells were then incubated with 10 µM peptide for 4 h at 26°C/ 5% CO₂ followed by a 1 h incubation at 37°C/ 5% CO₂. Next, 1 × 10⁶ peptide-pulsed RMA-S cells were stained with anti-H-2K^b PE (AF6-88.5.5.3, Invitrogen) and anti-H-2D^b APC (clone 28-14-8, Invitrogen) antibodies in FACS buffer for 30 min at 4°C and then evaluated using a FACSCalibur flow cytometer (BD Biosciences).

6.2.5 Synthetic long peptide vaccines

All peptides were synthesized by Genscript. The sequences of all tested SMA560 29mer SLPs, with the mutation at residue 15, are listed in Appendix A Table 9. The peptide sequences used for Lama4 and Alg8 vaccines were QKISFFDGFEVGFNFRTLQPNGLLFYYT and AVGITYTWTRLYASVLTGSLV (MHC I-restricted neoepitope underlined), respectively (Gubin et al., 2014). For P30-conjugated vaccines, furin-P30 (RVKRFNNFTVSFWLRVPKVSASHLE) was positioned after the carboxyl terminus of the MHC I epitope.

6.2.6 *In vivo* immune and tumor studies

For tumor studies, three days prior to tumor implantation, the right rear flank of VMDk mice was shaved. Mice were inoculated with 7.5×10^5 SMA560 tumor cells suspended in 1x PBS (Gibco). Tumor growth was determined using a digital caliper. In some experiments, mice were given 200 μ g α CD40L (clone MR-1, BioXCell), α CD8a (clone 2.43, BioXCell), or α CD4 (clone GK1.5, BioXCell) or corresponding isotypes (BioXCell) antibody intraperitoneally. For immune studies, all vaccines were comprised of 15 nmol peptide plus 100 μ g polyinosinic:polycytidylic acid (poly(I:C); InvivoGen) administered intravenously. In some experiments, VMDk mice were given 200 μ g α CD40L or isotype antibody intraperitoneally on days -1, 0, and 2 relative to a vaccine. CD40L antibody dose was 400 μ g for 129S6 mice, due to greater spleen cellularity.

6.2.6 Tumor-infiltrating lymphocyte processing

TIL processing for ELISpot analysis was performed as previously described (A. M. Swartz et al., 2018). Briefly, ~100 mg excised tumor was minced into small pieces in 15 mLs of T-cell medium and transferred into a paddle blender bag. Tumor was dissociated in a paddle blender for 45 min at 37°C, followed by straining through a 70 µm cell strainer. Following centrifugation at 350 g for 10 min, cells were resuspended in 40 mL T-cell medium and 2 x 20 mL seeded into T150 flasks. After a 12 h incubation at 37°C/ 5% CO₂, nonadherent cells were centrifuged, resuspended in 15 mL T-cell medium, and replated into a T75 flask. Cells were incubated at 37°C/ 5% CO₂ for an additional 2.5 days before harvesting for ELISpot analysis.

6.2.7 IFN γ ELISpot assay

Splenocytes or TILs were filtered using a 70 µm strainer. Cells were resuspended in 8mLs R10 medium, layered onto Lymphocyte-M (Cedarlane), and isopycnic centrifugation performed at 1,300 g for 25 min with no brake. The buffy coat layer was removed and cells washed twice with R10 medium before counting using a flow cytometer (Guava easyCyte, Millipore Sigma). 5×10^5 splenocytes or 1×10^5 TILs were plated per well of a 96-well PVDF plates (Multiscreen HTS, Millipore Sigma) that had previously been coated with $10\mu\text{g mL}^{-1}$ anti-IFN γ antibody (clone AN18, Mabtech). For TIL ELISpots, 2.5×10^4 naïve splenocytes were added to each well to provide a

source of APCs. Cells were stimulated with 1 μM peptide or 4 $\mu\text{g mL}^{-1}$ ConA. After an 18-22 h incubation period at 37°C/ 5% CO₂, IFN γ secretion was detected using 1 $\mu\text{g mL}^{-1}$ biotinylated anti-IFN γ (clone R4-6A2 antibody, Mabtech) followed by development with avidin-conjugated peroxidase (Vectastain ABC, Vector Laboratories) and AEC (Sigma). For MHC II-blocking ELISpots, 20 $\mu\text{g mL}^{-1}$ anti-MHC II antibody (M5/114, BioXcell) was incubated with cells for 2 h prior to peptide stimulation. For bead-based depletion ELISpots, CD4⁺ or CD8⁺ cells were removed by adding 6 $\times 10^6$ antibody-conjugated or unconjugated beads (M280 Dynabeads, Invitrogen) in 10 μL s to each sample, gently rotated for ~8 mins, and beads magnetically removed. All samples were evaluated in duplicates.

6.2.8 Statistical analysis

ELISpot data was evaluated using an unpaired two-sample *t*-test or one-way ANOVA with post hoc Tukey's test (Dittrich et al., 2012). Mean tumor volumes at the time point when the control cohort surpassed a mean tumor volume of 2000 mm³ were compared between two groups using an unpaired two-sample student's *t*-test. Asterisks indicate degree of significance (**P* < 0.05, ***P* ≤ 0.01, ****P* < 0.001, *P* > 0.05 not significant (ns)).

6.3 Results

6.3.1 Identification of expressed SMA560 neoantigens and candidate neoantigen selection

The spontaneous mouse malignant astrocytoma SMA560, from the VMDk mouse strain (H2^b haplotype), was selected for this study due to its immunological resemblance to human malignant astrocytoma stem cells, which are considered to be major drivers of tumor recurrence following standard-of-care therapy (Auffinger et al., 2015). Principle factors included low expression of MHC I and lack of MHC II expression (Di Tomaso et al., 2010; Sampson et al., 1997). Exome sequencing of SMA560 cells and healthy VMDk brain tissue revealed 1,414 single-nucleotide coding mutations (Table 4). Interestingly, SMA560 possesses a nonsense mutation in TP53 at exon 8 (~81% homology with human TP53 exon 8) – a locus that is frequently mutated in human glioblastoma (Ohgaki et al., 2004) – and may signify an early event in malignant transformation. 721 tumor-specific nonsynonymous mutations were identified, of which 673 resulted in amino acid substitutions (Table 4). To assess neoantigen expression within SMA560, we employed ribosome profiling (Reid et al., 2015), which sequences only transcripts with bound ribosomes and serves as a more reliable indicator of protein translation compared to mRNA sequencing. Using a conservative cut-off of FPKM > 0.25, 225 missense mutations were selected for further evaluation (Table 4).

Table 4: Single nucleotide coding mutations in SMA560, determined by exome sequencing and ribosome profiling

Single Nucleotide Coding Mutations (SMA560)	
Point Mutations	1,414
Synonymous	693
Nonsynonymous	721
Nonsense	48
Missense	673
Expressed (FPKM > 0.25)	225

MHC I- (Castle et al., 2012; Gubin et al., 2014; Ott et al., 2017) and MHC II-binding algorithms (Kreiter et al., 2015) have proven useful for candidate neoantigen selection for vaccine strategies. Though SMA560 does not express MHC II, even upon exposure to IFN γ (Sampson et al., 1997), studies have demonstrated that host antigen presenting cells can present tumor-derived MHC II epitopes to T cells (Perez-Diez et al., 2007), which can lead to indirect antitumor effects (Mumberg et al., 1999). Therefore, we did not disregard MHC II-restricted neoepitopes for this study. The region spanning the missense mutation for all 225 expressed neoantigens was evaluated using IEDB MHC I- and MHC II-binding algorithms. Sixty-three unique peptides, comprised of top-ranked peptides from both MHC I- and MHC II-prediction algorithms, were empirically tested for immunogenicity (Appendix A Table 9; see methods).

6.3.2 A 29mer SLP vaccine targeting the SMA560 neoantigen Odc1 elicits antitumor benefits in a therapeutic setting

The immunogenicity of candidate neoantigens was tested using 29mer SLPs, with the mutated residue at position 15, administered intravenously with the dsRNA analog poly(I:C). Seven days after a single vaccination, splenocytes were evaluated for their responsiveness to the immunizing mutant peptide and the corresponding wild-type peptide using the IFN γ ELISpot assay. Of the 63 tested SLPs, 8 (~13%) elicited a detectable immune response, with the response, in most cases, reacting predominantly to the mutant peptide (Figure 8).

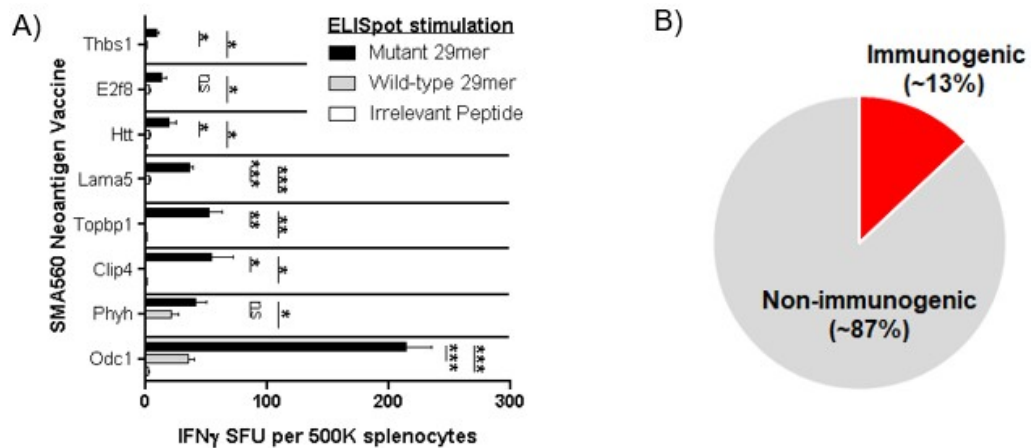


Figure 8: Immune responses engendered by SLPs targeting SMA560 missense mutations

A) IFN γ ELISpot: splenocyte response to the immunizing mutant 29mer peptide and the corresponding 29mer wild-type peptide assessed 7 days following mutant 29mer SLP vaccination (n = 3-4). One-way ANOVA with post-hoc Tukey's test. B) Percentage of 63 tested SLPs that are immunogenic versus non-immunogenic.

Next, we examined the therapeutic potential of each immunogenic SLP by immunizing syngeneic VMDk mice on days 1 and 8 following tumor implantation. A significant antitumor benefit was achieved only with the SLP targeting the Odc1 neoantigen (Figure 9).

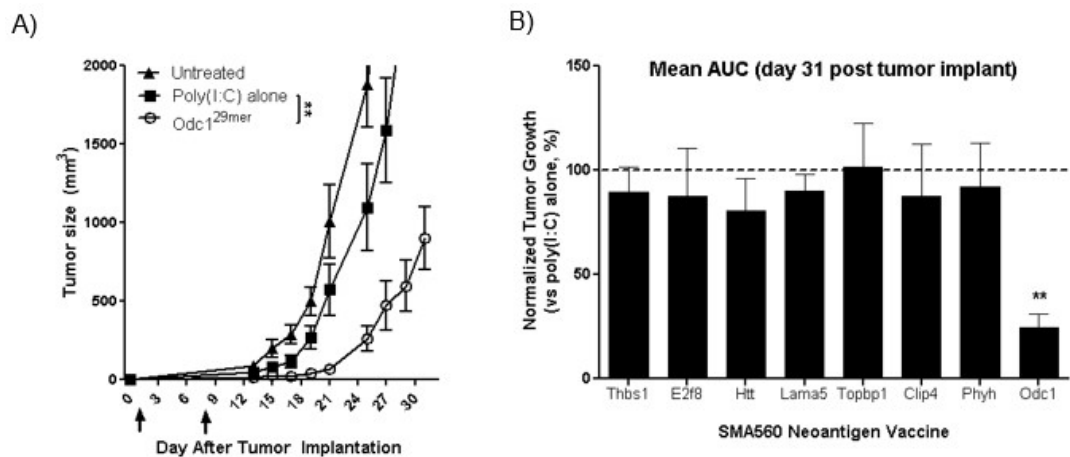


Figure 9: Antitumor responses engendered by SLPs targeting SMA560 missense mutations

A) Subcutaneous SMA560 tumor growth in mice (n = 8) immunized on days 1 and 8 with poly(I:C) alone or Odc1^{29mer} formulated in poly(I:C) or left untreated. Day 31 tumor volume, two-sample *t*-test. B) Mean area under tumor growth curve in SLP immunized mice, normalized to poly(I:C) alone-treated cohort. Day 31 volume, two-sample *t*-test.

We and others (Johanns et al., 2016) have observed Odc1-reactive tumor infiltrating lymphocytes (TILs) within untreated SMA560 tumors (Figure 10), demonstrating that the Odc1 neoantigen is spontaneously immunogenic upon tumor implantation. This effect was not seen with other immunogenic SMA560 neoantigens,

however. Given the favorable immunogenic and therapeutic profile of the Odc1 neoantigen, these findings created an opportunity to gain mechanistic insight into a therapeutically-relevant neoantigen vaccine, in hopes that this knowledge could be leveraged to enhance the effectiveness of other neoantigen-targeting SLP vaccines.

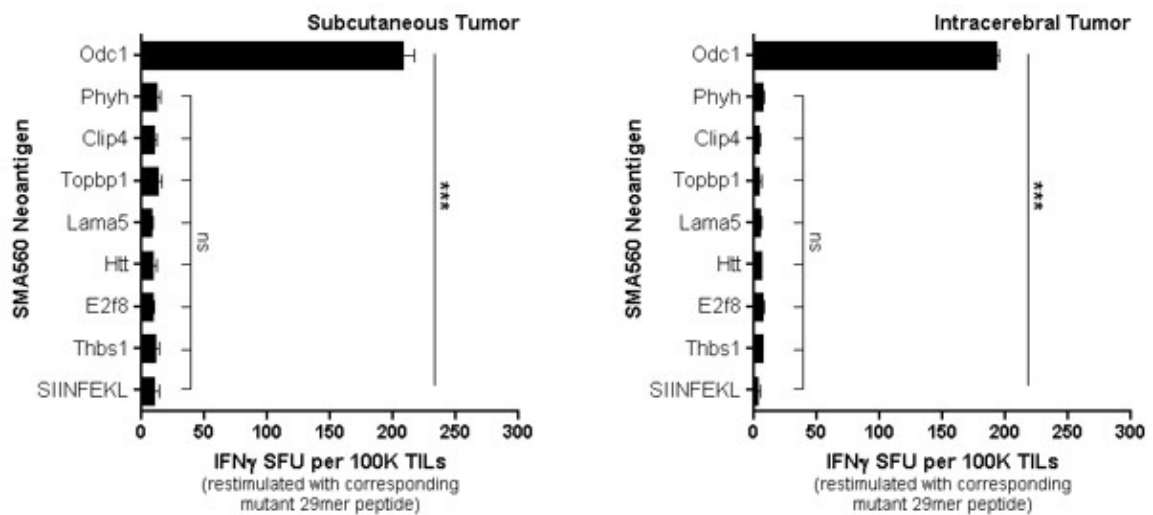


Figure 10: Odc1-reactive TILs detected within untreated subcutaneous and intracerebral SMA560

IFN γ ELISpot: TILs from day 27 subcutaneous (left, n = 2) or day 18 intracerebral (right, n = 2) SMA560 tumors were evaluated for their reactivity to 29mer SLPs spanning SMA560 missense mutations. Responses were compared to stimulation with the negative-control peptide SIINFEKL. One-way ANOVA with post-hoc Tukey's test. Representative data of two independent experiments.

6.3.3 The antitumor effects of the Odc1^{29mer} vaccine is mediated by a helper-dependent CTL response

Earlier studies have demonstrated that mutant Odc1-cognate CD8+ T cells are present within SMA560 tumors (Johanns et al., 2016; Woroniecka et al., 2018a); therefore, we hypothesized that the therapeutic effect of the Odc1^{29mer} vaccine was mediated by a potent induction of an Odc1-reactive CD8+ T-cell response. In an effort to characterize the immune response induced by the Odc1^{29mer} and other immunogenic SLPs, we first evaluated the ability of all highly-ranked predicted MHC I epitopes (i.e. IEDB MHC I percentile rank score of ≤ 1) within immunogenic 29mer SLPs to stabilize surface MHC I molecules using the RMA-S assay, which determined that only the top-ranked predicted MHC I epitope within the Odc1^{29mer} and 5 additional neoantigen SLPs was able to bind to MHC I (Figure 11A, Table 5). Both the Odc1^{29mer} and Topbp1^{29mer} vaccines elicited a detectable CD8+ T-cell response to their respective MHC I-restricted neoepitope (Figure 11B); however, strikingly, these responses accounted for only a portion of the total immunizing peptide restimulation response (Figure 11C). Therefore, we tested whether the remainder of the response was reactive towards an embedded MHC II epitope. Immunologically blocking MHC II (Kreiter et al., 2015) prior to restimulating splenocytes from vaccinated mice with the 29mer immunizing mutant peptide revealed that all immunogenic SLPs did, in fact, elicit a MHC II-specific immune response (Figure 11D), which was confirmed to be CD4+ T cells by depletion experiments (data not

shown). Altogether, these results provided evidence that the antitumor capabilities of the Odc1^{29mer} vaccine may be driven, at least in part, by the stimulation of Odc1-cognate CD8+ T cells.

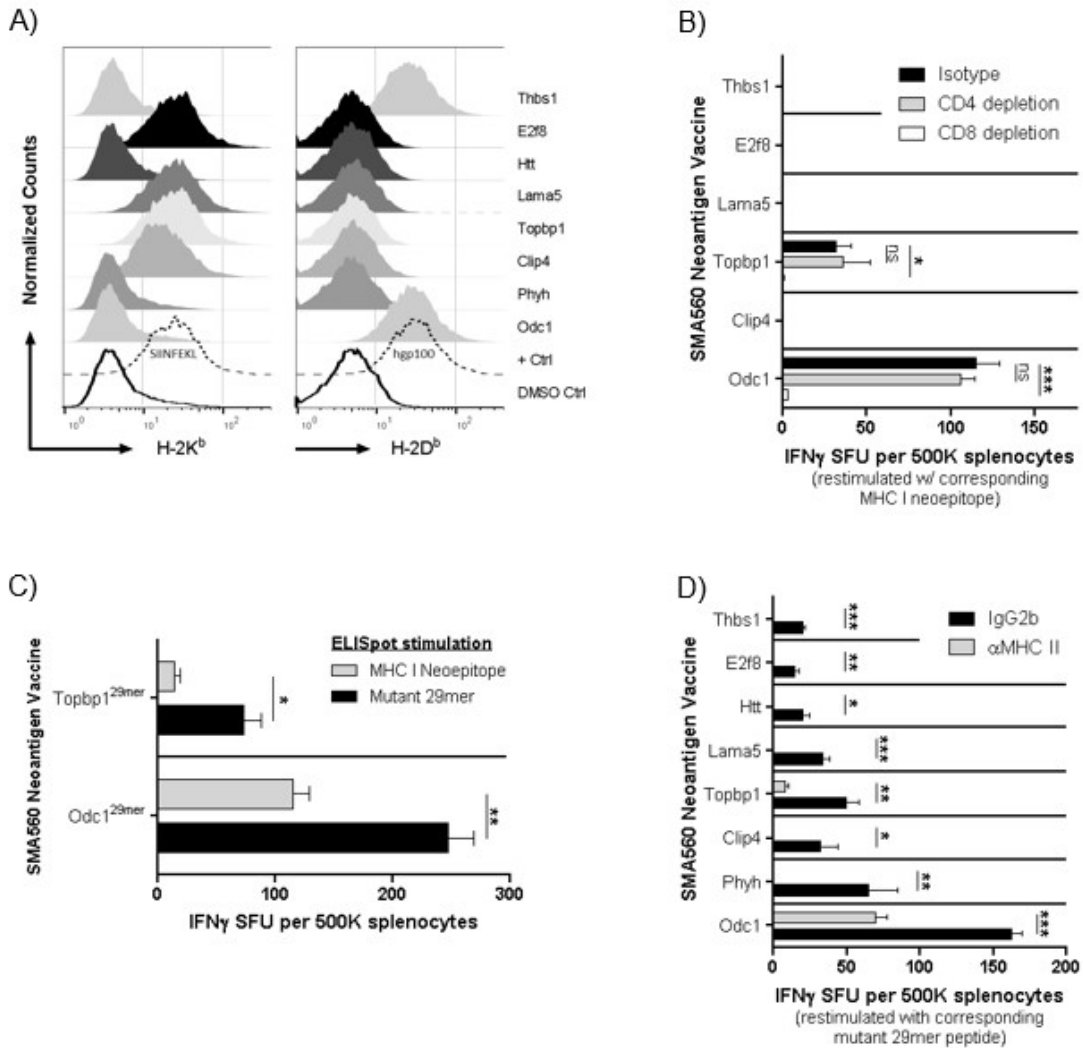


Figure 11: Most immunogenic 29mer SLPs possess a MHC I and MHC II epitope

A) IFN γ ELISpot: the contribution of MHC II to the 29mer SLP-cognate immune response determined by immunologically blocking MHC II prior to peptide

restimulation (n = 3-4). IgG2b vs. α MHC II, two-sample *t*-test. B) The presence of MHC I-binding epitopes within immunogenic 29mer SLPs evaluated using the RMA-S stabilization assay. Representative plots of 4 independent experiments. C) IFN γ ELISpot: 29mer SLP-mediated immune response to corresponding MHC I-restricted neoepitopes (n = 4). One-way ANOVA with post-hoc Tukey's test.

Table 5: Features of immunogenic SMA560 SLPs

Neoantigen ID	Mutation	29mer vaccine sequence (mutation in bold, MHC I epitope underlined)	Best MHC I score	Best MHC II score	MHC I/II restriction
Thbs1	D1008Y	DPGLAVGYDEFN AVYFSGTFF INTERDDD	1.0	21.39	D ^b + I-A ^b
E2f8	K272R	VNSRKDKSLRV MSQRFV MLFLVSTPQIVS	0.15	1.42	K ^b + I-A ^b
Htt	V2151M	ANGQKSPLFEAARG MIL NRVTSVVQQQ LPA	0.8	8.79	I-A ^b
Lama5	S2201F	LPAIREQLQGINAS FAAWARL HRLNASIA	0.3	8.79	K ^b + I-A ^b
Topbp1	R792I	KKAVTPLDMNRF QSI AFRAVISQQRGQDP	0.4	19.2	K ^b + I-A ^b
Clip4	S610Y	FAKTKTTLRRSW SSYTT AGGLEGTVKLHE	0.7	13.2	K ^b + I-A ^b
Phyh	G267V	GDTVFFHPLLIHG SV RNKTQGRKAISCH	3.5	7.97	I-A ^b
Odc1	Q129L	KQVSQIKYAASNG V LMMTFDSEIELMKVA	0.2	4.62	D ^b + I-A ^b

To gain insight into the effector functions mediated by the Odc1^{29mer} vaccine, we examined the recruitment of Odc1-reactive T cells into the tumor following vaccination. Odc1-cognate CD8⁺ TILs, but not Odc1-cognate CD4⁺ TILs, were detectable by ELISpot following Odc1^{29mer} immunization (Figure 12A), implicating their role as cytotoxic lymphocytes (CTLs). Consistent with this notion, the depletion of CD8⁺ T cells beginning 5 days after the initial vaccine completely abolished any antitumor benefit of the Odc1^{29mer} vaccine (Figure 12B). However, immunization with the Odc1 MHC I-

restricted neoepitope (Odc1^{MHC I}) alone was insufficient to induce efficacy (Figure 12C), likely owing to its inability to generate a significant Odc1-cognate CD8+ T-cell response (Figure 12D). Together, these data suggest that the Odc1^{29mer} vaccine exerts its antitumor effects through the robust stimulation of Odc1^{MHC I} CTLs.

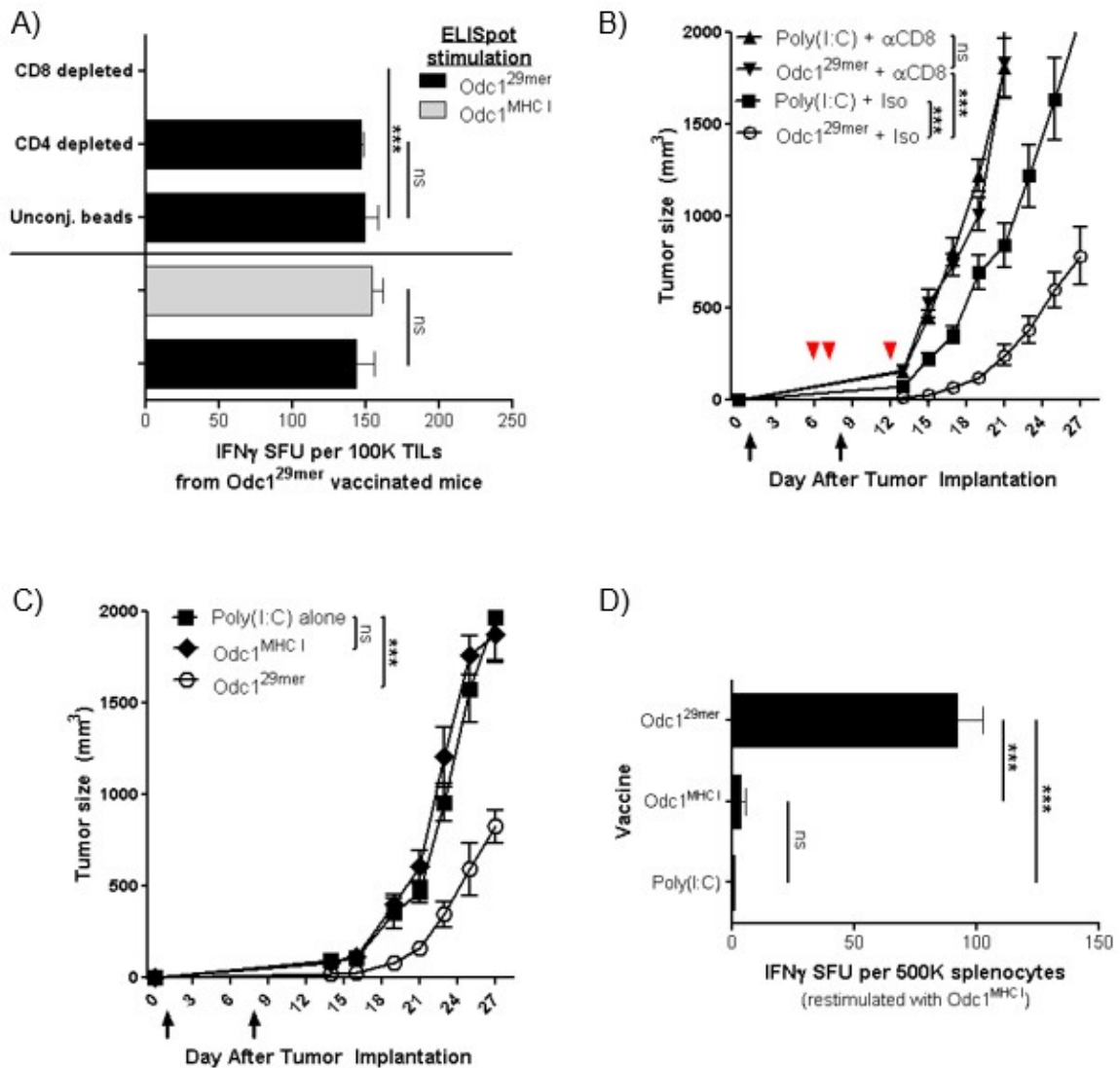


Figure 12: Odc1^{MHC I}-reactive CTLs drive the antitumor effect mediated by the Odc1^{29mer} vaccine

A) IFN γ ELISpot: the presence of Odc1-reactive tumor infiltrating lymphocytes (TILs) within day 27 subcutaneous SMA560 tumors (n = 3) from Odc1^{29mer}-immunized mice (irrelevant peptide-background subtracted). (Top) cells treated with CD8-depleting, CD4-depleting, or unconjugated beads prior to Odc1^{29mer} restimulation. One-way ANOVA with post-hoc Tukey's test. (Bottom) Odc1^{MHC I} vs. Odc1^{29mer} restimulation, two-sample *t*-test. Representative data of 2 independent experiments. B) Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1

and 8 with poly(I:C) alone or Odc1^{29mer} SLP in the context of CD8+ depletion (arrowheads). Day 21 tumor volume, two-sample *t*-test. C) Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1 and 8 with poly(I:C) alone, Odc1^{MHC I}, or Odc1^{29mer} SLP. Day 27 tumor volume, two-sample *t*-test. D) IFN γ ELISpot: splenocyte response to Odc1^{MHC I} 7 days following immunization with poly(I:C) alone, Odc1^{MHC I}, or Odc1^{29mer} SLP (n = 3). One-way ANOVA with post-hoc Tukey's test.

The elongated Odc1^{29mer} peptide, which we have previously shown contains a MHC I and MHC II epitope (Figure 11), was required to induce a substantial Odc1^{MHC I} CTL response (Figure 12D). We, therefore, hypothesized that Odc1-cognate CD4+ T cells augmented the Odc1^{MHC I} CTL response by functioning as helper T cells at the priming phase (Schoenberger et al., 1998). To address this question mechanistically, we immunologically blocked the dominant CD4+ T-cell help pathway mediated through CD40L:CD40 interactions, which promotes the maturation of dendritic cells (DCs) into potent activators of CTLs (Schoenberger et al., 1998). Concordant with our hypothesis, CD40L blockade significantly diminished the immune and antitumor capabilities of the Odc1^{29mer} vaccine (Figure 13A & B), the latter of which was associated with reduced levels of Odc1^{MHC I} CTLs infiltrating the tumor (Figure 13C). Interestingly, we found that depleting CD4+ T cells prior to immunization eliminated the requirement for T-cell help, even to the Odc1^{MHC I} vaccine (Figure 14A), which was likely due to the concomitant depletion of CD4+ Tregs as previously described (Ballesteros-Tato et al., 2013).

Nevertheless, *in vivo* blockade of MHC II significantly diminished the Odc1^{MHC I} CTL response engendered by the Odc1^{29mer} SLP vaccine (Figure 14B), implicating CD4+ T cells in this effect.

Together, these data demonstrate that the immune and antitumor responses generated by the Odc1^{29mer} vaccine are dependent on a native helper epitope. A CD40L-dependent mechanism was also required for the pronounced CTL response induced by two additional efficacious SLPs targeting the neoantigens Lama4 and Alg8 expressed in the mouse sarcoma d42m1-T3 (Gubin et al., 2014), demonstrating that these principles transcend the Odc1^{29mer} vaccine and, therefore, may be broadly applicable for neoantigen-targeting SLP vaccines (Figure 13D).

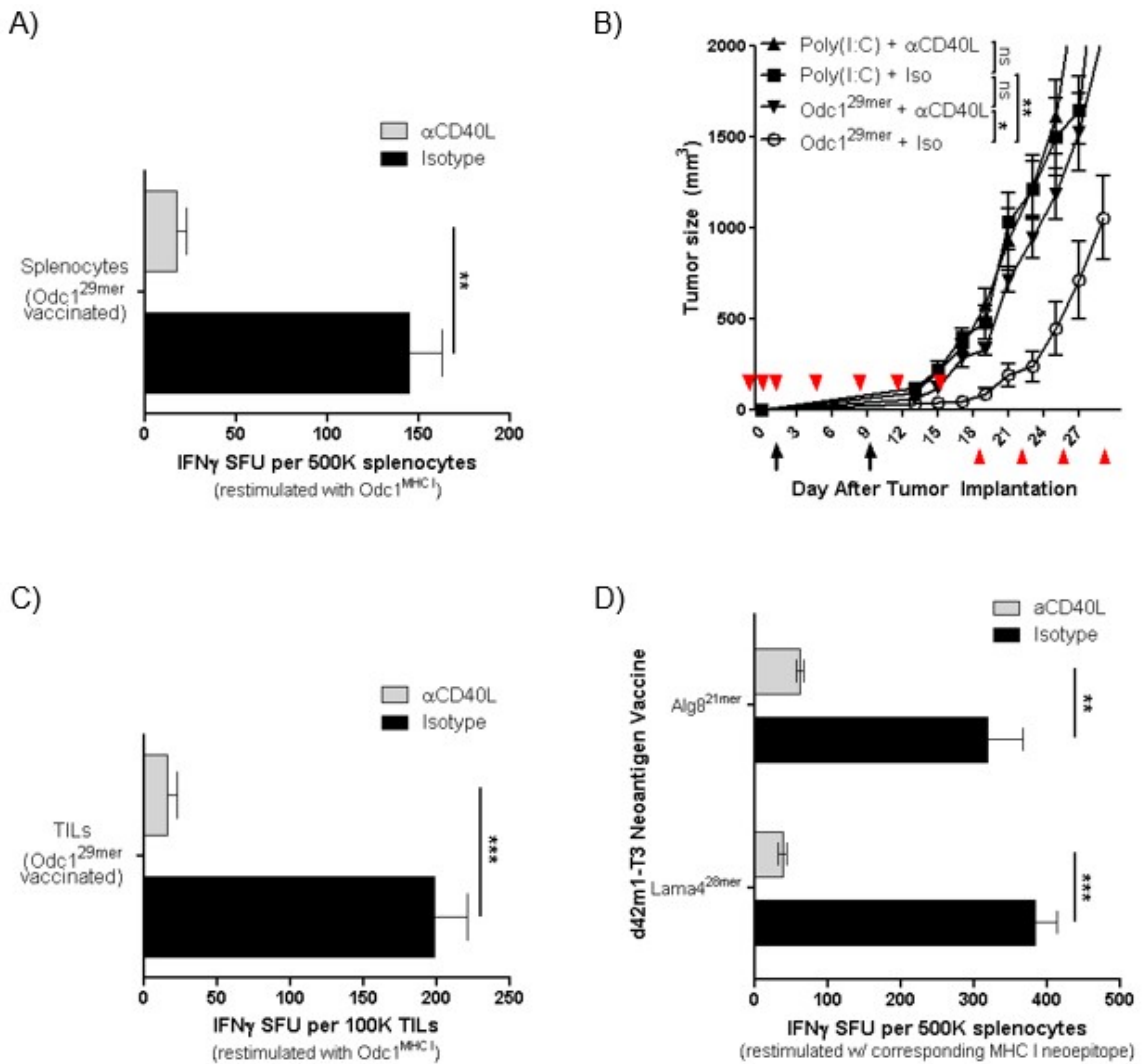


Figure 13: Efficacious neoantigen-targeting SLPs induce a potent CTL response in a CD40L-dependent manner

A) IFN γ ELISpot: splenocyte response to Odc1^{MHC I} 7 days following immunization with Odc1^{29mer} SLP in the context of CD40L-blockade (n = 3). Isotype vs. α CD40L, two-sample *t*-test. B) Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1 and 8 with poly(I:C) alone or Odc1^{29mer} in the context of CD40L-blockade (arrowheads). Day 29 tumor volume, two-sample *t*-test. C) IFN γ ELISpot: the presence of Odc1^{MHC I}-reactive TILs within day 29 subcutaneous SMA560 tumors (n = 4-5) from Odc1^{29mer} vaccinated mice treated with CD40L-blocking antibody or isotype

control (irrelevant peptide-background subtracted). Representative of 2 independent experiments. Isotype vs. α CD40L, two-sample *t*-test. D) IFN γ ELISpot: splenocyte response to the Lama4 or Alg8 MHC I-restricted neopeptide 7 days following immunization with the corresponding SLP in the context of CD40L-blockade (n = 3). Isotype vs. α CD40L, two-sample *t*-test.

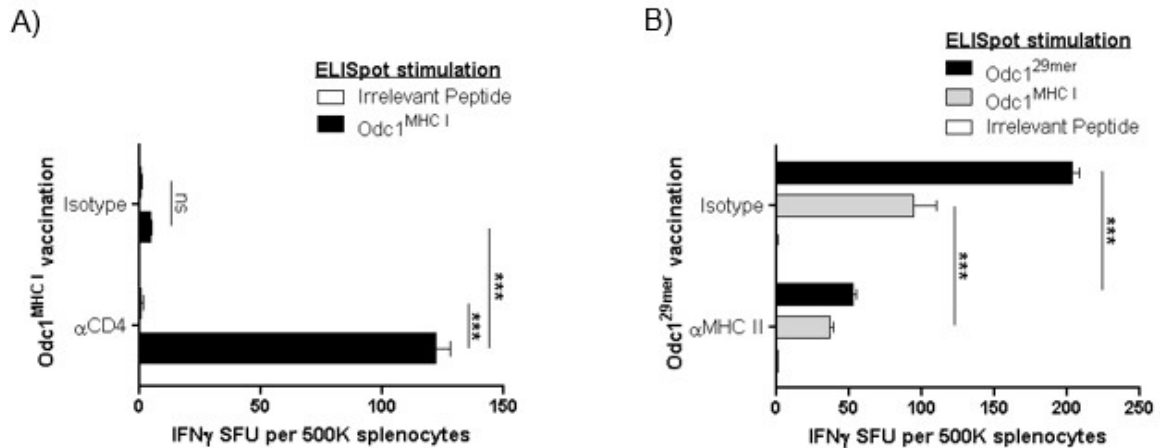


Figure 14: The enhanced Odc1-cognate CD8+ T-cell response engendered by the Odc1^{29mer} SLP vaccine is MHC II-dependent

A) IFN γ ELISpot: mice (n = 3) were given 3 doses of α CD4 or isotype antibody, administered once daily, beginning 3 days prior to Odc1^{MHC I} immunization. Splenocyte response to Odc1^{MHC I} assessed 7 days later. One-way ANOVA, post-hoc Tukey's test. Representative data of two independent experiments.

B) IFN γ ELISpot: mice (n = 3) were given 3 doses of α MHC II or isotype antibody, administered once daily, beginning 1 days prior to Odc1^{29mer} immunization. Splenocyte response to Odc1^{MHC I} and Odc1^{29mer} assessed 7 days later. One-way ANOVA, post-hoc Tukey's test. Representative data of two independent experiments.

6.3.4 A universal helper epitope can supplant a native helper epitope for enhanced neoantigen-specific CTL responses

The inclusion of a native helper epitope within the Odc1^{29mer} was fortuitous, considering that our data indicate that this was necessary for the therapeutic benefits rendered by this vaccine. To our knowledge, there are no computational or high-throughput *ex vivo* methods that enable the rapid and accurate determination of helper epitopes prior to immunization, posing a potential challenge to clinical neoantigen-vaccine design. Due to the tumor-agnostic nature of T-cell help, we asked whether this function could be provided by a more generalizable means, using a universal helper epitope.

We designed SLPs wherein the MHC I-restricted neoepitope of Odc1, Lama4, or Alg8 was conjugated to the universal helper epitope from tetanus toxin, P30 (FNNFTVSFWLRVLPKVSASHLE) (Panina-Bordignon et al., 1989). The MHC I-restricted neoepitope and P30 were separated with a furin cleavage site (RVKR) to ensure efficient proteolysis and to mitigate the formation of novel epitopes between the two peptides. Strikingly, these modified vaccines were capable of generating neoepitope-specific CTL responses comparable to that of SLPs spanning the native neoantigen sequence; however, this effect was only observed when P30 was physically conjugated to the MHC I-restricted neoepitope (Figure 15A). Furthermore, Odc1^{MHC I-P30} was as effective as the Odc1^{29mer} vaccine at treating subcutaneous SMA560 tumors (Figure 15B), suggesting a

nonessential role of Odc1-cognate CD4⁺ T cells at the effector phase. As anticipated, CD40L was required for the potent induction of Odc1^{MHC I} CTLs by the Odc1^{MHC I-P30} vaccine (Figure 15C). Together, these findings show that a universal helper epitope can confer the necessary T-cell help required for efficacious, neoantigen-specific CTL responses.

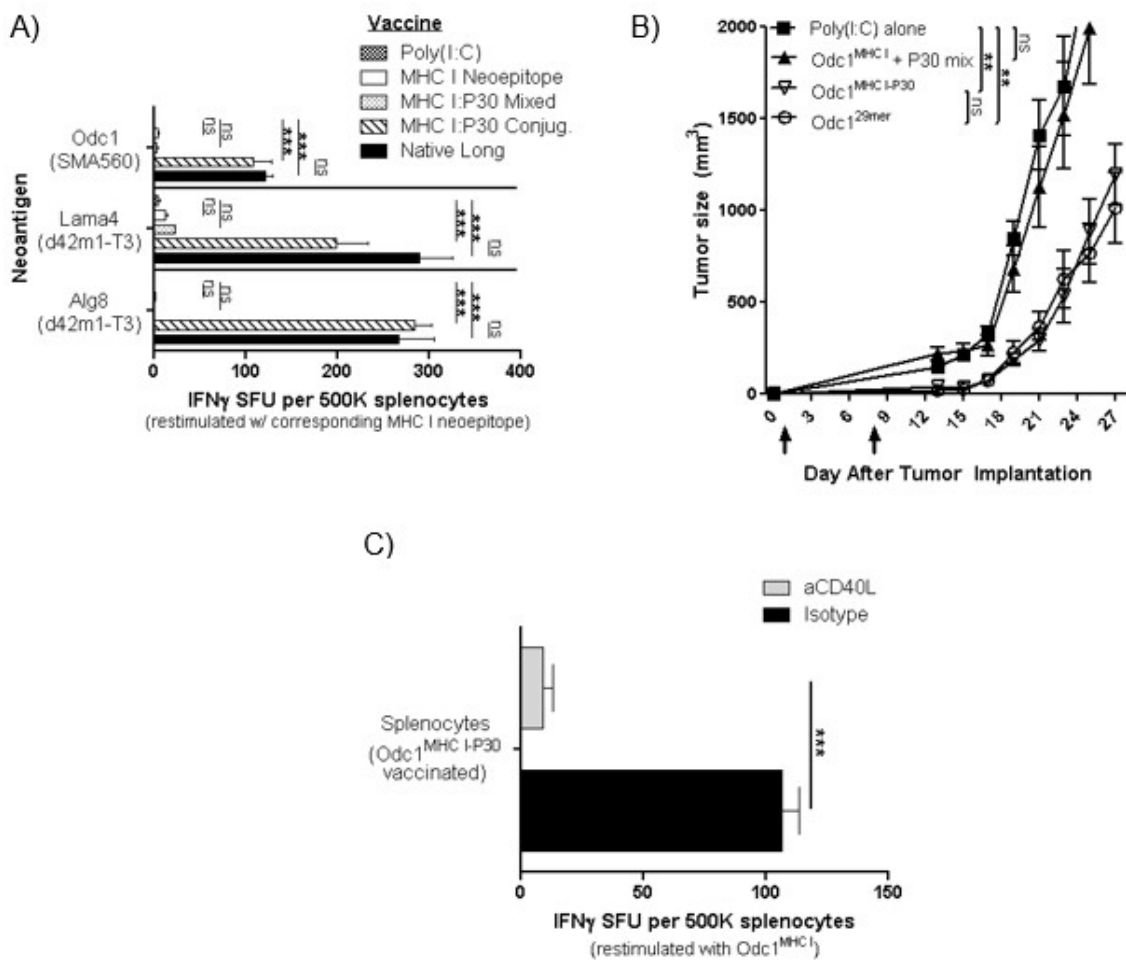


Figure 15: The universal helper epitope P30 can provide the necessary CD40L-mediated help for neoantigen-specific CTL responses

A) IFN γ ELISpot: for each neoantigen, mice (n = 3) were immunized with poly(I:C) alone, the MHC I-restricted neoepitope alone, the MHC I-restricted neoepitope mixed with the P30 helper epitope, the MHC I-restricted neoepitope conjugated to the P30 helper epitope, or the efficacious SLP spanning the endogenous neoantigen sequence (native long). Seven days later, the splenocyte response to the corresponding MHC I-restricted neoepitope was evaluated. One-way ANOVA with post-hoc Tukey's test. B)

Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1 and 8 with poly(I:C) alone, Odc1^{MHC I} mixed with P30, Odc1^{MHC I} conjugated to P30 (Odc1^{MHC I-P30}), or the Odc1^{29mer} SLP. Day 27 tumor volume, two-sample *t*-test. C) IFN γ ELISpot: splenocyte response to Odc1^{MHC I} 7 days following immunization with the Odc1^{MHC I-P30} SLP in the context of CD40L-blockade (n = 3). Isotype vs. α CD40L, two-sample *t*-test.

Excluding the Odc1^{29mer}, we identified 5 additional immunogenic 29mer SLPs spanning SMA560 missense mutations that contained a MHC I-restricted neoepitope (Figure 11A); however, vaccine-induced CD8+ T-cell responses to these were poor or undetectable (Figure 11B). Given that these SLPs also induced a CD4+ T-cell response (Figure 11D), which should be able to afford helper function, these data might suggest that these weak CD8+ T-cell responses were a consequence of alternative factors, such as poor MHC I-binding affinity or low CD8+ T-cell precursor frequency. Surprisingly, however, conjugation of P30 to the MHC I-restricted neoepitope of Lama5 (Lama5^{MHC I-P30}) or Topbp1 (Topbp1^{MHC I-P30}) prompted a dramatic enhancement in the neoepitope-specific, CD8+ T-cell response compared to the native 29mer SLP vaccine (Figure 16A). Notably, Topbp1^{MHC I-P30} induced an antitumor effect that was associated with the

infiltration of Topbp1^{MHC I} TILs, which was not realized with the Topbp1^{29mer} vaccine (Figure 16B & C).

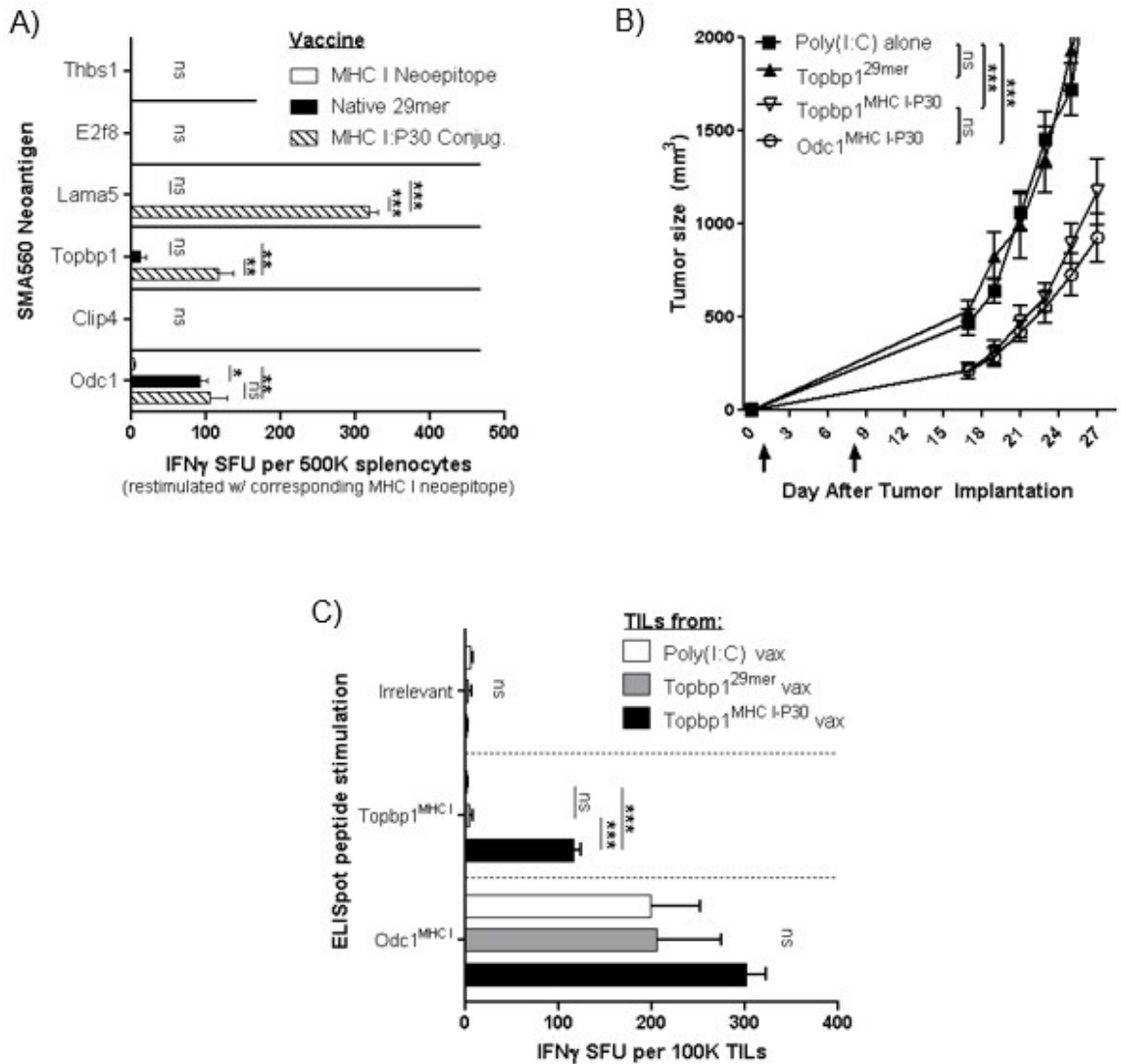


Figure 16: P30 conjugation can improve the therapeutic effects of neoantigen vaccines

A) IFN γ ELISpot: splenocyte response to the corresponding MHC I-restricted neoepitope 7 days following immunization with the MHC I-restricted neoepitope alone, the 29mer SLP spanning the endogenous neoantigen sequence (native 29mer), or the

MHC I-restricted neoepitope conjugated to P30 (n = 3). One-way ANOVA with post-hoc Tukey's test. B) Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1 and 8 with poly(I:C) alone, Topbp1^{29mer}, Topbp1^{MHC I-P30}, or Odc1^{MHC I-P30} SLP. Day 27 tumor volume, two-sample *t*-test. C) IFN γ ELISpot: the presence of Odc1^{MHC I}- and Topbp1^{MHC I}-reactive TILs within day 27 subcutaneous SMA560 tumors (n = 5-6) from poly(I:C), Topbp1^{29mer}, or Topbp1^{MHC I-P30} vaccinated mice (cells alone-background subtracted). One-way ANOVA with post-hoc Tukey's test.

Conversely, despite the induction of a potent CD8+ T-cell response (Figure 16A), immunization with Lama5^{MHC I-P30} did not result in an appreciable antitumor response or a detectable infiltration of Lama5^{MHC I} TILs (Figure 17A & B). These data suggested that the Lama5 neoantigen was not antigenic due to inadequate processing of the Lama5^{MHC I} from the native Lama5 neoantigen sequence. To test this, we evaluated whether the native Lama5^{29mer} peptide could restimulate Lama5-cognate CD8+ T cells generated by the Lama5^{MHC I-P30} vaccine using the IFN γ ELISpot assay. As expected, Lama5^{MHC I} and Lama5^{MHC I-P30} sufficiently stimulated Lama5-cognate CD8+ T cells; however, the Lama^{29mer} peptide was a very poor stimulator (Figure 17C).

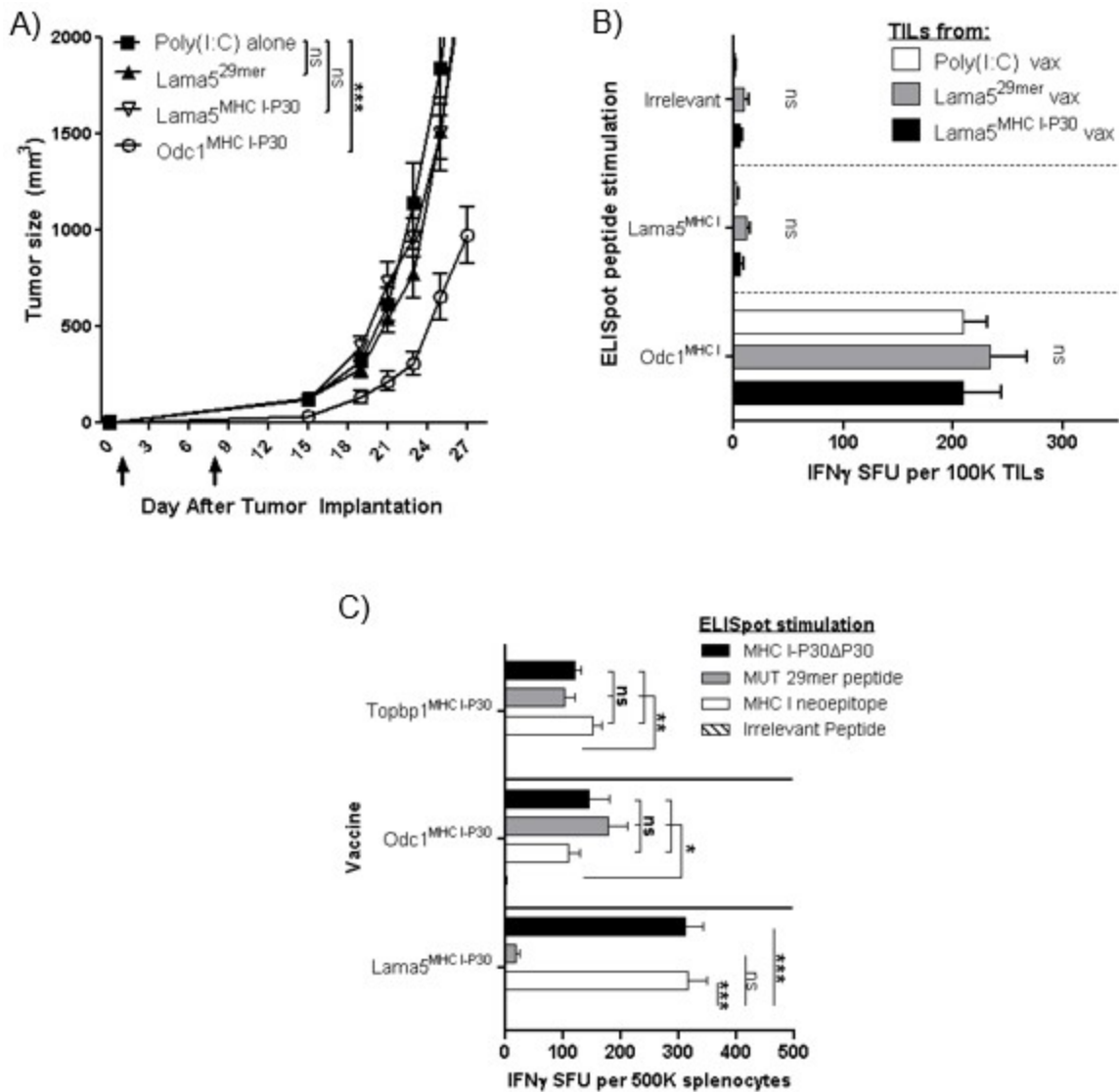


Figure 17: The Lama5 neoantigen from SMA560 is not antigenic

A) Subcutaneous SMA560 tumor growth in mice (n = 7) following therapeutic immunization on days 1 and 8 with poly(I:C) alone, Lama5^{29mer}, Lama5^{MHC I-P30}, or Odc1^{MHC I-P30} SLP. Day 27 tumor volume, two-sample *t*-test. B) IFN γ ELISpot: the presence of Odc1^{MHC I}- and Lama5^{MHC I}-reactive TILs within day 27 subcutaneous SMA560 tumors (n = 6) from poly(I:C), Lama5^{29mer}, or Lama5^{MHC I-P30} vaccinated mice (cells alone-background subtracted). One-way ANOVA with post-hoc Tukey's test. C) IFN γ ELISpot: mice (n = 3) were immunized with Topbp1^{MHC I-P30}, Odc1^{MHC I-P30}, or

Lama5^{MHC I-P30} in order to induce neoantigen-specific CD8+ T-cell response. The ability of the MHC I epitope, MHC I-P30 conjugate, and native 29mer peptides to restimulate vaccine-induced CD8+ T cells – an indicator of adequate antigen processing and presentation of the endogenous MHC I neoepitope – was assessed 7 days following immunization. The response to P30 alone was subtracted from the MHC I-P30 response (MHC I-P30 Δ P30). One-way ANOVA with post-hoc Tukey's test.

While there are major differences in the antigen processing that occurs within an ELISpot compared to that which occurs within tumor cells, extracellular SLPs used in the ELISpot are exposed to the phagosome-to-cytosol cross-presentation pathway (Kreer et al., 2011; Rosalia et al., 2013). This pathway utilizes the proteasome and the transporter associated with antigen processing (TAP) (Rosalia et al., 2013), which are the major early components of the classical mechanism that tumor cells, and other non-professional antigen-presenting cells (APCs), use to process MHC I epitopes from intracellular antigens. Thus, if professional APCs (e.g. DCs, macrophages, and B cells) within the ELISpot cannot adequately process and present the Lama5^{MHC I} from the Lama^{29mer}, it is highly unlikely that tumor cells will be capable. Altogether, these findings demonstrate that conjugation of a MHC I-restricted neoepitope to a universal helper epitope has the potential to enhance neoepitope-specific CD8+ T-cell responses relative to the native neoantigen sequence, which can promote therapeutic responses to antigenic neoantigens.

6.4 Discussion

In this study, we elucidate a CD40L-dependent mechanism of efficacious SLP vaccines spanning the native neoantigen sequence, mediated by an endogenous helper epitope. Additionally, we provide proof-of-concept that conjoining a universal helper epitope to a MHC I-restricted neoepitope can maintain, or even enhance, the effectiveness of SLP vaccines relative to the native neoantigen sequence. This effect required the physical linkage of these two epitopes, suggesting that, at least for SLPs administered intravenously with poly(I:C), T-cell help requires a three-cell interaction (i.e. DC, CD4+ T cell, CD8+ T cell) as opposed to a sequential two-cell interaction (i.e. DCs previously matured by CD4+ T cells persist to activate CD8+ T cells) (Ridge et al., 1998). For our studies, we utilized the universally immunogenic helper epitope P30, which contains several MHC II epitopes that bind to an array of MHC II haplotypes (Panina-Bordignon et al., 1989) and can be assumed to be safe for human use given the prevalent use of tetanus toxoid vaccines (Brander et al., 1996). However, alternative universal helper epitopes, such as PADRE, would likely also be safe and effective (La Rosa et al., 2012).

Our finding that most immunogenic SLPs spanning the native neoantigen sequence possess a conjoined MHC I and MHC II epitope mirrors results from a recent phase I trial evaluating neoantigen-targeting SLPs in patients with high-risk melanoma

(Ott et al., 2017). In this study, SLPs that were selected exclusively on MHC I-binding algorithms elicited an initial CD4+ T-cell response. Together, these data suggest that candidate neoantigen selection may be aided by consideration of native neoantigen sequences containing a proximal MHC I and MHC II epitope. Accordingly, we have found that several spontaneously, and thus highly, immunogenic neoantigens across several mouse tumor lines (i.e. Odc1:SMA560, Lama4 & Alg8:d42m1-T3 (Gubin et al., 2014), and Imp3:GL261 (Johanns et al., 2016)) have very favorable predicted MHC I and MHC II scores (i.e. IEDB percentile ranks scores of ≤ 0.2 and < 6 , respectively; Figure 18) within the native ± 14 amino acids spanning the missense mutation. We are currently exploring the fidelity of this strategy in alternative tumor models.

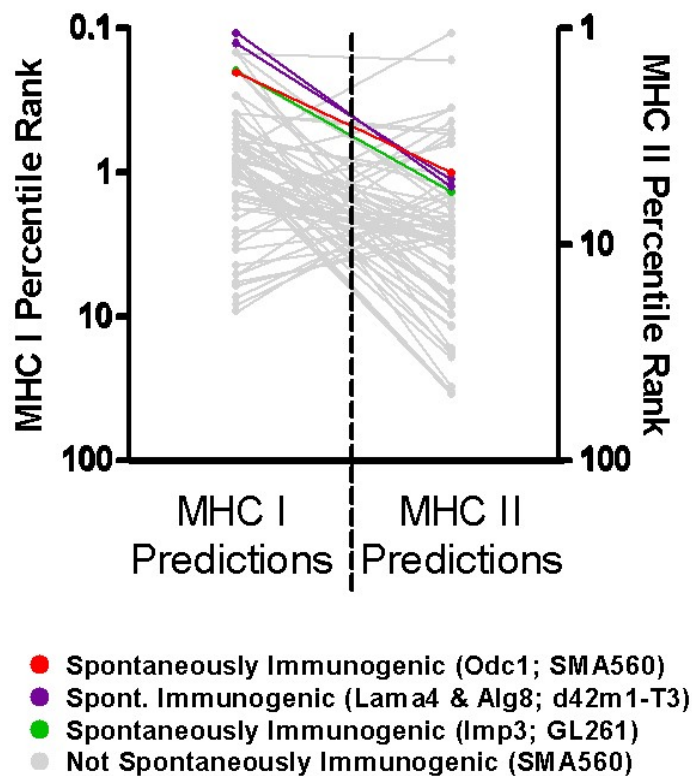


Figure 18: Favorable MHC I and MHC II-binding-prediction scores may reveal highly immunogenic neoantigens

Graph of MHC I and MHC II predicted binding scores, derived from *in silico* IEDB MHC I and MHC II-binding algorithms, for 29mer peptides spanning missense mutations from SMA560, d42m1-T3, and GL261 mouse tumors and their status as spontaneously immunogenic upon tumor implantation.

The enhanced immune and antitumor responses engendered by P30-conjugated SLPs were rather surprising considering that all immunogenic SMA560 SLPs that contained a MHC I-restricted neoepitope already possessed a native MHC II epitope. Thus, the inability of the native neoantigen sequence to induce a substantial immune

response, while the P30-conjugate was capable, could be interpreted as a defect in antigen processing and presentation, and, as such, it would be reasonable to assume that the native neoantigen expressed within the tumor may not be antigenic. This concern is exemplified in our studies with Lama5 vaccines. While the P30-conjugated vaccine was able to induce a potent Lama5-cognate CD8+ T-cell response, these cells likely cannot interact with the tumor since the Lama5 MHC I-restricted neoepitope is not efficiently liberated from the endogenous Lama5 neoantigen. Nevertheless, studies with Topbp1 SLPs demonstrate that poor vaccine-mediated CD8+ T-cell responses with the native neoantigen sequence do not always dictate a lack of antigenicity.

While we have not rigorously examined the mechanism governing the improved immunogenicity of P30-conjugate SLPs, one possibility is that the inclusion of a furin-cleavage site (RXKR) facilitates enhanced antigen processing and subsequent MHC:epitope presentation by host dendritic cells. Modified peptides containing furin sites are efficiently cleaved into their constituent MHC epitopes within the trans-Golgi network, a component of the secretory pathway (Lu et al., 2004). Alternatively, SLPs spanning the native neoantigen sequence that lack furin sites primarily utilize the conventional intracellular proteasome/TAP-dependent cross-presentation pathway to liberate MHC I-restricted epitopes (Rosalia et al., 2013), as well as phagolysosomal proteases for the release of MHC II-restricted epitopes. Any insufficiency in these

processes could lead to inadequate MHC:epitope presentation required for necessary T-cell receptor crosslinking and, thus, T-cell activation. These factors appear to contribute to the inability of the Lama^{29mer} vaccine to induce a detectable CD8+ T-cell response. Another possibility is that P30 simply provides a qualitatively and/or quantitatively better T-cell help function. The average response to P30 alone following MHC I-P30 immunization was approximately 80 IFN γ SFU, which is roughly double the CD4+ T-cell response induced by the native Lama^{5^{29mer}} and Topbp1^{29mer} vaccines.

We did not observe evidence of antitumor effects against SMA560 by neoantigen-reactive CD4+ T cells. Though our vaccine regimen was likely not optimized for efficacy, a similar strategy with a SLP containing a MHC II-restricted neoepitope administered intravenously with poly(I:C) was capable of mediating significant antitumor benefits against mouse B16F10 melanoma (Castle et al., 2012). However, a critical difference between these models is that B16F10 can express MHC II and SMA560 cannot. While the expression of MHC II on GBM tumors remains a controversial issue (reviewed in (A. H. Friedman et al., 2016)), studies have demonstrated CD4+ T cells can promote antitumor effects even against MHC II-deficient tumors, for example, through indirect effects of IFN γ (Mumberg et al., 1999). In fact, recent evidence has implicated neoantigen-reactive CD4+ T cells in GBM tumors. In this study, GBM patients were immunized with up to 20 neoantigen-targeting SLPs, and CD4+ T-cell clonotypes

responsive to the immunizing peptides were detected within the TIL compartment (Anandappa et al., 2018). Thus, MHC II-restricted neoepitopes may ultimately have utility in neoantigen vaccines for GBM.

Though Odc1-reactive TILs were detected within intracerebral SMA560 tumors, indicating a functioning network between the immune system and the CNS-resident tumor, we did not observe antitumor benefits against orthotopic SMA560 tumors with the Odc1^{29mer} or Odc1^{MHC I-P30} vaccines. This was rather unsurprising, given the modest antitumor effects we achieved against an ectopic, subcutaneous SMA560 tumor and the quasi-immune-privileged nature of the CNS. Furthermore, an earlier study has shown that the spontaneously-arising Odc1^{MHC I} CTLs within an intracerebral SMA560 tumor are in an exhausted state (Woroniecka et al., 2018a), which vaccine-stimulated T cells likely also succumb to. Together, these data suggest that vaccines alone will likely be insufficient to treat human GBM tumors, and combination therapies that can potentiate the T-cell response are required. Correspondingly, we are currently evaluating native and P30-conjugated SLP neoantigen vaccines in a phase I trial for ND GBM. One treatment arm will include co-therapy with anti-CD27 (varlimumab), which we have coincidentally shown enhances the effectiveness of SLP vaccines containing bipartite MHC I and MHC II epitopes against intracerebral tumors in mice (submitted manuscript).

To our knowledge, this is the first demonstration that a native neoantigen sequence exhibiting weak immunogenicity and antitumor functions can be enhanced through rational vaccine design. With an average of only 35 somatic mutations, maximizing the therapeutic potential of each GBM neoantigen is essential for the potential management of this disease using neoantigen-targeting strategies. The approach set forth in the current study is highly adaptable to the clinic due to the relative accuracy of *in silico* MHC I-binding algorithms and can be further aided by the empirical confirmation of MHC I-binding neoepitopes using HLA-matched, TAP-deficient cell lines (e.g. T2 cells), as we have shown using the RMA-S cell line. Collectively, our work here is encouraging as it offers a clinically-tractable approach with the potential to enhance the therapeutic breadth of neoantigen-targeting vaccines.

6.5 Future Directions

SMA560 is derived from male VMDk mice. Therefore, X chromosomal mutations should be near homozygous. However, we identified a few X chromosomal mutations that exhibited allelic frequencies of less than 0.5 by next-generation sequencing, suggesting that bulk SMA560 cells are genetically heterogeneous. The genomic evaluation of one these mutations, Rlim, in 56 SMA560 single cell clones revealed that this was, in fact, the case (Figure 19), together suggesting that SMA560 is a better model for malignant glioma than previously thought.

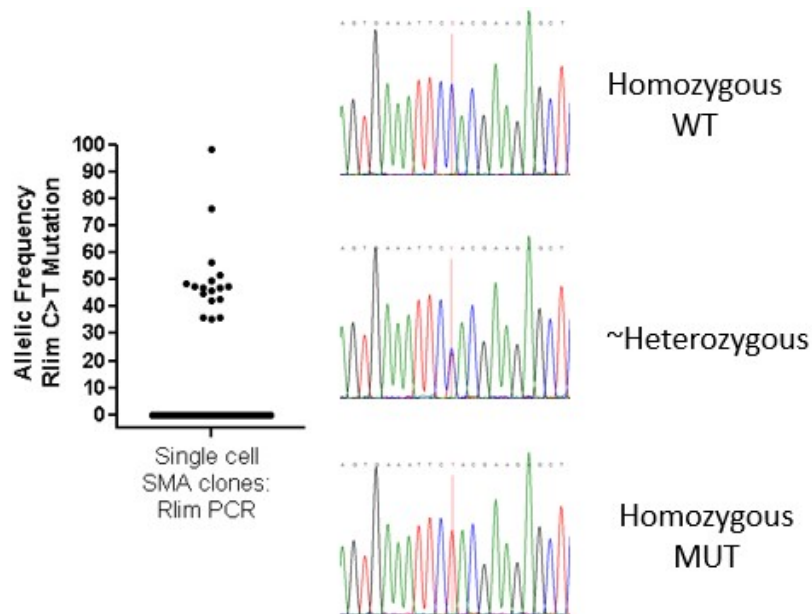


Figure 19: Heterogeneous allelic frequency of mutant Rlim in SMA560 cells

The gene for Rlim resides on the X chromosome. Plot of mutant Rlim allelic frequency, compared to the wild-type allele, derived from PCR performed on genomic DNA from 56 SMA560 single cell clones.

The heterogeneity of bulk SMA560 cells could potentially affect the therapeutic response to immunotherapy. Correspondingly, we found that the mutant Odc1 allele, too, was heterogeneous among SMA560 clones (Figure 20). While most clones exhibited near heterozygosity for the mutant allele, several SMA560 clones displayed low mutant Odc1 allelic frequency. Therefore, it is may be possible that Odc1 vaccinations exert a

selective pressure on SMA560 tumors, favoring SMA560 cells with suppressed or absent mutant Odc1 protein expression.

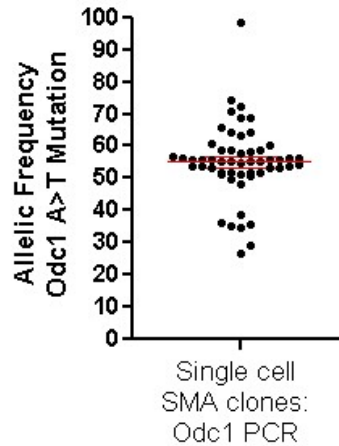


Figure 20: Heterogeneous allelic frequency of mutant Odc1 in SMA560 cells

Plot of mutant Odc1 allelic frequency, compared to the wild-type allele, derived from PCR performed on genomic DNA from 56 SMA560 single cell clones.

While this may occur to a degree, we do not view this as the primary mechanism of vaccine failure. Using an antibody that detects wild-type Odc1, we were able to demonstrate Odc1 protein expression in escape tumors following immunization with Odc1^{29mer} plus poly(I:C) (Figure 21A) or poly(I:C) alone (data not shown). Furthermore, conducting RT-PCR on the region flanking the mutation site revealed the presence of the mutant Odc1 transcript in escape tumors (Figure 21B). Together, these data strongly

suggest that mutant Odc1 expression is still present in tumor outgrowths following vaccination against mutant Odc1. However, this is certainly not a rigorous examination of this mechanism, which may be very spatiotemporally dependent, and more conclusive studies would be helpful to rule out this possibility.

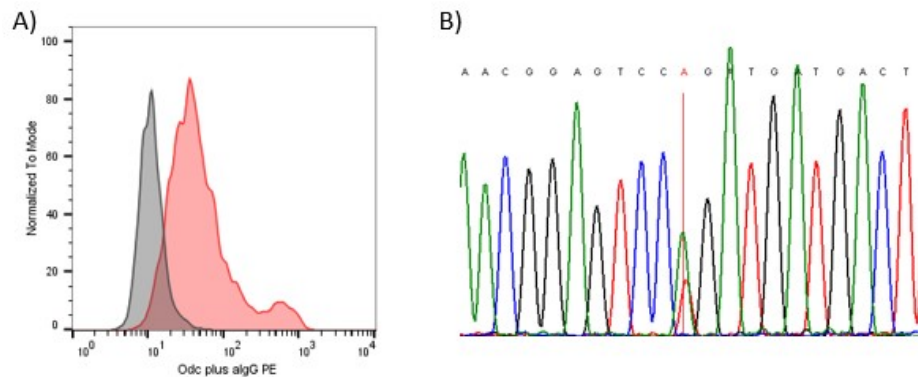


Figure 21: Mutant Odc1 expression is evident in escape tumors following Odc1^{29mer} vaccination

A) Flow cytometric analysis of intracellular Odc1 protein expression in tumor excised on day 25 from mouse that received two vaccinations with Odc1^{29mer}, gated on CD45^{neg} cells. B) RT-PCR-derived electropherogram of Odc1 transcript assessed in tumor from A). Red line denotes mutated nucleotide (A = WT; T = MUT).

Immune and antitumor responses with SLPs targeting mutant Odc1 and Topbp1 were able to maintained or enhanced by conjugating their MHC-restricted epitope to P30. Therefore, we have evaluated whether P30-conjugation could enhance the immune response to other highly-predicted MHC I epitopes that were not immunogenic in their native 29mer form. Of the 14 predicted MHC I epitopes we tested, all having an IEDB

percentile rank score of ≤ 0.6 , only the immune response to the Sh3yl MHC I epitope was modestly enhanced upon P30 conjugation (Figure 22). However, like we have shown with Lama5, the native 29mer sequence was unable to stimulate Sh3yl-cognate CD8+ T cells, suggesting that the Sh3yl neoantigen is not antigenic.

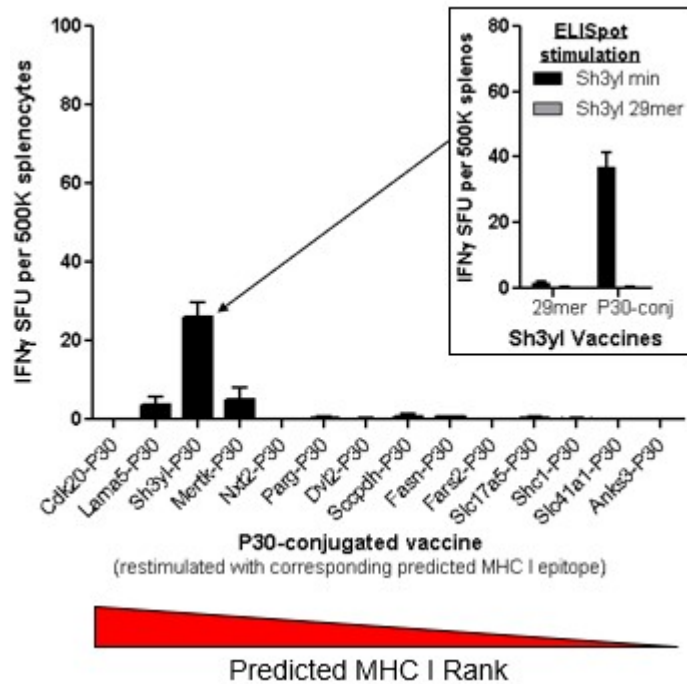


Figure 22: The immunogenicity of predicted SMA560 MHC I neoepitopes from nonimmunogenic 29mer SLPs is not greatly improved by P30-conjugation

Predicted MHC I neoepitopes, having an IEDB MHC I rank score of ≤ 0.6 , from SMA560 that were not at all immunogenic in their native 29mer form were synthesized in conjunction with P30. MHC I neoepitope-specific response within splenocytes of immunized mice ($n = 3$) was evaluated on day 7 by IFN γ ELISpot assay. Inset shows results of Sh3yl^{29mer} stimulation of splenocytes from Sh3yl^{MHC I-P30} immunized mice ($n = 3$).

While the low therapeutic success rate we have observed with P30-conjugated vaccines in SMA560 (2 of 20, or 10%) may not be an issue for newly-diagnosed GBM, in which all neoantigens can likely be immunized against, this does present a concern for tumors containing hundreds to thousands of somatic mutations, as can be the case with recurrent GBM (Johnson et al., 2014). For these tumors, additional neoantigen selection criteria will likely be required. One potential prospect is the empirical determination of MHC I-binding affinity, which has been shown to govern the therapeutic success of T-cell-based immunotherapies (Kammertoens et al., 2013). Supporting this notion, we identified Odc1 and Topbp1 MHC I-restricted neoepitopes as having that highest MHC I-binding affinity of all identified SMA560 MHC I-restricted epitopes (Figure 23). In order for this to be a reliable refinement criterion, it will be important to determine how these results compare to the MHC I affinities of other highly-predicted MHC I neoepitopes from SMA560, such as those tested in Figure 22, as well as those from neoantigens within other tumor models.

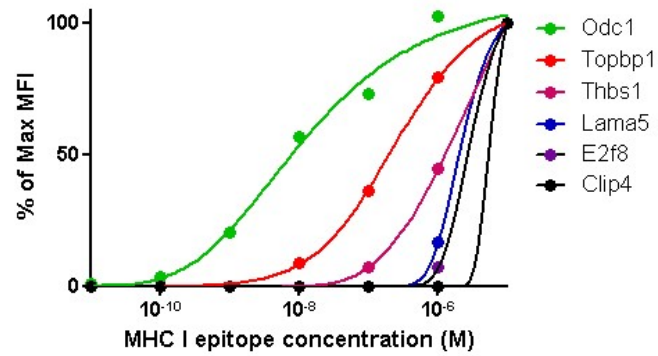


Figure 23: MHC I -binding affinities of SMA560 MHC I-restricted neoepitopes

RMA-S cells were pulsed with various concentrations of MHC I-restricted peptides derived from SMA560 neoantigens (Table 5). A saturation curve was generated by plotting the median fluorescence intensity (MFI) detected by flow cytometry upon staining with α MHC I (i.e. α K^b or α D^b) antibodies.

7. The Evaluation of Minigene-Transfected Dendritic Cell Vaccines Targeting Tumor Antigens Reveals Limitations of GM-CSF + IL-4-Differentiated DC Vaccines

7.1 Introduction

In the previous chapter, we demonstrated that efficacious neoantigen vaccines require CD4⁺ T-cell help for the activation of tumor-killing CTLs. With respect to translating this into the clinic, generating a number of SLP vaccines possessing universal helper epitopes will likely not be a logistical challenge for newly-diagnosed GBM due to its relatively few mutations. However, this approach becomes problematic for tumors that contain hundred to thousands of somatic mutations, as can be the case for recurrent GBM treated with the mutagen TMZ (Johnson et al., 2014). This challenge arises from the current inability to accurately predetermine immunogenic neoantigens from long lists of expressed neoantigens.

Messenger RNA-transfected DC vaccines may offer a vaccine platform that can overcome this challenge. First and foremost, *ex vivo*-derived DCs have the inherent ability to confer CD4⁺ T-cell help due to their natural expression of MHC II, which can be loaded with immunogenic epitopes contained within culture media (Faiola et al., 2002). Furthermore, mRNA represents the ideal antigen delivery vehicle since it can be easily manufactured to encode a significant number of potential antigens without an underlying knowledge of precise epitopes or patient-specific MHC haplotypes.

Moreover, mRNAs can be rationally designed to efficiently traffic antigen to the MHC I and MHC II antigen processing and loading compartments (Figure 24). Thus, mRNA-transfected DCs can expose the immune system to a very large number of neoantigen-derived epitopes from a single, consolidated vaccine.

In this study, we examined the immunotherapeutic potential of minigene-transfected DCs for the treatment of tumors. Minigenes encoding ubiquitinated 15 mer peptides spanning a MHC I-restricted epitope were used for these experiments, since we have shown in the previous chapter that CD8⁺ CTLs are the major drivers of the antitumor response against malignant astrocytomas; however, minigenes can be easily designed to activate CD4⁺ T-cell responses (Figure 24) (Kreiter et al., 2008). Though our data yielded a few promising therapeutic responses, they also highlighted several fundamental drawbacks of GM-CSF + IL-4-derived DC vaccines. This includes an inability of booster vaccines to enhance or maintain an antigen-specific immune response, as well as their activation of an immunosuppressive IL-10⁺ Tr1 response.

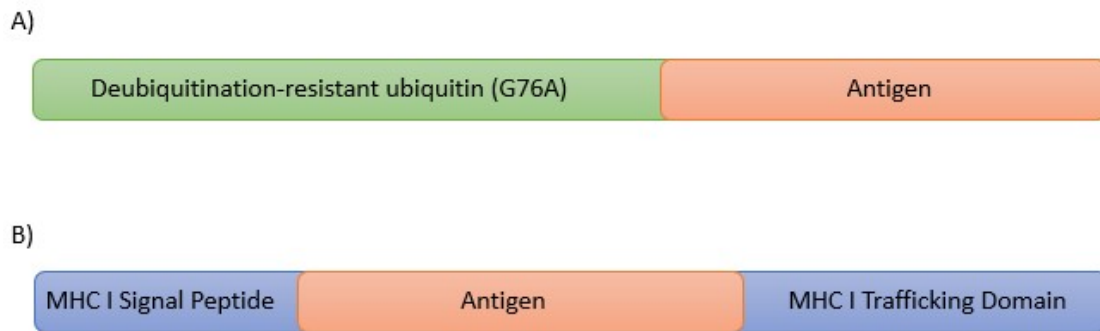


Figure 24: Minigene constructs that promote antigen presentation

A) An antigen coding sequence preceded by deubiquitination-resistant ubiquitin coding sequence promotes epitope presentation on MHC I by shuttling antigen to the proteasome. B) An antigen coding sequence flanked by the coding sequence for the signal peptide of MHC I and the N-terminal trafficking domain of MHC I promotes epitope presentation on MHC I and MHC II by shuttling antigen to endolysosomal compartments (Kreiter et al., 2008).

7.2 Methods and Materials

7.2.1. Cells, lines, and media

All animal experiments were performed in accordance with Duke University's Institutional Animal Care and Use Committee-approved protocol. Wild-type VMDk mice were bred in-house under pathogen-free conditions at Duke University Medical Center. Female C57BL/6 mice were obtained from Charles River Laboratories. All experiments were conducted on 6-12-week-old mice. SMA560 astrocytoma cells were cultured in IMEM-Zinc Option medium (Gibco) containing 10% FBS (Gemini Bio-Products) and 1x penicillin-streptomycin (Gibco). B16OVA melanoma (B16 expressing

chicken ovalbumin) cells were cultured in D10 medium, which consisted of DMEM (Gibco), 10% FBS (Gemini Bio-Products), and 1x penicillin-streptomycin. Dendritic cells were cultured in either AIM-V (Gibco) containing 50 μ M beta-mercaptoethanol (Gibco), 20 ng mL⁻¹ GM-CSF (Gemini Bio-Products), and 20 ng mL⁻¹ IL-4 (Gemini Bio-Products), with and without 5% FBS (Gemini Bio-Products), or RPMI containing 1x penicillin-streptomycin (Gibco), 1x NEAA (Gibco), 1 mM glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 50 μ M beta-mercaptoethanol (Gibco), 20 ng mL⁻¹ GM-CSF (Gemini Bio-Products), and 20 ng mL⁻¹ IL-4 (Gemini Bio-Products), with and without 5% FBS. FACS buffer consisted of 1x PBS (Gibco) plus 2% FBS (Gemini Bio-Products).

7.2.2 Differentiation of bone marrow-derived dendritic cells.

Tibias, femurs, sternums, and humerus were harvested from sacrificed mice. End caps of bones were removed with autoclaved surgical scissors and bone marrow flushed out using a 25-gauge syringe needle and a 10 mL syringe filled with RPMI. Sternum were compressed with surgical forceps to extract bone marrow. Bone marrow was dissociated by angling the opening of a 10 mL syringe at the bottom of petri dish and gently withdrawing the plunger to create high pressure suction. Dissociated bone marrow was filtered through a 70 μ M strainer and pelleted at 350 g for 10 min. Upon removal of supernatant, red blood cells (RBCs) were lysed using RBC lysis buffer

(Pharm Lyse, BD Biosciences) for 1 min and the reaction neutralized with RPMI. After pelleting, bone marrow cells were counted and resuspended at 1×10^6 cells mL^{-1} in DC medium. 50 mL was plated in a sterile T150 flask. After incubating at $37^\circ\text{C}/5\% \text{CO}_2$ for 3 days, nonadherent cells were poured off and 50 mL of DC medium was replaced. Cells were incubated for an additional 4 days at $37^\circ\text{C}/5\% \text{CO}_2$, at which time they were harvested for experiments.

7.2.3 Generation of *in vitro*-transcribed mRNA

In vitro-transcription plasmids were designed using the pGEM®-4Z vector (Promega; Figure 25). Minigenes used in this study were composed of a deubiquitination-resistant ubiquitin (G76A) (Pickart et al., 1994) coding sequence placed upstream of a minigene encoding a 15 mer peptide spanning a known MHC I-restricted tumor-rejection epitope. These included ovalbumin's (B16OVA) SIINFEKL, Trp2's (B16F10) SVYDFVWL, and Odc1's (SMA560) YAASNGVLM; the region flanking the MHC I epitope was derived from the native antigen sequence (Appendix A Table 8). The transgene was flanked by the 5' and 3' UTRs of *Xenopus* beta-globin, which has been shown to enhance translational efficiency (Kruys et al., 1987), and the start codon preceded by a Kozak sequence (GCCACC) – all downstream of the T7 promoter. Plasmids were linearized by an overnight digestion with Spe1 restriction enzyme (NEB) and purified (DNA clean and concentrator, Zymo Research). *In vitro* transcription was

carried out using the mMessage mMachine™ T7 transcription kit (Invitrogen) using their protocol. Reactions were carried out for 2+ hours in a 37°C water bath. Following a 15 min DNase treatment, mRNAs were polyadenylated using the Poly(A) tailing kit (Invitrogen), which adds at least 150 nucleotides to the 3' terminus. Polyadenylated *in vitro*-transcribed mRNAs were purified using the RNeasy mini kit (Qiagen), concentration quantified using a NanoDrop™ spectrophotometer (Thermo Scientific) and the remained sample stored at -80°C until needed. Plasmid linearization and *in vitro* transcription were verified using agarose gel electrophoresis (Figure 26).

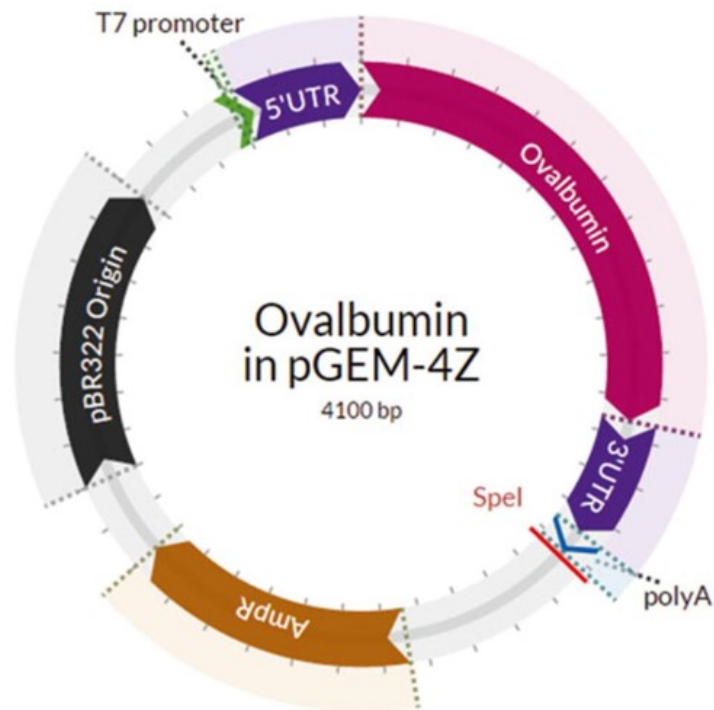


Figure 25: Example plasmid map of *in vitro*-transcription vector.

Figure reproduced with permission (Batich et al., 2016)

The cDNA-derived sequence for chicken ovalbumin was cloned between UTRs obtained from *Xenopus laevis* β -globin cDNA. The poly(A) tail is encoded by a 62 base pair sequence, which is immediately followed by a SpeI restriction enzyme cleavage site to allow for plasmid linearization.

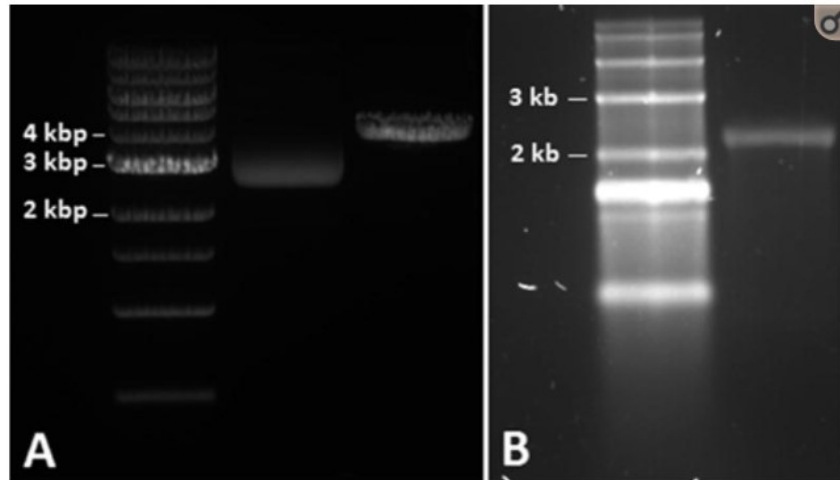


Figure 26: Confirmation of plasmid linearization and single *in vitro*-transcribed mRNA product using gel electrophoresis

Figure reproduced with permission (Batich et al., 2016)

A) Verification of plasmid linearization using 1 % agarose/TAE gel electrophoresis. From *left to right*: NEB 1 kb DNA ladder, 1 μ g undigested ovalbumin in pGEM-4Z, and 1 μ g Spe1-digested ovalbumin in pGEM-4Z (expected MW = 4100 bp). B) Verification of single IVT mRNA product using 1.5 % denaturing agarose gel electrophoresis. From *left to right*: Invitrogen 0.5–10 kb RNA ladder and 1 μ g IVT ovalbumin mRNA (expected MW = 2054 bases)

7.2.4 Tumor studies

For subcutaneous tumor studies, three days prior to tumor implantation, the right rear flank of C57BL/6 mice or VMDk mice was shaved. Mice were inoculated with

2.5×10^5 B16OVA cells or 7.5×10^5 SMA560 cells suspended in 1x PBS (Gibco). Tumor growth was determined using a digital caliper.

For intracerebral tumor studies, B16OVA cells were resuspended to 2×10^6 cells mL⁻¹ in 1x PBS tumor cells and mixed thoroughly in an equal volume of 10% methylcellulose in PBS using a sterile 1 mL syringe. 6 to 12-month-old C57BL/6 mice were premedicated with buprenorphine SR and anesthetized in an isoflurane chamber. Anesthetized mice were then placed into a stereotactic frame (Kopf), and a ~1 cm sagittal incision was made to reveal the skull. One drop of bupivacaine was added to the incision site. A 25-gauge injection needle was placed 2 mm right of the bregma and inserted to a depth of 4 mm below the surface of the skull. 5×10^3 cells in a volume of 5 μ L were delivered into the caudate nucleus. Following injection, the hole was sealed using bone wax and the incision closed with a surgical staple. C57BL/6 mice inoculated intracerebrally with B16OVA cells become moribund in ~18-22 days. All animal experiments were performed in accordance with Duke University Institutional Animal Care and Use Committee-approved protocols.

7.2.5 Dendritic cell electroporation and immunization

Day 7 DCs were pelleted at 350 g for 10 min and resuspended in 10 mL Opti-MEM medium (Gibco) for counting. After a single wash in Opti-MEM, DCs were

resuspended to 25×10^6 cells/ mL and electroporated at 300 V/500 μ s/2 pulses with 10 pmol *in vitro*-transcribed mRNA per 1×10^6 cells. Electroporated cells were incubated at 37°C/ 5% CO₂ for 2 h in DC medium to allow for mRNA translation. Cells were then washed thrice in cold 1x PBS (Gibco) and resuspended to 10×10^6 cells/ mL in 1x PBS for vaccinations. Mice received a total of 1×10^6 DCs administered with 50 μ L on both sides of the inner thigh.

7.2.6 *In vitro* T cell: dendritic cell cultures

RF3370 cells were a kind gift from Dr. Smita Nair. For RF3370 stimulation experiments, BMDCs were electroporated with 10 pmol per 1×10^6 cells Ubi mRNA or UbiOVA mRNA. Next, 1×10^5 RF3370 cells and 1×10^4 transfected BMDCs were added to a U-bottom 96 well plate (Falcon) in 200 μ L RPMI1640 DC media without cytokines. Plate was incubated at 37°C/ 5% CO₂ for 24 h, at which point it was centrifuged at 350 g x 10 min and 160 μ L supernatant removed for ELISA assay. IL-2 ELISA (LegendMax, BioLegend) was performed according to the manufacturer's protocol. For Trp2 and Odc1 *in vitro* stimulations, splenocytes from mice immunized 7 days prior with either the Trp2 180-188 MHC I-restricted epitope (SVYDFFVWL, Anaspec) or the Odc1 29mer SLP (KQVSQIKYAASNGVLMMTFDSEIELMKVA, Genscript) were enriched using RBC lysis buffer (ACK lysis buffer, Lonza) followed by isopycnic centrifugation (Lympholyte-M, Cedarlane). BMDCs were electroporated with 10 pmol per 1×10^6 cells

Ubi mRNA, UbiTrp2 mRNA, or UbiOdc1 mRNA. In a non-TC-treated 24 well plate (Falcon), 2.5×10^5 splenocytes and 2.5×10^4 transfected BMDCs were added to each well in 500 μ L RPMIifbs media without cytokines, and the plate incubated at 37°C/ 5% CO₂ for 3 days before half the media was replaced with fresh. On day 6, cells were harvested for analysis by IFN γ ELISpot.

7.2.7 CD11c+ cell enrichment

BMDC cultures were stained with anti-CD11c PE antibody (clone N418, BioLegend). CD11c+ cells were conjugated to anti-PE microbeads (Miltenyi Biotec) per the manufacturer's protocol and enriched with the AutoMACS cell separator (Miltenyi Biotec) using the "Possel" program.

7.2.8 Tr1-inducing-factor blocking experiments

For IL10 and TGF- β blocking experiments, 200 μ g anti-IL-10 (clone JES5-2A5, BioXCell), -IL-10R (1B1.3A, BioXCell), -TGF- β (1D11.16.8, BioXCell), or their corresponding isotype controls (BioXCell) were administered intraperitoneally 4 h prior to DC immunization and every 2 days thereafter until day 6. For ICOSL blocking experiments, 300 μ g anti-ICOSL (clone HK5.3, BioXCell) or its corresponding isotype control (BioXCell) were given intraperitoneally on days -1 and 0 relative to DC immunization. Also, for this experiment, 3×10^6 RPMIifbs DCs were incubated with 50

$\mu\text{g mL}^{-1}$ anti-ICOSL or isotype antibody in 1x PBS for 30 min prior to washing and subsequent immunization.

7.2.9 Flow cytometry

All antibody staining was performed using 0.5-1 μL antibody per $\sim 1 \times 10^6$ *in vitro*-derived cells or splenocytes or 50 μL RBC-lysed (FACS lysing solution, BD) blood and incubated at room temperature for 30 min in FACS buffer. For tetramer staining, cells were stained with anti-SIINFEKL/K^b PE tetramer (MBL) and anti-CD8 APC (clone 53-6.7, eBioscience). For BMDC phenotyping, cells were stained anti-CD11c APC (clone N418, BioLegend) and PE-conjugated anti-CD40 (clone 3/23, BioLegend), -CD80 (clone 16-10A1, BD Biosciences), -CD86 (clone GL1, BD Biosciences), or -IAb (clone AF6-120.1, BD Biosciences). For identification of the IL-10 producing subset, cells were stained with viability dye (Zombie Green[™], BioLegend) and anti-IL10 PE (clone JES5-16E3, BioLegend) plus APC-conjugated anti-CD8 (clone 53-6.7, eBioscience), -CD4 (clone GK1.5, eBioscience) plus FITC-conjugated anti-FoxP3 (clone R16-715, BD Biosciences), -CD19 (clone 6D5, BioLegend), -NK1.1 (clone PK136, BD Biosciences), -F4/80 (clone BM8, eBioscience), or -CD11b (clone M1/70, BioLegend). Anti-CD11c APC (clone N418, BioLegend) and anti-SIINFEKL/K^b PE (clone 25-D1.16, BioLegend) were used to detect the surface expression of SIINFEKL bound to K^b.

7.2.10 Western blot

CD11c+ cells were enriched from AIM-V and RPMI fibs DC cultures using the aforementioned method. Cells were lysed using RIPA buffer (Thermo Scientific) containing proteases (cOmplete™ mini, Roche Diagnostics) according to the manufacturer's specifications. Lysate was centrifuged through a QIAshredder (Qiagen) twice at full speed. Flow through was centrifuged at maximum speed and supernatant collected, taking care not to disturb any potential pellet. Protein concentration was determined using a BCA protein assay kit (Pierce) and lysate stored at -20°C until needed. 25 µg protein was mixed with NuPAGE LDS loading buffer (Invitrogen), boiled at 95°C for 5 min, and chilled on ice. Sample was loaded into a 10% bis-tris gel (Invitrogen) alongside a Precision Plus Protein™ Kaleidoscope™ molecular weight standard (Bio-Rad). Gel was ran at 200V until dye front left bottom of gel. Gel was then transferred onto a PVDF membrane (Invitrogen) and subsequently stained with anti-Irf4 (clone M-17, Santa Cruz), -Irf8 (clone C-19, Santa Cruz), -BATF (clone WW8, Santa Cruz), or -GAPDH (clone GAPDH-71.1, Sigma-Aldrich) antibodies. Following secondary antibody staining (HRP-conjugates, Invitrogen) and addition of substrate (ECL Plus, Pierce), membranes were imaged on a ChemiDoc™ imager (Bio-Rad). GAPDH blots were exposed for 20 sec and Irf4/Irf8/BATF blots for 5 min.

7.2.11 IFN γ and IL-10 ELISpot assay

Splenocytes were dissociated using the blunt end of a 10 mL syringe plunger and filtered using a 70 μ M strainer. Cells were resuspended in 8 mL R10 medium, layered onto Lymphocyte-M (Cedarlane), and isopycnic centrifugation performed at 1,300 g for 25 min with no brake. The buffy coat layer was removed and cells washed twice with R10 medium before counting using a flow cytometer (Guava easyCyte, Millipore Sigma). 2.5×10^5 or 5×10^5 splenocytes were plated per well of a 96-well PVDF plates (Multiscreen HTS, Millipore Sigma) that had previously been coated with $10 \mu\text{g mL}^{-1}$ anti-IFN γ (clone AN18, Mabtech) or anti-IL-10 (clone MT60, Mabtech) antibody. Cells were stimulated with 1 μ M peptide, AIM-V medium containing 5% FBS, or $4 \mu\text{g mL}^{-1}$ ConA. After an 18-22 h (IFN γ) or 48 h (IL-10) incubation period at 37°C/ 5% CO $_2$, IFN γ or IL-10 secretion was detected using 1 $\mu\text{g mL}^{-1}$ biotinylated anti-IFN γ (clone R4-6A2, Mabtech) or -IL-10 (clone 51E6, Mabtech) antibody, respectively, followed by development with avidin-conjugated peroxidase (Vectastain ABC, Vector Laboratories) and AEC (Sigma). For MHC II-blocking ELISpots, 20 $\mu\text{g mL}^{-1}$ anti-MHC II antibody (M5/114, BioXCell) was incubated with cells for 2 h prior to antigen restimulation. All samples were evaluated in duplicates.

7.2.12 Statistical analysis

ELISpot data was evaluated using an unpaired two-sample *t*-test or one-way ANOVA with post hoc Tukey's test (Dittrich et al., 2012). Mean tumor volumes at the time point when the control cohort surpassed a mean tumor volume of 2000 mm³ or at the predetermined end of the experiment were compared between two groups using an unpaired two-sample student's *t*-test. Asterisks indicate degree of significance (**P* < 0.05, ***P* ≤ 0.01, ****P* < 0.001, *P* > 0.05 not significant (ns)).

7.3 Results

7.3.1 Comparison of BMDCs generated in serum-free and serum-containing medium

DCs are conventionally differentiated in serum-containing medium; however, recent studies have demonstrated that DCs generated in serum-free CellGro medium exhibited superior immunogenicity (Napoletano et al., 2007; Warncke et al., 2006). We typically use serum-free AIM-V medium as the base for human DC vaccines generated for clinical studies, though 5% human serum is added. Therefore, we wished to evaluate whether AIM-V alone could generate highly-immunogenic DC vaccines.

A hallmark feature of *in vitro*-generated dendritic cells is the formation of loosely adherent DC precursors by day 3 (Inaba et al., 1992). Clusters of these cells are also evident on day 7. Culture in serum-free RPMI or AIM-V medium dramatically reduced the presence of these clusters on day 7 (Figure 27). Interestingly, AIM-V base medium

appeared to result a more substantial blanketing of adherent cells on day 7 compared to DC medium containing an RPMI base.

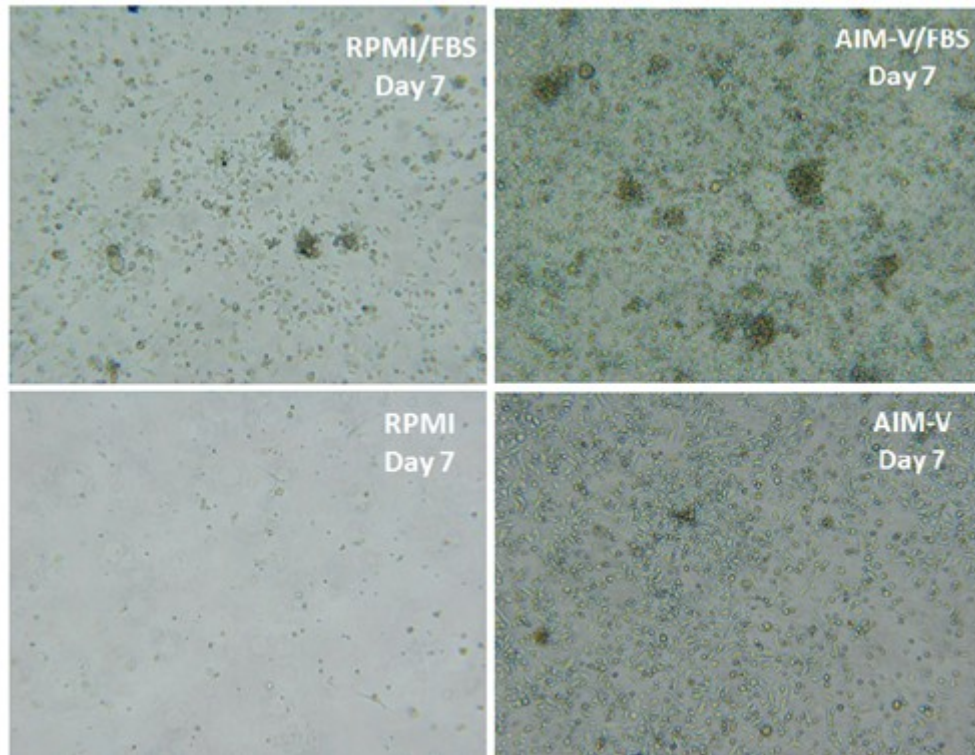


Figure 27: Adherent cells observed on day 7 in BMDC cultures with various media

Bright-field image of cells bound to the bottom of T150 flask at day 7 of BMDC differentiation in GM-CSF + IL-4-containing serum-free RPMI, RPMI + 5% FBS, serum-free AIM-V, or AIM-V + 5% FBS.

Media containing FBS generated much greater yields compared to serum-free media (Figure 28A). This was unsurprising considering the growth advantage that FBS has been known to afford. This did not equate, however, to diminished viabilities in all

serum-free conditions, as DCs generated in AIM-V alone had a similar average viability as that of AIMV+FBS and RPMI+FBS. RPMI alone fared the worst of all tested media, yielding very few cells with low viability (Figure 28B).

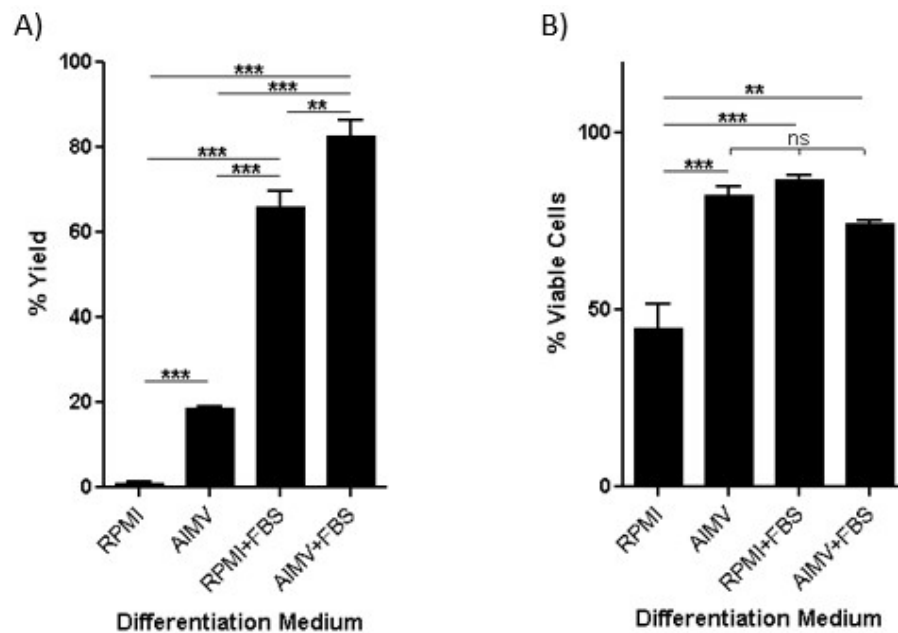


Figure 28: Cell yields and viabilities with different serum-free and serum-containing BMDC-culture media

A) Bar graph showing cell yield on day 7 of BMDC differentiation in various media ((viable cells on day 7/ bone marrow cells plated on day 0) * 100), determined using trypan blue exclusion assay. Average of 3 independent experiments. One-way ANOVA with Tukey's post-hoc test. B) Bar graph showing percent of viable cells in day 7 BMDC cultures, determined flow cytometrically using Zombie Green™ viability dye. Average of 3 independent experiments. One-way ANOVA with Tukey's post-hoc test.

Interestingly, RPMI-based media resulted in the generation of higher percentages of CD11c⁺ DCs, with FBS-free RPMI being the best (Figure 29A). Of note, serum-free media generated DCs with a larger and more granular morphology, as indicated by high forward and side scatter by flow cytometry (Figure 29B).

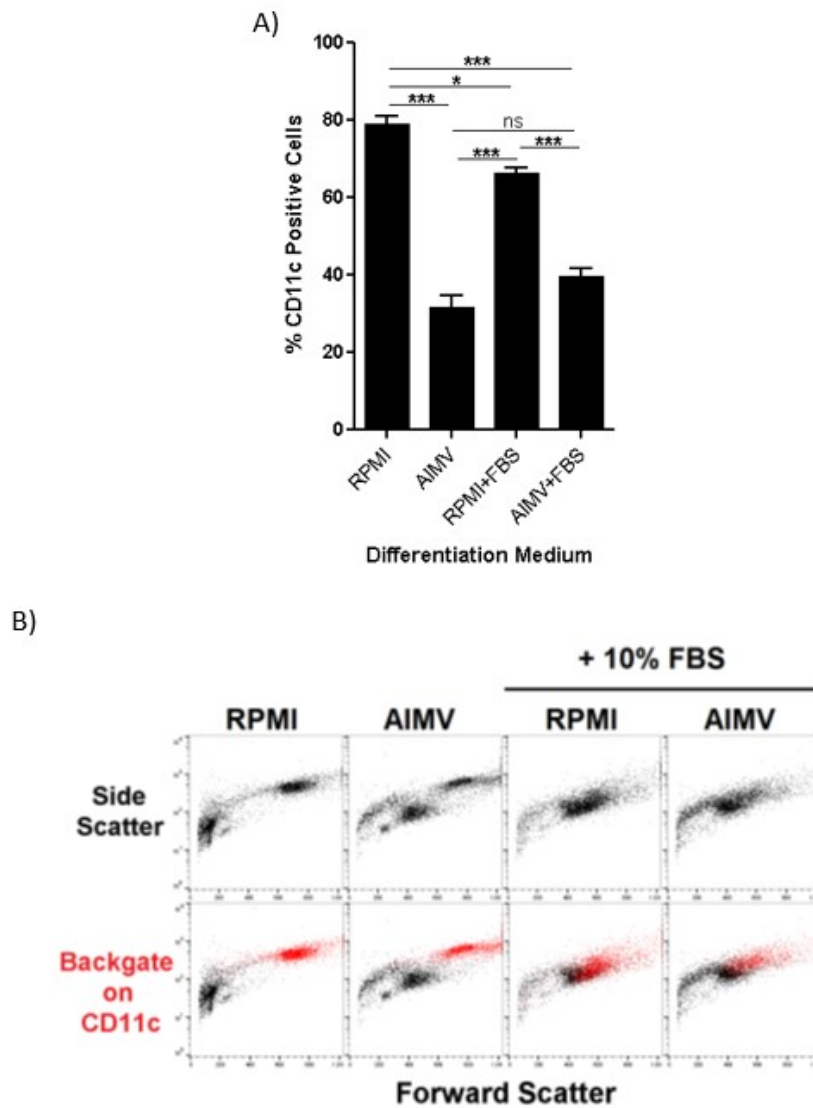


Figure 29: Base medium influences percent of CD11c⁺ cells in BMDC cultures

A) Bar graph showing percent CD11c+ cells in day 7 BMDC cultures with various serum-free and serum-containing media, determined by flow cytometry. Average of 3 independent experiments. One-way ANOVA with Tukey's post-hoc test. B) Representative side scatter vs forward scatter of day 7 BMDC cultures (top & bottom), including a backgate on CD11c+ cells (bottom).

DCs exist in several functional states that govern their immunological function. Immature DCs likely are not desirable for cancer vaccine, as they have been shown to induce tolerance towards an antigen or an otherwise tolerogenic state (Dhodapkar et al., 2001; Hawiger et al., 2001; Jonuleit et al., 2000; Mahnke et al., 2003). For this reason, DCs used for cancer vaccines are generally activated *ex vivo* with maturation cocktails to induce a mature, T-cell activating phenotype (Jonuleit et al., 1997; Mailliard et al., 2004). A defining feature of activated DCs is the upregulation of maturation markers, such as MHC II, CD80, CD86, and CD40. Phenotypic analysis revealed that BMDCs cultured in serum-free media exhibited a very high frequency of mature DCs, approaching 100%. On the other hand, DCs generated in FBS-containing media displayed bimodal expression of maturation markers, suggesting the presence of both immature and mature DCs (Figure 30). Collectively, these data suggest that the maturation phenotypes of BMDCs are similar among those generated in serum-free or serum-containing media, regardless of the base medium, with FBS-containing media generating a high frequency

of immature DCs. Furthermore, the presence of serum appears to influence cell yield, while the base medium seems to affect the percentage of CD11c+ DCs.

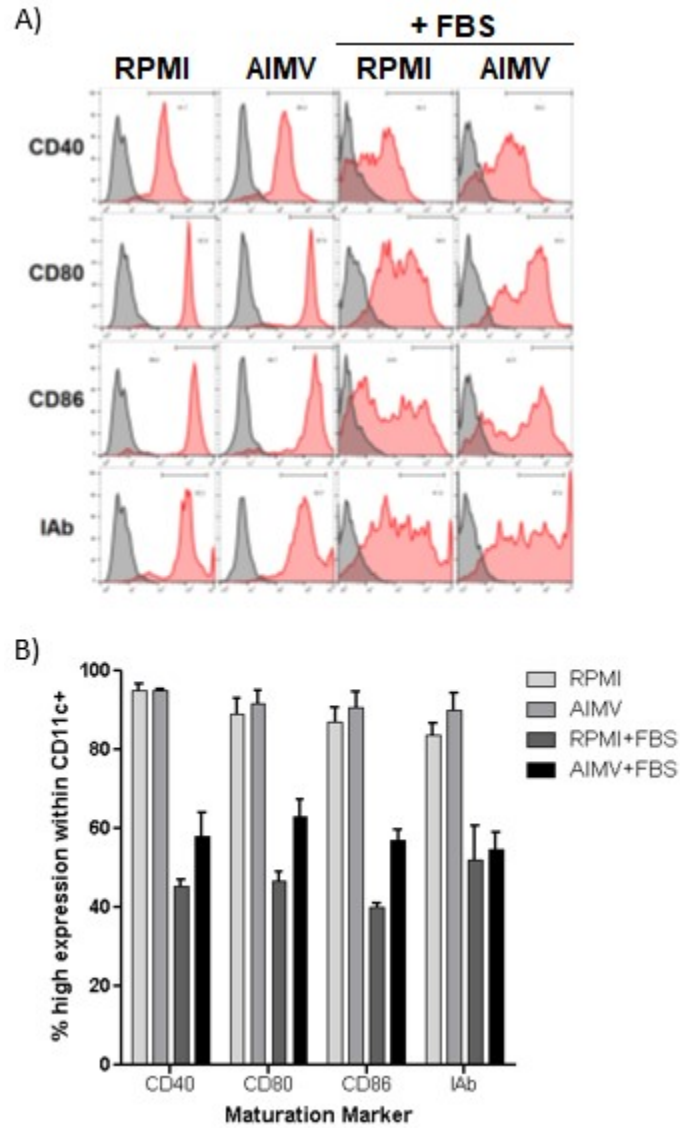


Figure 30: BMDCs cultured in serum-free medium exhibit a more mature phenotype

A) Representative histograms of DC maturation-marker expression on CD11c+ BMDCs grown in various serum-free and serum-containing media, determined by flow

cytometry. **B)** Bar graph showing the percent of CD11c+ BMDCs grown in various media exhibiting high expression of maturation markers, determined by flow cytometry. Average of 3 independent experiments.

7.3.2 Minigene-transfected BMDCs can stimulate T cells in an antigen-dependent manner

Moving forward, we compared the immunogenicity and efficacy of BMDCs generated in AIM-V alone and RPMI + FBS DC medium. RPMI alone DC medium was eliminated due to poor cell yields and AIMV + FBS was dropped due to the phenotypic similarity of the resulting BMDCs to those grown in RPMI + FBS.

Given the availability of commercial tools for investigating ovalbumin-specific immunological effects, a minigene encoding a ubiquitinated 15 mer peptide containing the SIINFEKL epitope was developed (UbiOVA; Appendix A Table 8), which should enable enhanced trafficking of SIINFEKL to the MHC I-presentation pathway (Figure 24). Sure enough, transfection of UbiOVA into either AIM-V or RPMIfbs DCs led to the surface presentation of SIINKFEL in K^b, which was evident in >80% of CD11c+ cells (Figure 31A). Next, we investigated the degree of SIINKFEL saturation on all surface MHC I molecules. To this end, the α SIINFEKL/K^b signal was compared to a standard curve created using SIINKFEKL peptide-pulsed DCs. Interpolation of signal values from 3 independent tests with UbiOVA-transfected DCs revealed that UbiOVA

prompted the surface expression of SIINFEKL/K^b to an equivalent degree as that of pulsing with between 24-46 nM SIINFEKL peptide (Figure 31B).

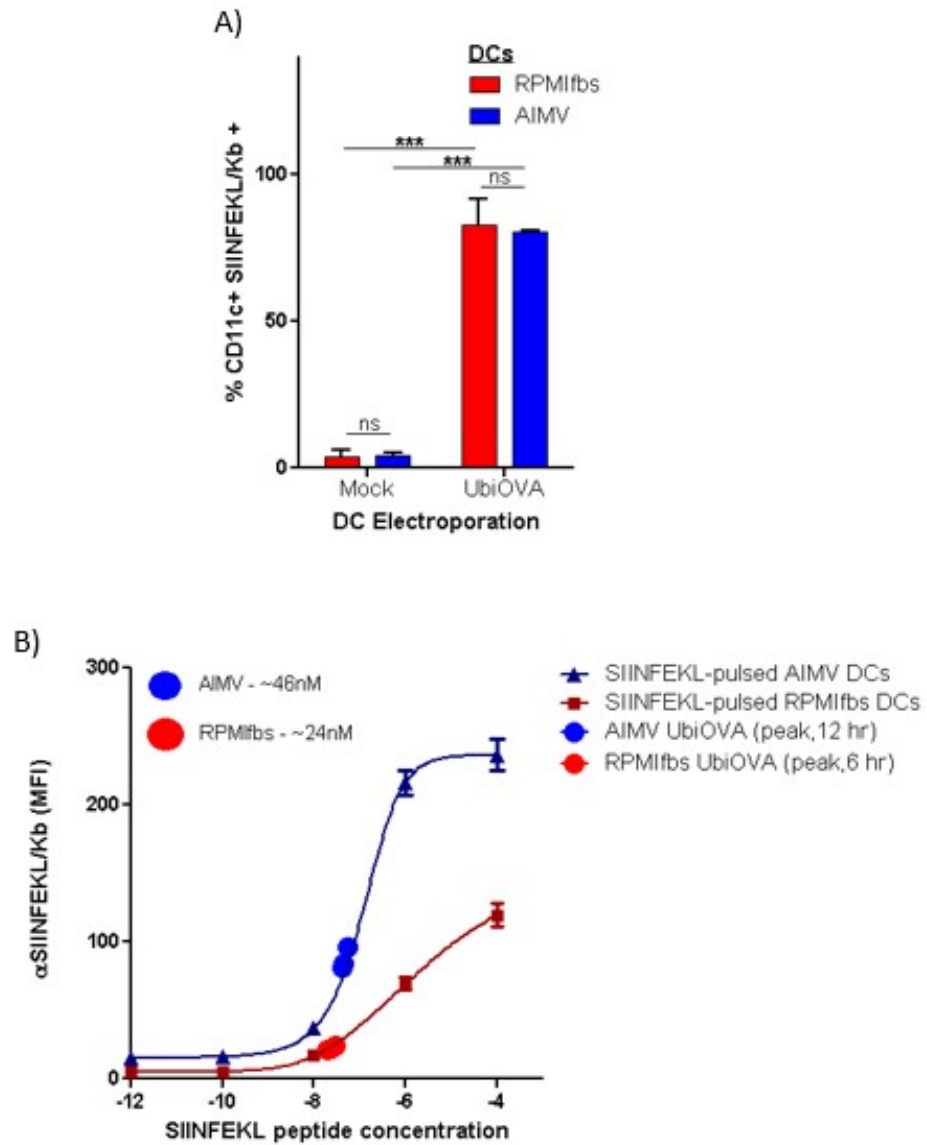


Figure 31: BMDCs generated in serum-free or FBS-containing media are efficiently electroporated and present minigene-derived MHC I epitopes

A) Bar graph showing percent of CD11c⁺ cells from BMDC cultures in RPMI₁₆₄₀ or AIM-V media exhibiting detectable surface presentation of SIINFEKL bound to K^b at 4 h upon mock electroporation or electroporation with the UbiOVA minigene, determined using flow cytometry. Average of 3 independent experiments. Two-way ANOVA with Bonferroni post-hoc test. B) RPMI₁₆₄₀ and AIM-V BMDCs were pulsed with various concentrations of SIINFEKL peptide, and the median fluorescence intensity (MFI) at each concentration was determined flow cytometrically with α SIINFEKL/K^b. The peak α SIINFEKL/K^b MFI upon UbiOVA-transfection was interpolated into the standard curve in order to determine the equivalent SIINFEKL peptide-pulsed concentration. Average of 3 independent experiments.

We next asked whether minigene-transfected DCs could stimulate T cells in an antigen-dependent manner. RF3370 is a T-cell hybridoma that possesses a TCR recognizing SIINFEKL in the context of K^b. Upon antigen-dependent crosslinking of their TCRs, RF3370 secretes IL-2 (D. A. Mitchell et al., 1998). Both AIM-V and RPMI₁₆₄₀ DCs transfected with UbiOVA mRNA were capable of stimulating RF3370 cells. DCs transfected with equimolar amounts of an IVT mRNA encoding ubiquitin alone (i.e. Ubi, Appendix A Table 8) were not able to stimulate T cells, suggesting that stimulation was not an indirect consequence of mRNA-transfected DCs and required the translation of the peptide containing the SIINFEKL epitope (Figure 32A). Interestingly, BMDCs cultured in AIM-V were better stimulators of RF3370 cells relative to RPMI₁₆₄₀ DCs, which may be a consequence of an enriched population of mature DCs in serum-free conditions.

SIINFEKL is derived from the model antigen chicken ovalbumin; therefore, there are obvious concerns with its relevance to cancer immunotherapy. Therefore, we tested whether BMDCs electroporated with minigenes encoding *bona fide* tumor antigens were capable of stimulating cognate T cells in an antigen-dependent manner. To this end, minigenes encoding a ubiquitinated 15 mer peptide spanning the B16F10 melanoma tumor-associated antigen Trp2 (UbiTrp2) or the SMA560 neoantigen Odc1 (UbiOdc1; Appendix A Table 8) were generated. When administered as a peptide vaccine, the MHC I-restricted epitope of Trp2 (Cho et al., 2013; Llopiz et al., 2013) and a 29mer peptide spanning the Odc1 MHC I epitope (Figure 12D) are capable of inducing a significant antigen-specific CD8⁺ T-cell response. Antigen-specific T cells from spleens of mice immunized with Trp2^{MHC I} or Odc1^{29mer} peptide 7 days prior were able to be maintained within *in vitro*-cultures with minigene-transfected DCs; however, mock-transfected DCs did not enable these cells to persist (Figure 32B). Together, these data indicate that minigene-transfected DCs can stimulate tumor antigen-specific T cells in an antigen-dependent manner.

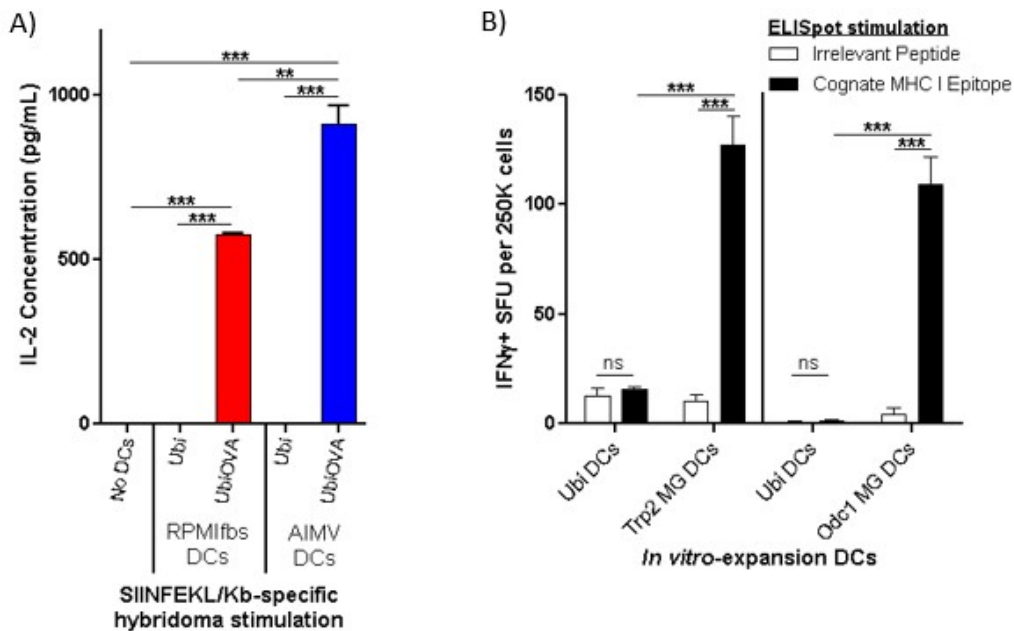


Figure 32: Minigene-transfected BMDCs stimulate T cells in an antigen-dependent manner

A) IL-2 secretion from RF3370 T-cell hybridoma cells upon stimulation with BMDCs transfected with Ubi mRNA or UbiOVA mRNA. Transfected DCs alone did not generate a detectable IL-2 response (not shown). One-way ANOVA with Tukey's post-hoc test. B)

Antigen-specific CD8⁺ T cells were induced in mice by vaccinating with an immunogenic peptide vaccine (i.e. Trp2¹⁸⁰⁻¹⁸⁸ or Odc1^{29mer}). Splenocytes from immunized mice were cocultured with BMDCs transfected with Ubi mRNA (Ubi DCs) or cognate minigene-transfected BMDCs for 6 days. The ability of minigene-transfected BMDCs to sustain antigen-specific CD8⁺ T-cell survival was assessed by IFN γ ELISpot. Two-way ANOVA with Bonferroni post-hoc test.

7.3.3 Minigene-transfected DCs are not universally efficacious

Concordant with the enhanced *in vitro* T-cell stimulatory capacity of UbiOVA AIM-V DCs (Figure 33A), these DCs were capable of activating a SIINFEKL-specific T-

cell response *in vivo* to a significantly greater degree than that of UbiOVA RPMI fbs DCs. We have shown the BMDC cultures are a highly heterogeneous population of cells, many of which are not CD11c⁺ DCs (Figure 29B). To confirm that the SIINFEKL-specific immune response was engendered by DCs, BMDC cultures were enriched for CD11c-negative and CD11c-positive cells prior to electroporation. As expected, CD11c-positive, but not CD11c-negative, UbiOVA-transfected cells were capable of eliciting the antigen-specific immune response (Figure 33B). Furthermore, activated SIINFEKL-cognate T cells were capable of effector function, as demonstrated by IFN γ ELISpot analysis (Figure 33C). Together, these results suggest that minigene-transfected DCs may be relevant for cancer immunotherapy, given their ability to activate antigen-specific, effector T-cell responses.

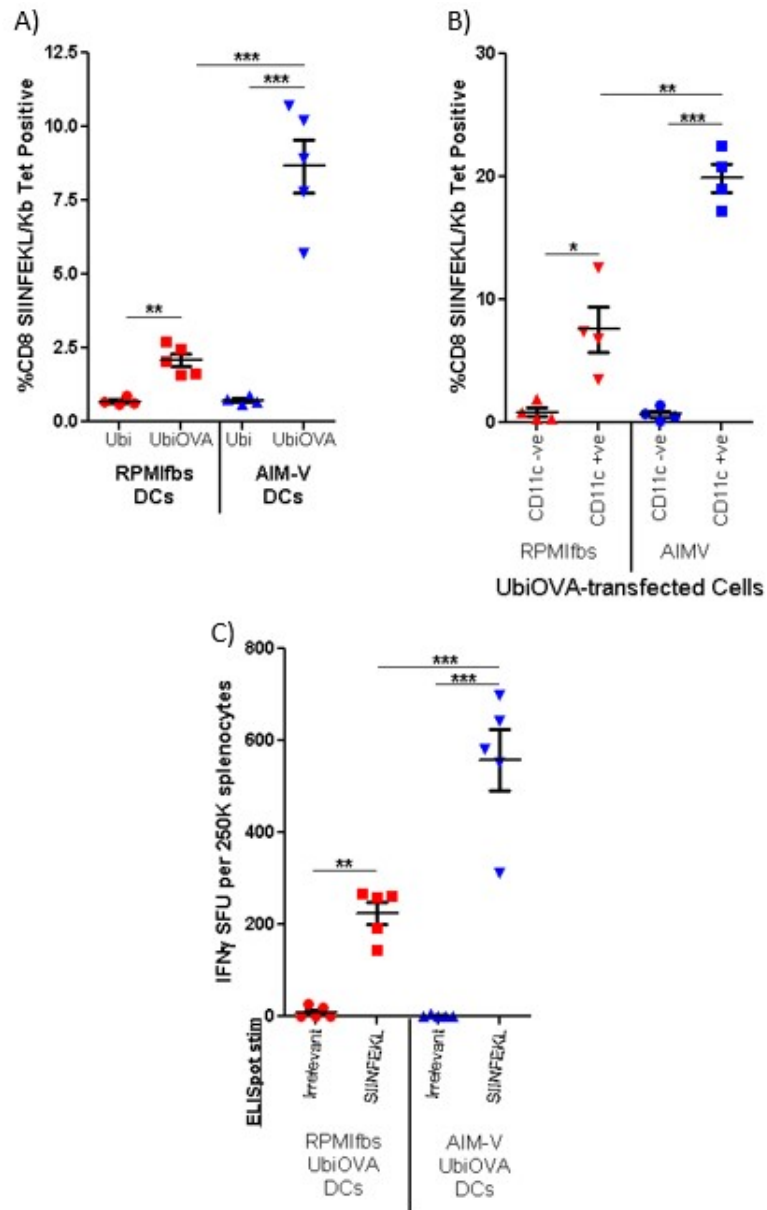


Figure 33: Minigene-transfected serum-free AIM-V BMDCs are more immunogenic than RPMIfbs BMDCs

A) Mice (n = 4-5) were immunized once with 1×10^6 bulk AIM-V or RPMIfbs BMDCs electroporated with Ubi mRNA or UbiOVA mRNA. The SIINFEKL-cognate CD8⁺ T-cell response was assessed at day 7 in blood using flow cytometric tetramer analysis. Graph depicts percent of CD8⁺ T cells that are SIINFEKL/K^b tetramer positive. One-way

ANOVA with Tukey's post-hoc test. B) Mice (n = 4) were immunized once with 5×10^5 CD11c-negative or CD11c-positive cells, derived from AIM-V or RPMIfbs BMDC cultures, electroporated with Ubi mRNA or UbiOVA mRNA. The SIINFEKL-cognate CD8+ T-cell response was assessed at day 7 in blood using flow cytometric tetramer analysis. Graph depicts percent of CD8+ T cells that are SIINFEKL/K^b tetramer positive. One-way ANOVA with Tukey's post-hoc test. C) Mice (n = 5) were immunized once with 1×10^6 bulk AIM-V or RPMIfbs BMDCs electroporated with Ubi mRNA or UbiOVA mRNA. The SIINFEKL-cognate effector CD8+ T-cell response was determined at day 7 in splenocytes by IFN γ ELISpot analysis. One-way ANOVA with Tukey's post-hoc test.

We next sought to determine the therapeutic relevance of minigene-transfected DCs. Strikingly, mice bearing 7-day subcutaneous B16OVA tumors experienced a significant survival benefit upon a single vaccination with either UbiOVA- or UbiTrp2-transfected DCs (Figure 34A). Interestingly, though AIM-V UbiOVA DCs elicited a greater SIINFEKL-cognate effector T-cell response (Figure 33), the antitumor response they elicited against B16OVA was similar to that of RPMIfbs UbiOVA DCs. Moreover, UbiOVA-transfected DCs – both RPMIfbs (Figure 34B) and AIM-V (data not shown) – were capable of eliciting a modest antitumor effect against intracerebrally-implanted B16OVA, altogether demonstrating the minigene-transfected DCs can promote therapeutic effects against tumors.

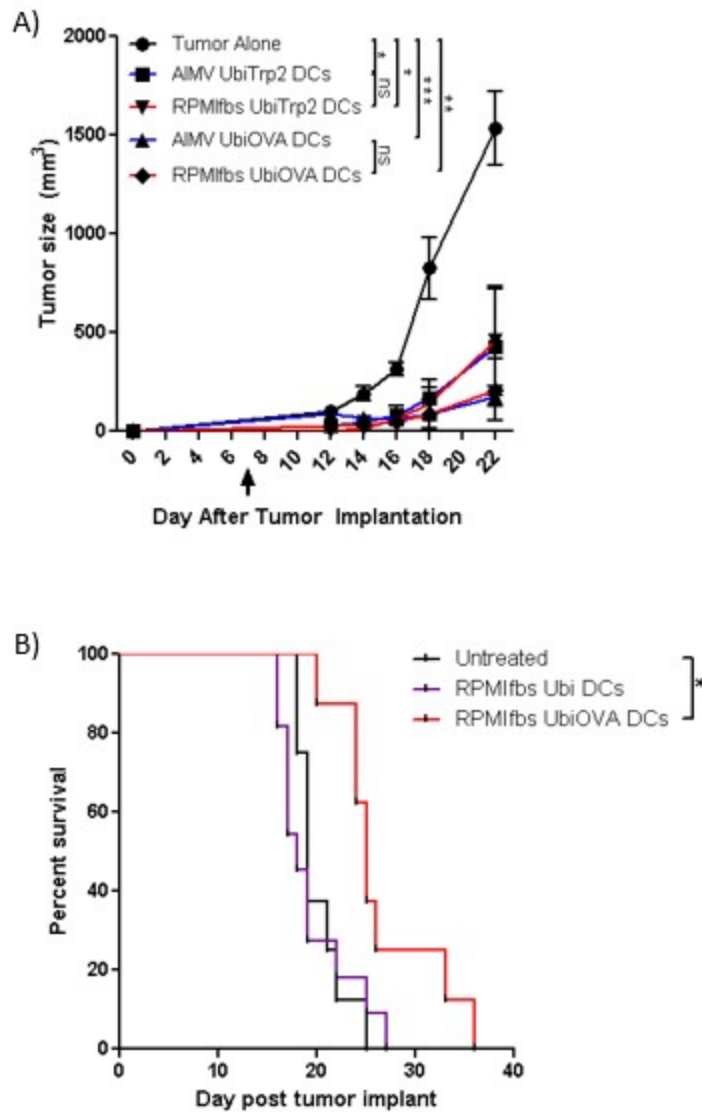


Figure 34: Minigene-transfected DCs are efficacious against subcutaneous and intracerebral B16OVA tumors

A) Mice (n = 5) were inoculated with B16OVA melanoma cells subcutaneously and then vaccinated on day 7 with UbiTrp2 or UbiOVA-transfected BMDCs from AIM-V or RPMIfbs cultures. Tumor growth was measured periodically. Day 22 tumor volume, two-sample *t*-test. B) Mice (n = 8-11) were inoculated with B16OVA melanoma cells intracerebrally and then vaccinated on day 1 with Ubi or UbiOVA-transfected RPMIfbs DCs. Log-rank test.

We have previously shown that a standard prime-boost regimen with a 29mer SLP vaccine or a P30-conjugated SLP vaccine (Figure 15B) targeting the SMA560 neoantigen Odc1 was capable of generating a modest, but significant, antitumor benefit against subcutaneous SMA560. However, a similar immunization regimen with UbiOdc1-transfected DCs was not capable of engendering therapeutic effects against subcutaneous SMA560 (Figure 35). Though the Odc1 MHC I-restricted epitope requires CD4+ T-cell help in order to generate antitumor responses (Figure 13A), this is likely not the culprit, given that BMDCs inherently activate CD4+ helper responses reactive to MHC II epitopes derived from proteins contained within DC-culture medium (e.g. FBS, albumin, etc.) (Faiola et al., 2002). As proof, the SIINFEKL epitope requires CD4+ T-cell help to generate a pronounced immune response (unpublished data from Dr. Katherine Riccione), and UbiOVA-transfected DCs can satisfy this requirement. Collectively, these results demonstrate that minigene-transfected DCs can mediate antitumor responses against some, but not all, tumor antigens.

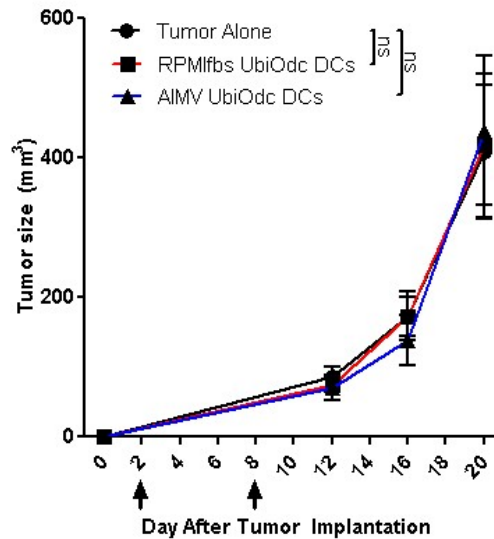


Figure 35: UbiOdc1-transfected DCs are not efficacious against subcutaneous SMA560

VMDk mice (n = 8) were inoculated with 7.5×10^5 SMA560 astrocytoma cells subcutaneously and then vaccinated on days 2 and 8 with UbiOdc1-transfected RPMIfbs. Tumor growth was measured periodically. Day 20 tumor volume, two-sample *t*-test.

7.3.4 GM-CSF + IL-4 DC vaccines suppress immune responses to subsequent vaccines

A single OVA mRNA-transfected DC vaccine was capable of promoting antitumor effects against B16OVA; however, multiple vaccines did not improve the antitumor response (Figure 36).

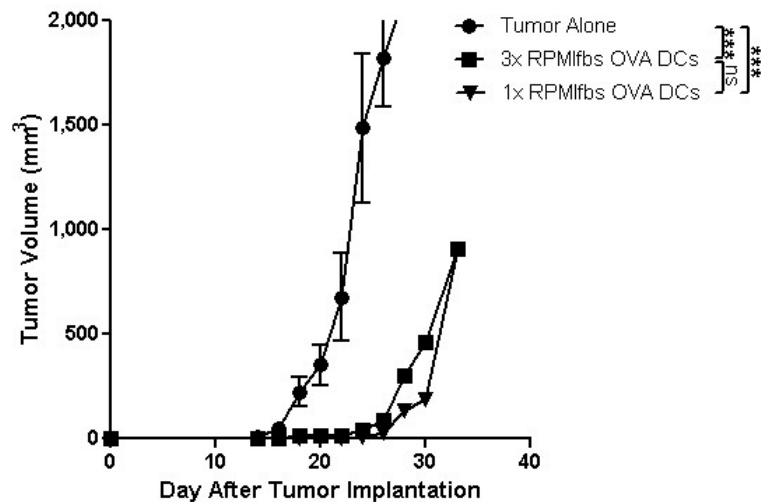


Figure 36: Booster mRNA-transfected DC vaccines do not improve antitumor efficacy

Mice (n = 6-8) were inoculated with B16OVA melanoma cells subcutaneously and then vaccinated on days 7, 14, and 21 with OVA mRNA-transfected RPMI fbs DCs. Tumor growth was measured periodically. Day 30 tumor volume, two-sample *t*-test.

This data led to the hypothesis that these DC vaccines were incapable of boosting the antigen-specific immune response. Such an effect may explain the lack of antitumor benefits bestowed by the UbiOdc1 DC vaccine against subcutaneous SMA560 (Figure 35). In accordance with this hypothesis, a booster RPMI fbs UbiOVA-transfected DCs could not sustain or enhance the SIINFEKL-cognate, effector T-cell response (Figure 37). These effects were also observed with AIMV UbiOVA-transfected DCs (data not shown).

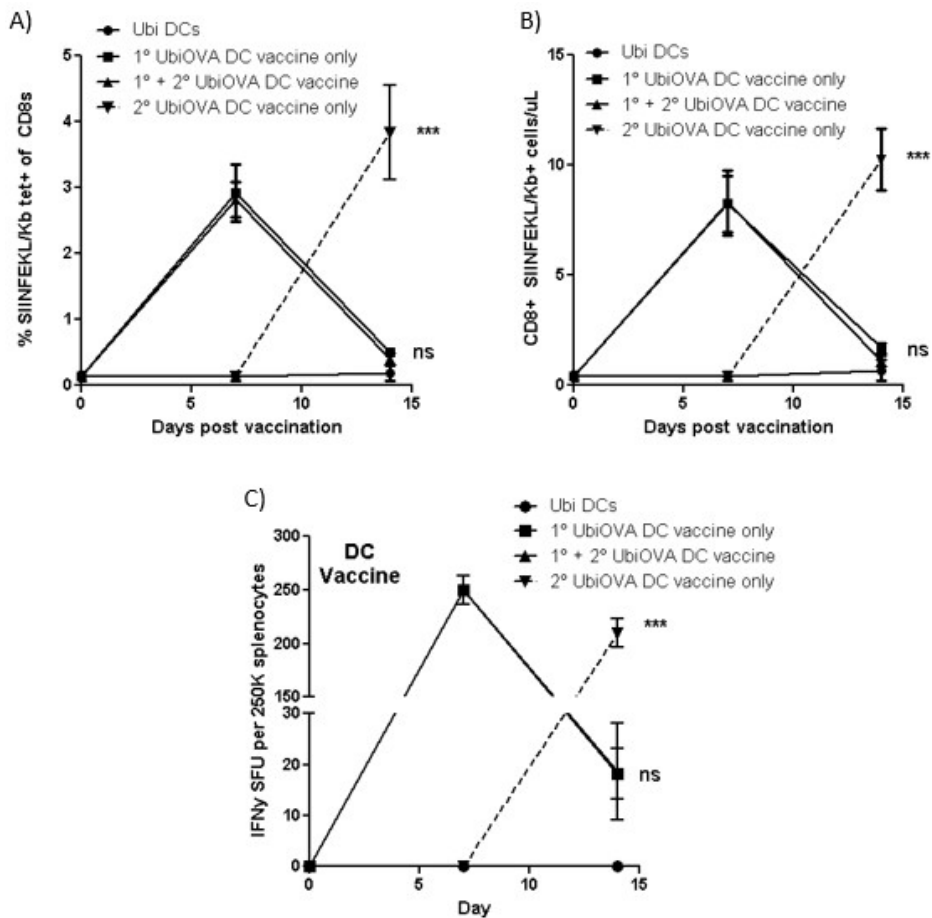


Figure 37: Booster minigene-transfected DC vaccines do not enhance or sustain antigen-specific T-cell responses

Mice (n = 3) were immunized with Ubi or UbiOVA-transfected RPMifbs DCs. Booster DC vaccines were administered 7 days later. SIINFEKL-cognate CD8+ T-cell A) percentages and B) counts in the blood were assessed at days 0, 7, and 14 using flow cytometric tetramer analysis. C) SIINFEKL-cognate effector responses in the spleen were determined by IFN γ ELISpot analysis, stimulating with the SIINFEKL peptide, on days 0, 7, and 14. One-way ANOVA with Tukey's post-hoc test.

This outcome was not a consequence of an inherent inability of SIINFEKL-specific T-cell responses to be maintained following repetitive immunizations. This was evidenced in experiments with the SIINFEKL peptide vaccine, wherein a second vaccine could maintain the SIINFEKL-cognate, effector T-cell response (Figure 38).

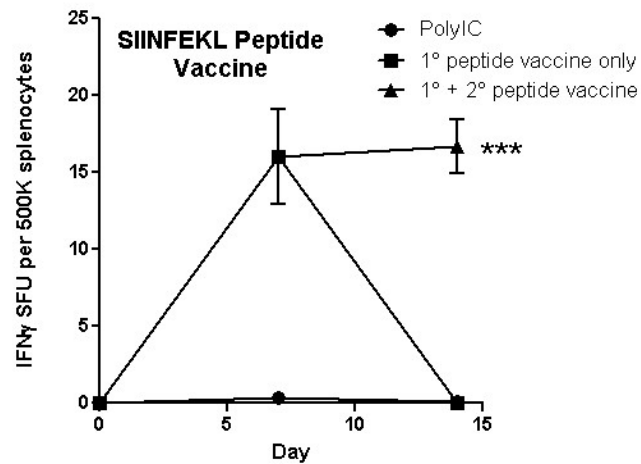


Figure 38: SIINFEKL-cognate effector responses are maintained by booster peptide vaccines

Mice ($n = 3$) were immunized 50 μg SIINFEKL peptide plus 100 μg poly(I:C), or poly(I:C) alone, and boosted with the same vaccine 7 days later. SIINFEKL-cognate effector responses in the spleen were determined by IFN γ ELISpot analysis, stimulating with the SIINFEKL peptide, on days 0, 7, and 14. One-way ANOVA with Tukey's post-hoc test.

The inability of UbiOVA-transfected DCs to induce even the slightest booster response was rather surprising considering the immunogenic potency of UbiOVA-transfected DCs. This behavior seemed to suggest that the initial vaccine response was

promoting an immunological milieu that was not conducive to T-cell activation or stimulation. This did, in fact, seem to be the case, given that that preconditioning mice with a mock-transfected DC vaccine significantly diminished the ability of the UbiOVA-transfected DC vaccine to induce a SIINFEKL-cognate, effector T-cell response (Figure 39). Together, these results demonstrate that GM-CSF + IL-4-generated DC vaccines are incapable of boosting antigen-specific T-cell responses, which may be a consequence of an immunosuppressive environment induced by these vaccines.

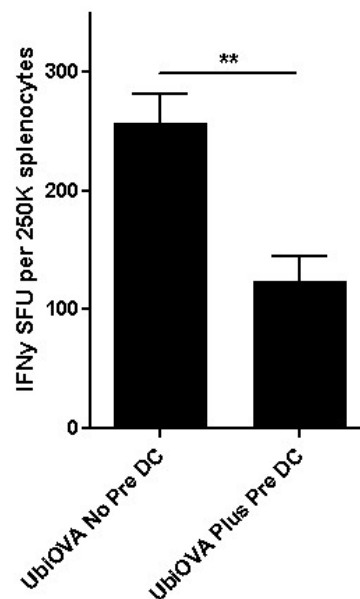


Figure 39: Primary DC vaccines suppress the antigen-specific immune response elicited by booster DC vaccines

Mice (n = 3) received immunizations with PBS (No Pre DC) or mock-transfected RPMIfbs DCs (Plus Pre DC) and then treated with 1×10^6 UbiOVA-transfected RPMIfbs DCs 7 days later. The SIINFEKL-cognate effector responses in the spleen was determined 7 days later by IFN γ ELISpot analysis. Background from the irrelevant

peptide stimulation was subtracted from the SIINFEKL-specific response. Two-sample *t*-test.

7.3.5 GM-CSF + IL-4 DC vaccines generate a pronounced IL-10+ CD4+ Tr1 response reactive to media-resident proteins

There exists a vast array of mechanisms that can suppress antigen-specific T-cell responses. However, we focused our attention on the major immunosuppressive cytokine IL-10, which was initially identified as a Th1-inhibitory cytokine produced by Th2 cells (Fiorentino et al., 1989) and has been associated with poor immune responses from splenic DCs (Zhang et al., 2005). Interestingly, we found that a single RPMifbs DC vaccination generated IL-10-producing cells reactive to antigens contained within FBS (Figure 40A). BSA was identified as the major component of FBS that stimulated this response (Figure 40B). This effect was also observed with AIM-V DCs; however, in this case, the response was towards AIMV-resident components and not to FBS (data not shown). IL-10-producing cells were detected in the draining inguinal lymph node and the spleen, indicating that the DC vaccine-induced IL-10 effect was systemic, and the response appeared to follow the transient kinetics of a normal vaccine-induced T-cell response, which was greatly diminished by day 14 (Figure 40C). Altogether, these data demonstrate the GM-CSF + IL-4 DCs activate IL-10-producing cells reactive to media-

resident proteins in a manner that is independent of the BMDC-culture conditions (i.e. serum-free vs serum-containing).

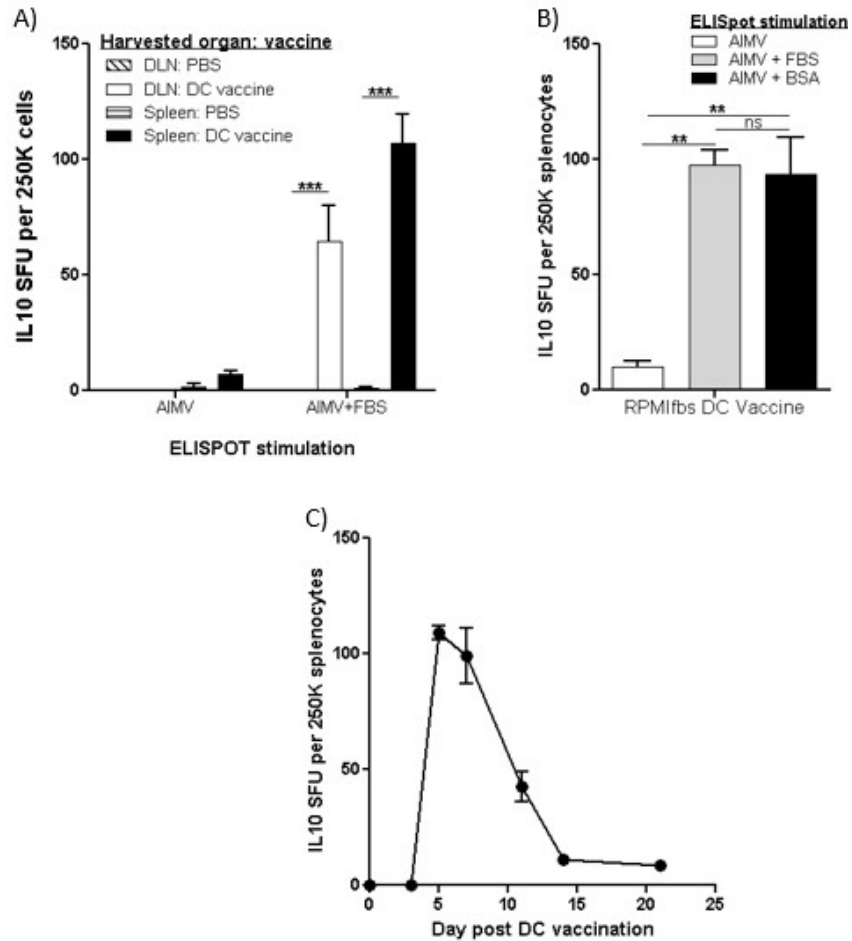


Figure 40: RPMIfbs DC vaccines elicit a transient IL-10+ response reactive to BSA

A) Mice (n = 3) were vaccinated with PBS or 1×10^6 mock-transfected RPMIfbs DCs. Seven days later, splenocytes and draining inguinal lymph node cells were evaluated for their responsiveness to FBS by IL-10 ELISpot assay. One-way ANOVA with Tukey's post-hoc test. B) Using splenocytes from A), the IL-10 response to FBS was compared to BSA by IL-10 ELISpot analysis. One-way ANOVA with Tukey's post-hoc test. C) Time

course of the FBS-specific IL-10 response, determined by IL-10 ELISpot, following a single immunization with 1×10^6 mock-transfected RPMIifbs DCs (n = 3).

We next sought to determine the immune cell subset responsible for the IL-10 response. To this end, splenocytes from DC immunized mice were stimulated with AIMV + FBS and the presence of intracellular IL-10 was evaluated in CD4⁺ cells, CD8⁺ cells, B cells, myeloid cells, NK cells, and macrophages by flow cytometry. Only CD4⁺ cells exhibited a noticeable upregulation of intracellular IL-10 upon AIMV + FBS stimulation (Figure 41A). The involvement of CD4⁺ T cells in this effect was substantiated by a complete abrogation of IL-10 secretion upon blockade of MHC II prior to restimulation (Figure 41B). While FoxP3⁺ Tregs are able to produce IL-10, only 7.1% of IL-10-producing CD4⁺ T cells were FoxP3 positive. These data suggest that the prominent IL-10-producing CD4⁺ subset is the CD4⁺ T regulatory type 1 (Tr1) cell.

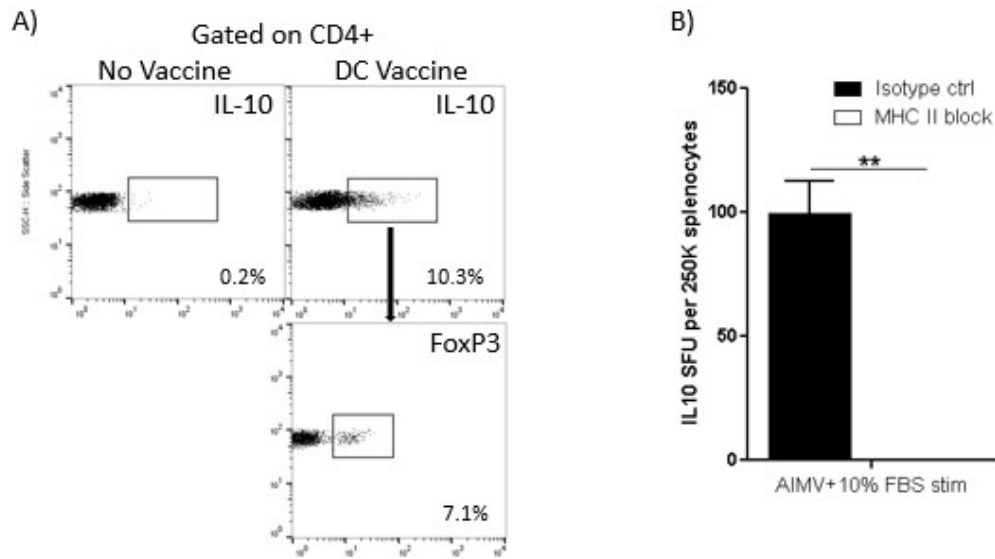


Figure 41: The FBS-specific IL-10 response is produced by the CD4+ T-cell compartment

A) Splenocytes were harvested from mice ($n = 3$) immunized 7 days prior with mock-transfected RPMI_{fbs} DCs or mice that were unimmunized. Cells were stimulated with AIM-V + FBS for 36 h, at which point immune cell subsets (i.e. CD4+ T cells, CD8+ T cells, B cells [CD19], NK cells [NK1.1], macrophages [F4/80], and myeloid cells [CD11b]) were examined for their production of IL-10 by intracellular staining and flow cytometric analysis. Flow cytometry plot showing intracellular IL-10 in the CD4+ T-cell compartment of RPMI_{fbs} DC vaccinated mice, but not unvaccinated mice. Only 7.1% of IL-10-producing CD4+ T cells were Tregs (FoxP3+). B) The FBS-specific IL-10 response was completely ablated upon immunological MHC II blockade prior to IL-10 ELISpot analysis. Two-sample *t*-test.

7.3.6 Blockade of Tr1-inducing factors does not profoundly affect the DC vaccine-mediated immune response

Tr1 cells have been shown to have a negative impact on the antitumor capabilities of DC vaccines (Zhang et al., 2005). Therefore, logic would dictate that impeding their development could enhance DC vaccine-mediated therapeutic

responses. DCs can drive the differentiation of Tr1 cells through various mechanism. These include, but are likely not limited to, the secretion of TGF- β 1, IL-27, and IL-10, as well as conveyance of signals mediated through ICOS:ICOSL interactions (Kushwah et al., 2011). Transcripts encoding all of these factors were identified in both RPMIfbs DCs and AIM-V DCs by mRNA sequencing (data not shown). Thus, we asked whether blocking these signals could suppress the IL-10⁺ CD4⁺ T-cell response engendered by UbiOVA-transfected DC vaccines.

Phenotypic analysis confirmed the expression of ICOSL on RPMIfbs DCs (Figure 42A). Therefore, UbiOVA DCs were incubated with α ICOSL blocking antibody or isotype antibody for the 2 h resting period following electroporation. Treatment with α ICOSL blocking antibody did not affect the SIINFEKL-cognate T-cell response or the IL-10⁺ CD4⁺ T-cell response engendered by UbiOVA DCs (Figure 42B & C).

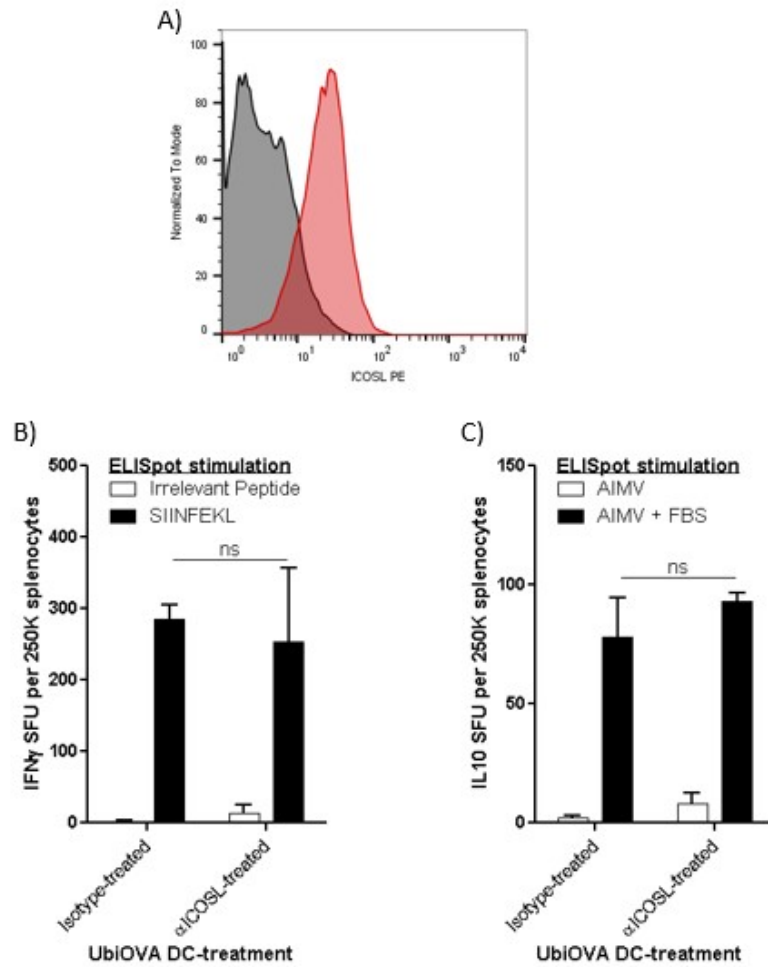


Figure 42: Blockade of ICOSL does not affect the antigen-specific T-cell response or Tr1 response induced by a minigene-transfected DC vaccine

A) Histogram showing ICOSL expression on CD11c+ BMDCs grown in RPMIifbs. B) SIINFEKL-cognate T-cell response and C) IL-10+ Tr1 response to FBS at day 7 following a single vaccine in mice (n = 3) with RPMIifbs UbiOVA-transfected DCs in the context of ICOSL blocking or isotype antibodies. One-way ANOVA with Tukey's post-hoc test.

Next, IL-10, IL-10 receptor (IL-10R), or TGF β blocking/neutralizing antibodies were evaluated for their ability to enhance the booster response of UbiOVA DC

vaccines. Interestingly, adjuvant therapy with α TGF β antibody slightly increased the SIINFEKL-cognate T-cell response (Figure 43A), but this effect was not associated with a decreased IL-10+ CD4+ T-cell response (Figure 43B). Conversely, α IL-10R greatly enhanced the IL-10+ CD4+ T-cell response; however, this was not accompanied by a diminished SIINFEKL-cognate T-cell response. Collectively, these data indicate that blockade of common Tr1-inducing pathways does not improve the immunogenicity of minigene-transfected DC vaccines. It may be that there are redundant pathways involved in the induction of Tr1 cells by DC vaccines, which would consequently require the simultaneous blockade of multiple Tr1-inducing factors; however, this remains untested.

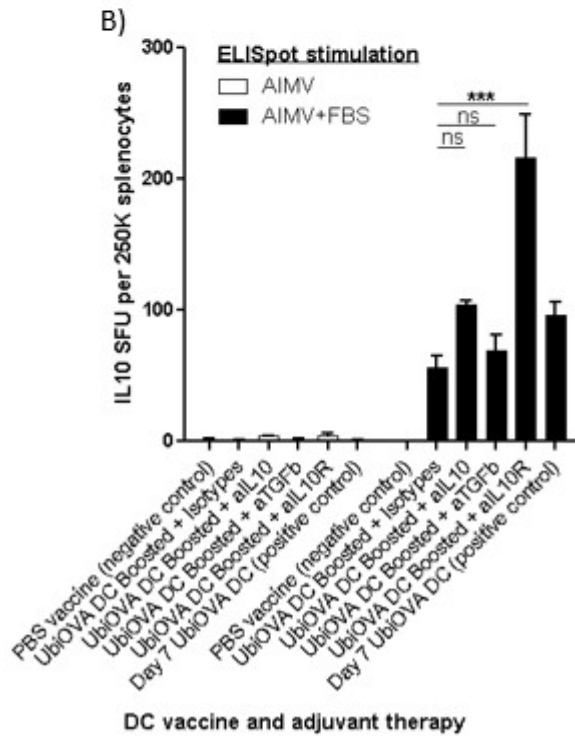
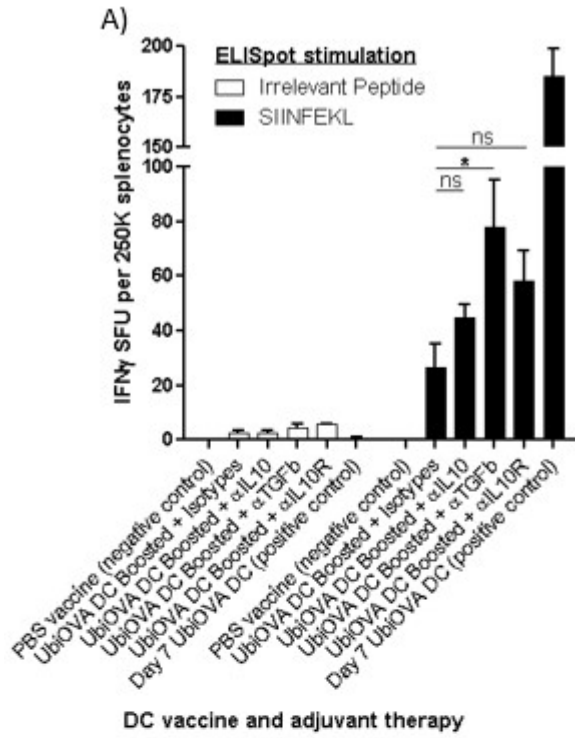


Figure 43: Blockade of known Tr1-inducing factors does not dramatically affect the antigen-specific T-cell response or Tr1 response induced by a minigene-transfected DC vaccine

A) SIINFEKL-cognate T-cell response and B) IL-10+ Tr1 response to FBS at day 14 following a prime (day 0)/boost (day 7) vaccine regimen in mice (n = 3) with RPMI-fbs UbiOVA-transfected DCs in the context of blocking or isotype antibodies. One-way ANOVA with Tukey's post-hoc test.

As a final attempt, we aimed to repolarize the CD4+ T-cell compartment away from a Tr1 phenotype. Our lab has previously demonstrated the preconditioning of mice with tetanus/diphtheria (Td) toxoid is able to enhance DC vaccine migration to the lymph nodes via the induction of CCL3+ CD4+ T cells, which yields better antitumor responses (Batich et al., 2016; D. A. Mitchell et al., 2015). Preconditioning with Td toxoid did not, however, dramatically enhance the booster function of UbiOVA DC vaccines or alter the IL-10+ CD4+ response (Figure 44), suggesting this is not a mechanism through which Td toxoid mediates enhanced antitumor effects.

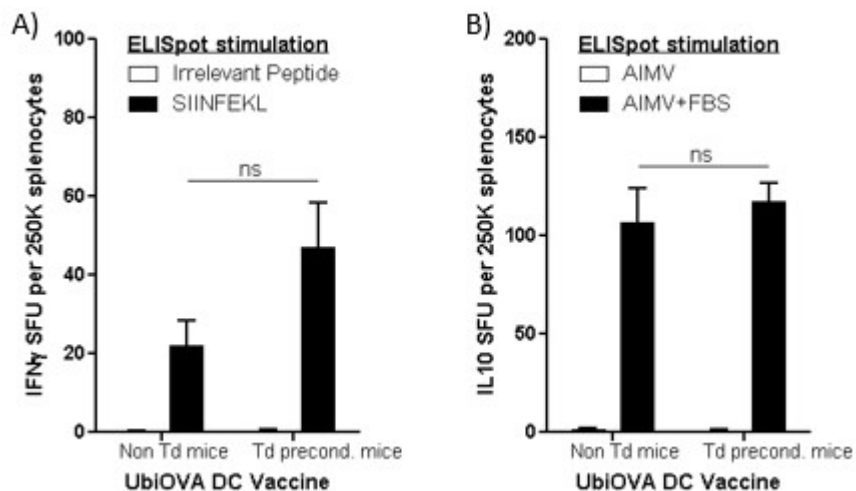


Figure 44: Tetanus/diphtheria toxoid preconditioning does not affect the antigen-specific T-cell response or Tr1 response induced by a minigene-transfected DC vaccine

Mice ($n = 4$) were immunized with PBS (negative control) or Td toxoid at -4 weeks, -2 weeks, and -1 day relative to DC vaccine, per a pre-described protocol (D. A. Mitchell et al., 2015). A) SIINFEKL-cognate T-cell response at day 14 following a prime (day 0)/boost (day 7) vaccine regimen with RPMIfbs UbiOVA-transfected DCs. One-way ANOVA with Tukey's post-hoc test. B) IL-10 $^{+}$ Tr1-response to FBS at day 7 following a single vaccination with RPMIfbs UbiOVA-transfected DCs. One-way ANOVA with Tukey's post-hoc test.

7.3.7 GM-CSF + IL4 DCs are likely polarized to a phenotype not optimal for DC-based cancer vaccines

A previous study compared the antitumor capabilities of various dendritic cell subsets within the spleen: CD8 $^{+}$ CD4 $^{-}$ DCs, CD8 $^{-}$ CD4 $^{+}$ DCs, and CD8 $^{-}$ CD4 $^{-}$ DCs (Zhang et al., 2005). While CD8 $^{+}$ CD4 $^{-}$ DCs and CD8 $^{-}$ CD4 $^{+}$ DCs were capable of engendering profound antitumor benefits, CD8 $^{-}$ CD4 $^{-}$ DCs required the depletion of

CD4⁺ cells before an antitumor effect could be observed. This was demonstrated to be a result of the activation of Tr1 cells by CD8⁻ CD4⁻ DCs, and genetic ablation of IL-10 within the CD4⁺ T-cell compartment was able to recover the antitumor benefit.

In many ways, CD8⁻ CD4⁻ splenic DCs mirror GM-CSF + IL-4 DCs. GM-CSF + IL-4 BMDCs are characterized by an absence of CD4 and CD8 (data not shown) and, like CD8⁻ CD4⁻ DCs, they also induce a prominent IL-10⁺ Tr1 response. Extrapolating other lessons from the aforementioned study, it would appear as though CD8⁺ CD4⁻ DCs are superior for cancer vaccines. Splenic CD8⁺ DCs are typified by the expression of BATF and IRF8 (Aliberti et al., 2003). GM-CSF + IL-4-induced BMDCs lack the expression of both (Figure 45, BATF western blot not shown). Alternatively, they express Irf4, which is associated with the CD8⁻ CD4⁻ DC phenotype (Bajana et al., 2016). Altogether, these data suggest that GM-CSF + IL-4 DCs model, at least in part, CD8⁻ CD4⁻ splenic DCs in their transcription-factor expression and immunological stimulation. Identifying methods that can generate Irf8/BATF⁺ DCs from blood-borne precursors will likely be more favorable for the field of DC-based cancer vaccines.

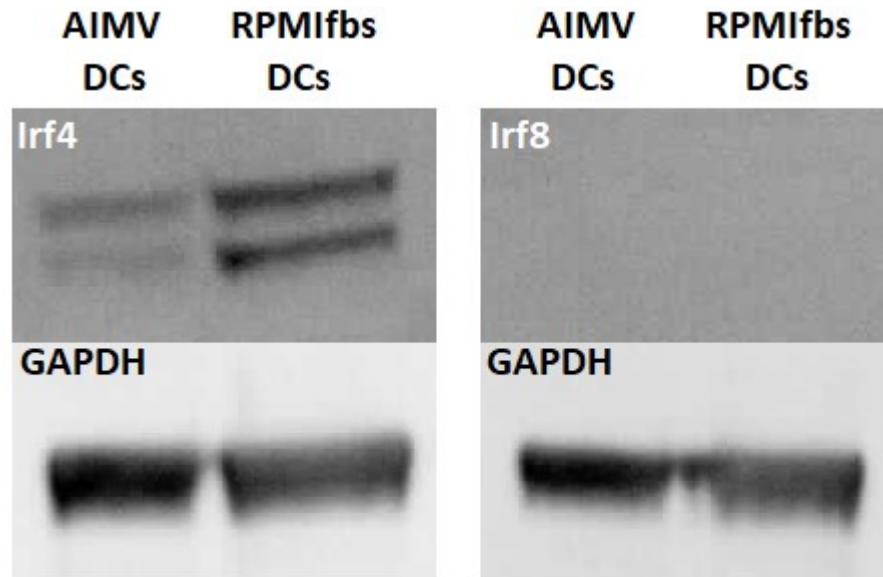


Figure 45: GM-CSF + IL-4 DCs express Irf4 but not Irf8

Western blot showing Irf4, but not Irf8, expression in CD11c+ DCs generated in GM-CSF + IL-4-containing AIM-V or RPMIfbs medium. GAPDH served as a protein loading control.

7.4 Discussion

DCs represent a promising cancer vaccine platform due to their unique ability to activate cytotoxic lymphocytes (CTLs), which are considered to be major drivers of the antitumor immune response. This process functions through 3 necessary signals. Signal 1 involves the engagement of the T-cell receptor (TCR) with a peptide:MHC complex. Signal 2 is provided by costimulatory molecules, such as CD80 and CD86 on DCs, that interact with CD28 expressed on T cells, prompting several T-cell activating mechanisms such as the stabilization of IL-2 mRNA (Umlauf et al., 1995). Finally, cytokines secreted

by DCs, including IL-12 and interferons (IFNs), embody signal 3 and are required for the robust activation and polarization of CTLs.

In this study, we revealed that mouse BMDCs cultured in serum-free conditions exhibited a more universally mature phenotype, which corroborates findings from an earlier study (Warncke et al., 2006). Furthermore, we found that serum-free AIM-V DCs were more immunogenic than RPMI fbs DCs. Thus, these data would conform to widely-accepted notion that DC maturation is required for the potent induction of antitumor CTL responses. However, the superior immune responses engendered by AIM-V DCs did not translate into better antitumor effects. To complicate things further, a recent study has demonstrated that it is, in fact, the MHC II^{low} RPMI fbs DC population that mediates the most potent antitumor effects (Ebrahimi-Nik et al., 2018). Thus, the requirement for total DC maturation is in question, and DCs that exist in a semi-mature state may be optimal for cancer vaccine strategies. While further work is necessary to clarify these mechanisms, this may ultimately lead to a paradigm shift in how DCs are prepared for immunotherapy.

We have found that minigene-transfected DC vaccines are incapable of boosting using our standard preclinical DC vaccine regimen (i.e. weekly treatments). These findings are similar to other published results, which have demonstrated that GM-CSF + IL-4 DC boosting served no advantage in a therapeutic setting against B16F1 or a

transgenic adenocarcinoma and, in some cases, was even detrimental (Ricupito et al., 2013). It is difficult to extrapolate our data here to clinical studies using DC vaccines, in which booster vaccines are regularly administered (Batich et al., 2017; Liau et al., 2018; D. A. Mitchell et al., 2015) and partial maintenance of antigen-specific T-cell responses has been observed. (Batich et al., 2017). However, clinically-administered DCs are typically given in the context of TMZ-mediated host lymphodepletion, which can have counterintuitive benefits for vaccines (D. A. Mitchell et al., 2011; Sampson et al., 2011). Our data here are also demonstrated with weekly DC vaccines, rather than biweekly as used in clinical testing; however, Trp2-pulsed DCs given biweekly did not improve the immune or antitumor response against B16 tumors (Ricupito et al., 2013).

Our data show that a primary DC vaccine conditions the host immune system in a manner that is not conducive to antigen-specific T-cell activation, and this effect is associated with the induction of an IL-10-specific CD4⁺ T-cell response. While we have not conclusively demonstrated that the two are incontrovertibly connected, it would be reasonable to assume that profound DC vaccine-mediated IL-10 production would have a devastating effect on the adaptive immune response, given the extensive literature on this topic [reviewed in (Rojas et al., 2017)]. In support of their association, we have shown that a booster peptide vaccine administered in poly(I:C) is able to maintain the SIINFEKL-cognate effector T-cell response, and this vaccine formulation has been shown

to not stimulate an IL-10 response, unlike other adjuvants (e.g., imiquimod and CpG) (Llopiz et al., 2016). For this reason, the immune and antitumor responses mediated by poly(I:C)-adjuvanted vaccines did not improve upon α IL-10R blockade, while imiquimod- and CpG-adjuvanted vaccines were augmented in this context. Conversely, we have shown in this study that blockade of IL-10R did not enhance the SIINFEKL-cognate T-cell response by booster UbiOVA DCs, suggesting that GM-CSF + IL-4 DC vaccines may exert immunosuppression through additional IL-10-independent mechanisms.

A major assertion of this study is that rather than attempting to overcome the multiplicity of mechanisms that govern the immunosuppressive nature of GM-CSF + IL-4 DCs, developing a more appropriately polarized Th1-activating DC vaccine would likely be more beneficial for the field of DC-based immunotherapy. DC vaccines are classically derived from the differentiation of bone marrow precursors or monocytes using GM-CSF \pm IL-4 or FLT3L cytokines (Y. Xu et al., 2007). The prior generates Irf4⁺ DCs, as we have shown, which are primarily involved in Th2 activation (Williams et al., 2013) and mirror splenic CD8⁻ CD4⁻ DCs in their inferior immunotherapeutic capabilities (Zhang et al., 2005). FLT3L generates DCs primarily of the plasmacytoid subset (Brawand et al., 2002), which have demonstrated limited success for immunotherapy (Dey et al., 2015). On the other hand, Irf8⁺ DCs, including BATF⁺ CD8⁺

splenic DCs and migratory CD103⁺ DCs (Watowich et al., 2010), have repeatedly been shown to be indispensable for the Th1-skewed immune and antitumor response (Desch et al., 2014; Spranger et al., 2015; Spranger et al., 2017; Zhang et al., 2005). Thus, identifying methods that can differentiate Irf8⁺ DCs from common hematopoietic progenitors, whether through cytokine exposure, cellular reprogramming, or trans-differentiation, would likely address many of the limitations of traditional DC vaccines.

The diversity and complexity of dendritic cells makes their optimization for DC-based immunotherapy a seemingly inextricable task. However, given the unique ability of DCs to activate CTLs, DC vaccines continue to attract interest for GBM immunotherapy. In fact, some of the most impressive clinical responses against GBM have been observed with DC vaccines (Liau et al., 2018; D. A. Mitchell et al., 2015; Polyzoidis et al., 2014). Nevertheless, recent insights, such as those presented in this study, suggest that there is still room for improvement.

8. Conclusions and Future Directions

8.1 Improving the effectiveness of cancer vaccines for GBM

Using active immunization to induce potent antitumor responses in patients with GBM is a challenging endeavor, especially considering the wide-spread immunosuppression and immunological dysfunction observed in these individuals. Furthermore, GBM's status as a "cold" tumor (i.e. low levels of T-cell infiltration) are suggestive of a deficiency in the immunological recognition of these tumors. Nevertheless, the objective responses reported across several clinical trials with GBM vaccines provide encouragement for this area of study. It has been well-established that cancer vaccines can successfully stimulate tumor-specific immune responses; however, the use of vaccines alone may be insufficient to promote complete GBM rejection. The following section briefly discusses several factors that may limit the effectiveness of GBM vaccines and approaches that are being considered to modulate these aspects.

8.1.1 Overcoming immunosuppression

GBM cells can express an array of suppressive molecules that interfere with immune cell function. Two well-described examples include TGF- β and IDO (Nduom et al., 2015). TGF- β is a multifunctional cytokine that engenders a host of immunosuppressive effects, many of which can restrict effector T cell function (reviewed in detail (J. Han et al., 2015)). IDO is the rate-limiting enzyme involved in tryptophan

catabolism, which generates kynurenine byproducts that impair the survival and growth of T cells (Mbongue et al., 2015). Therapeutics targeting these immunosuppressive molecules are currently being evaluated in the clinic against glioma and other solid tumors; however, on their own, there have yet to be any striking clinical outcomes. Nevertheless, given their known antagonistic effects on T-cell function, combination of these modalities with cancer vaccines is deserving of future study.

While GBM cells, themselves, can convey molecules that directly facilitate immunological evasion, they may also co-opt cells of the immune system for this purpose. As an example, GBM cells have been shown to secrete chemotactic signals that result in the recruitment of immunosuppressive Tregs into the tumor environment (Jordan et al., 2008). Within this niche, Tregs suppress T-cell function in a variety of ways, including the elaboration of immunosuppressive molecules (e.g., TGF- β , IL-10, cAMP), and consumption of the key T cell survival cytokine IL-2 (Sakaguchi et al., 2009). The latter is mediated through constitutive Treg expression of the high affinity IL-2 coreceptor CD25. Specific Treg depletion has been heavily pursued as an anti-cancer treatment, and CD25 has been a target for various Treg-depleting modalities for glioma (D. A. Mitchell et al., 2011). However, CD25 is also expressed on activated T cells, and poses a risk for deleting vaccine-induced T cells upon treatment. A more recent approach to targeting Tregs may be through the use of therapies targeting cytotoxic T-

lymphocyte-associated protein 4 (CTLA-4) – an immunological checkpoint molecule constitutively expressed on Tregs (Simpson et al., 2013). CTLA-4 exerts its effects during the T-cell activation stage by outcompeting CD28 for binding to the costimulatory molecules CD80 and CD86, which provide necessary signals for naïve T-cell priming. Antibody-based therapies targeting CTLA-4 have been shown to enhance vaccine responses (Sotomayor et al., 1999), as well as eliminate Tregs via antibody-dependent cellular cytotoxicity (Simpson et al., 2013). Therefore, targeting CTLA-4 may be advantageous for enhancing the effectiveness of vaccine-mediated T-cell responses by working at both the priming and effector phase of the T-cell response. A phase I trial is set to begin testing the safety of anti-CTLA4 (Ipilimumab) in combination with a personalized neoepitope vaccine and anti-programmed cell death protein 1 (anti-PD-1: Nivolumab) for patients with newly diagnosed GBM (NCT03422094).

Immunosuppression may also arise from conventional therapies used in the treatment of malignant brain tumors. For example, corticosteroids, such as dexamethasone, are routinely used to alleviate cerebral edema associated with brain tumors but are profoundly immunosuppressive. Studies have shown that dexamethasone suppresses naïve T-cell proliferation and may, therefore, compromise vaccine-mediated immune responses (Giles et al., 2018; Hinrichs et al., 2005). Utilization of anti-VEGF therapies, such as the monoclonal antibody bevacizumab, may represent

an alternative to dexamethasone since bevacizumab has been shown to reduce cerebral edema and severe neurological impairment in patients with GBM (Darmon et al., 2017). In addition, incorporation of bevacizumab could have beneficial effects on vaccines considering that it has been shown to be capable of reducing circulating Tregs (Thomas et al., 2017) and enhance DC stimulatory capacity (Osada et al., 2008).

Temozolomide (TMZ) is an integral component of the standard-of-care treatment for patients with newly diagnosed GBM, especially in MGMT-methylated patients. Utilization of TMZ to combat immunosuppression present in GBM patients has been proposed, as it has the capacity to eliminate Tregs and prolong survival of mice with intracranial tumors undergoing vaccination and adoptive T-cell therapy (Sanchez-Perez et al., 2014; L. A. Sanchez-Perez et al., 2013). However, these effects are dose dependent and could be associated with a detrimental effect on host T-cells responses (Litterman et al., 2013). Interestingly, new studies have revealed that utilization of TMZ can modulate other immune cell responses. While T cells tend to decrease after TMZ treatment, NK cells are resistant to TMZ due to increased expression of the multidrug-resistance transporter ABCC3 (Pellegatta et al., 2018; Pessina et al., 2016). In addition, humoral responses have been demonstrated to be increased in patients receiving higher doses of TMZ (Sampson et al., 2011; L. Sanchez-Perez et al., 2013). Therefore, careful dosing and timing of TMZ administration could be beneficial against the immunosuppression

observed in GBM patients by decreasing Tregs, increasing the frequency of potentially tumoricidal NK cells, and promoting tumor-specific humoral responses. In the context of vaccination aimed at raising T-cell responses, it would be hypothesized that TMZ could best be employed prior to vaccination and then terminated upon vaccination. This schedule, however, has to be carefully examined since the standard-of-care treatment calls for at least 6 months of maintenance TMZ.

8.1.2 Enhancing T-cell function

The PD-1 axis is another immunological checkpoint that has attracted significant attention in recent years. Binding of PD-1, expressed on T cells, with its cognate ligands initiates intracellular events that ultimately disrupt TCR signaling (Parry et al., 2005). Though GBM tumors are not typically enriched with lymphocytes (S. Han et al., 2014), studies have detected high numbers of PD-1⁺ TILs with the tumor microenvironment (Woroniecka et al., 2018b). Furthermore, the expression of programmed death-ligand 1 (PD-L1) was found to be low but detectable on GBM cells (Nduom et al., 2016). This low expression may be a consequence of a lack of an invasive T-cell front, which was a region of remarkably prominent PD-L1 expression in mismatch-repair deficient cancers (Le et al., 2015). A recent phase III trial in patients with recurrent GBM revealed that anti-PD-1 (Nivolumab), on its own, was unable to extend OS (D. Reardon et al., 2017). Though there are several potential explanations for this treatment failure, one possibility

is a dearth of tumor-specific T cells for the treatment to sufficiently act upon. With this in mind, several trials have commenced whereby anti-PD-1 will be given in conjunction with vaccines (NCT02529072 and NCT03422094), as well with oncolytic viruses (NCT02798406) to purposely enhancing the duration and efficacy of the tumor-specific T cell response.

Activated T cells are not a uniform entity. T cells can differ dramatically in their ability to proliferate, elicit cytotoxicity, and avoid cell death depending on the signals they receive throughout their existence. T cells express a number of costimulatory receptors that are not directly required for T-cell activation but, upon signaling, can supercharge T-cell function. Several examples of these costimulatory receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, including 4-1BB, OX40, and CD27. Varlilumab, a CD27 agonist antibody, has been shown to enhance T cell responses and selectively deplete Tregs while preserving antigen-experienced CD4⁺ T cells (Wasiuk et al., 2017). The use of co-therapies targeting TNFR-family receptors in combination with cancer vaccines is an emerging area of research that is yielding encouraging results in preclinical studies. It remains to be seen if these agonists can enhance vaccine responses and mediate antitumor efficacy against human intracranial tumors.

8.2 Final thoughts on cancer vaccines for GBM

GBM remains largely recalcitrant to non-specific traditional therapies, such as those that constitute the current standard-of-care regimen, due to the highly infiltrative nature of these tumors. Thus, immunotherapy has been heralded as a promising means to treat these tumors, given the cytotoxic potential and molecular-guided precision of cells of the immune system. Many forms of immunotherapy are currently being evaluated for their ability to treat GBM; however, a major appeal of cancer vaccines is the relative ease by which they can be personalized to an individual patient. This has become a significant factor considering the widespread intertumoral and intratumoral heterogeneity observed in GBM tumors.

Intertumoral heterogeneity is clearly observed, as seen in the current genetic classification of GBM tumors (Patel et al., 2014; Verhaak et al., 2010). Immunotherapy against these genetically diverse tumors could require prior identification of the genetic make-up and antigenicity in order to develop targeted vaccines. However, upon targeting these antigens, there is a potential for tumor escape due to the inherent intratumoral heterogeneity observed in glioblastoma. Results from phase II and III clinical trials with the EGFRvIII-targeting vaccine rinodopepimut have highlighted this possible effect considering that most recurrent tumors were largely comprised of EGFRvIII-loss variants, likely a consequence of the subclonal expression of EGFRvIII.

Thus, strategies that target a broader array of GBM cells, whether by targeting clonal tumor antigens or a larger number of tumor antigens, are needed. Approaches such as DCVax®-L or the utilization of oncolytic viruses to initiate immune responses remain potentially therapeutic since they allow the host immune system to scan and select all antigens naturally present in the tumor. Another approach that has garnered significant interest in recent years is the use of multivalent vaccines targeting neoantigens arising from somatic GBM mutations in order to promote a potent, tumor-specific cytolytic T-cell response to GBM tumors, which we have explored in this dissertation. A concern with this approach, however, is that it has been proposed that mutational load positively correlates with the objective response rate to checkpoint blockade therapy targeting T-cell immunity (Yarchoan et al., 2017), and newly-diagnosed GBM possesses, on average, only ~35 somatic mutations (Alexandrov et al., 2013). Recurrent GBM treated with TMZ, on the other hand, can express several thousand somatic mutations (Johnson et al., 2014); however, cancer vaccines targeting these likely subclonal mutations may suffer the same fate as rindopepimut. Nevertheless, at this time, we cannot justly deduce from earlier studies that potentiation of a multivalent cytolytic T-cell response to GBM antigens using cancer vaccines will not have an appreciable therapeutic effect on GBM tumors. Furthermore, the work described in this dissertation reveals a rational approach for

enhancing the therapeutic potential of neoantigen vaccines, which may have to potential to enhance the therapeutic breadth of neoantigen vaccines.

Despite the many challenges associated with the development and testing of cancer vaccines for GBM, hopeful clinical responses have been observed, and several encouraging pursuits lay on the horizon of GBM vaccine strategies. The clinical evaluation of neoantigen vaccine strategies are currently underway, including GAPVAC-101 – the results of which are highly anticipated. It has recently been shown that vaccine-stimulated, neoantigen-reactive T cells can, in fact, traffic to GBM tumors, lending credence to this overall approach (Anandappa et al., 2018). Additionally, combination therapy with treatments that mitigate immunosuppression (e.g., anti-CTLA4, immunosuppressive molecule inhibitors) or enhance T-cell function (e.g., anti-PD-1/L1, costimulatory molecule agonists) are also being ardently explored. Thus, these next few years are met with hopefulness and a reasonable degree of optimism that cancer vaccines may have their place in the armamentarium of GBM therapies.

Appendix A

Table 6: Flow Cytometry Antibody List – Quick Reference

Target Antigen	Clone ID	Isotype	Vendor
CD11c	N418	A. Hamster IgG	BioLegend
CD80	16-10A1	A. Hamster IgG	BD Biosciences
CD86	GL1	Rat IgG2a, κ	BD Biosciences
CD40	3/23	Rat IgG2a, κ	BioLegend
IAb (MHC II)	AF6-120.1	Mouse IgG2a, κ	BD Biosciences
SIINFEKL/K ^b	25-D1.16	Mouse IgG1, κ	BioLegend
CD3	145-2C11	A. Hamster IgG	eBioscience
CD8	53-6.7	Rat IgG2a, κ	eBioscience
CD4	GK1.5	Rat IgG2b, κ	eBioscience
CD19	6D5	Rat IgG2a, κ	BioLegend
NK1.1	PK136	Mouse IgG2a, κ	BD Biosciences
CD11b	M1/70	Rat IgG2b, κ	BioLegend
F4/80	BM8	Rat IgG2a, κ	eBioscience
FoxP3	R16-715	Rat IgG2a, κ	BD Biosciences
IL-10	JES5-16E3	Rat IgG2b, κ	BioLegend
α SIINFEKL/K ^b TCR	N/A	N/A	MBL
CD45	30-F11	Rat IgG2b, κ	BD Biosciences

K ^b (MHC I)	AF6-88.5.5.3	Mouse IgG2a, κ	eBioscience
D ^b (MHC I)	28-14-8	Mouse IgG2a, κ	eBioscience
Wild-type Odc1	ODC1/486	Mouse IgG2a, κ	Novus Biologicals
Mouse IgG	N/A	Goat IgG	Jackson Immunoresearch

Table 7: Blocking/Neutralizing Antibody List – Quick Reference

Target Antigen	Clone ID	Isotype	Vendor
CD40L	MR-1	A. Hamster IgG	BioXCell
CD8	2.43	Rat IgG2b, κ	BioXCell
CD4	GK1.5	Rat IgG2b, κ	BioXCell
MHC II	M5/114	Rat IgG2b	BioXCell
TGF-β	1D11.16.8	Mouse IgG1, κ	BioXCell
IL-10	JES5-2A5	Rat IgG1, κ	BioXCell
IL-10R	1B1.3A	Rat IgG1, κ	BioXCell
ICOSL	HK5.3	Rat IgG2a, κ	BioXCell

Table 8: Minigene Protein Sequences

Minigene	Protein Sequence (mouse codon optimized, ubiquitin in red , MHC I epitope underlined)
Ubi	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQ LEDGRTLSDYNIQKESTLHLVLRRLGA
UbiOVA	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQ LEDGRTLSDYNIQKESTLHLVLRRLGAEQLES <u>IINFEKL</u> TEW
UbiTrp2	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQ LEDGRTLSDYNIQKESTLHLVLRRLGAANCSVYDFFVW <u>L</u> HYY
UbiOdc1	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQ LEDGRTLSDYNIQKESTLHLVLRRLGAKY <u>YAAS</u> NGVLM <u>MT</u> FDS

Table 9: Empirically-tested SMA560-neoantigen-targeting SLPs

GeneID	FPKM	Mut Residue	AA From	AA to	Best MHC I Score	Best MHC II Score	Synthetic Long Peptide Vaccine	Pred MHC I IC50 (ANN)	Pred MHC I IC50 (SMM)
Emp1	120.26	131	S	R	0.65	14.36	THHYAHSEGNFNSSRHQGYCFILTWICFC	1073.39	2643.93
Eif4a2	41.659	281	N	K	1.1	9.71	LYETLTITQAVIFLKTRRKVDWLTEKMHA	651.4	2230.13
Mdh2	36.3368	162	N	S	6.25	8.87	IPITAEVFKKHGVYSPNKIFGVTTLDIVR	1879.39	1791.06
Shc1	26.3216	201	R	L	0.55	5.42	AQDVISTIGQAFELLFKQYLRNPPKLVTP	68.02	113.17
Zyx	20.474	391	R	H	8.45	6.4	GKCNQPLARAQPAVHALGQLFHITCFTCH	9025.88	2304.73
Nmt1	13.6027	411	P	Q	2.85	3.27	FLSFYTLPTIMNHQTHKSLKAAYSFYNV	9148.29	1594.48
Ddx39	11.4983	164	C	F	1.8	9.95	GLSIKKDEDVLLKKNFPHVVVGTGPRILAL	1058.31	433.25
Pdk1	11.1528	337	A	S	0.7	1.06	LRKIDRLFNYMYSTSPRPRVETSRAVPLA	275.71	100.4
Ip6k1	7.12376	311	I	F	1	24.33	LHNGLDLRRDLFEPFLSKLRGLKAVLERQ	3119.73	794.53
Nup188	6.8105	326	W	C	0.9	17.59	TLGDIPHHAPVLLACALLRHTLSPEETSS	299.95	200.79
Sccpdh	6.39732	380	Q	H	0.4	3.12	GPEAGYVATPIAMVHAAMTFLSDASDLPK	17.78	103.93
Phyh	6.25987	267	G	V	3.5	7.97	GDTVFFHPLLIHGSRNKTQGFRKAISCH	11898.62	2446.92
Ttc7	6.03583	581	K	T	0.8	31.55	ANALHLLALLFSAQTYQHALDVINMAIT	55.33	1723.18
Mfsd1	6.03005	407	A	S	1	24.07	QSIQNLGLAVIAILSGMILDSKGYLLLEV	2174.92	1576.23
Smad5_p1	5.88487	402	A	S	1.6	9.92	FAQLLAQSVNHGFESVYELTKMCTIRMSF	115.66	375.61
Vcl	4.13678	323	G	A	1	49.96	VGELCAGKERREILATCKMLGQMTDQVAD	3724.77	1910.34
Pus10	3.53627	161	V	L	0.95	49.53	PPQLSVREHAAWLLLKQEMGKQSLSLGRN	4488.74	6430.58
Ep300	3.36595	434	A	T	1.5	9.89	NAGDKRNQQSILTGTVPVGLGNPSSLGVGQ	1277.72	1344.68
Xpo5	3.15794	446	V	L	9.4	4.93	FNAFFNSSRAQHGELVRCVCRLDPKTSFQ	1152.58	830.06

Parg	2.97412	516	V	L	0.3	46.3	KHVKMPCEQNLYPLEDENGERTAGSRWE	89.19	125.49
Nxt2	2.90821	109	F	V	0.2	14.86	VTSGVVKFDGNKQHVFNQNFLLTAQSTPN	144.33	4029.49
Odc1	2.50028	129	Q	L	0.2	4.62	KQVSQIKYAASNGVLMMTFDSEIELMKVA	9.09	58.29
Dlg1	2.41637	888	T	S	1.8	7.5	QGDTLEDIYNQVKQSIEEQSGPYIWVPAK	14517.03	2548.3
Hsf1	2.3602	204	G	W	0.85	17.2	IQFLISLVQSNRILWVKRKIPLMLSDSNS	498.88	228.62
Mylip	2.28997	398	T	I	0.95	48.68	AMLCMACCEEEINSIFCPCGHTVCCESCA	2461.05	509.03
Hmha1	2.07233	231	L	F	1.45	9.77	MPLLSIYSLALEQDFEFGHGMVQAAGTLQ	10967.29	2585.95
Adck4	2.04782	152	G	A	1	33.66	ANAERIVQTLCTVRAAALKIGQMLSIQDN	608.19	320.44
Rbm19	2.01243	461	V	L	1.2	9.76	TFMFPEHAVKAYAELDGQVFQGRMLHVLP	178.38	245.32
Topbp1	1.84552	792	R	I	0.4	19.12	KKAVTPLDMNRFQSIAFRAVISQQRGQDP	30.29	95
Cad	1.71896	1218	R	H	0.6	11.34	IAKDDQLKVICNVHVSRSFPFVSKTLGV	329.16	213.36
Mertk	1.51034	682	R	P	0.2	6.21	FMKYGDLHTFLLYSPLNTGPKYIHLQTL	13.08	13.42
Fars2	1.50528	262	R	L	0.5	9.2	TRLVTHLFGDGLVWLWDCYFPFTHPSFE	419.5	174.63
Pias3_p2	1.41663	552	Q	H	3.2	2.73	ITSLDEQDTLGHFFQYRGTPSHFLGPLAP	9806.13	8876.67
Dvl2	1.26082	385	A	T	0.4	2.99	IQPIDPAAWVSHSATLTGAFPAYPGSSSM	186.01	147.27
Zbtb17	1.15526	72	Q	K	2.6	8.12	DVVHLDISNAAGLGKVLFFMYTAKLSLSP	1279.37	1020.33
Mtor	1.15404	2040	G	W	4.5	9.14	MWHEGLEEASRLYFWERNVKGMFEVLEPL	1230.96	4419.06
Lama5_p1	1.13509	2201	S	F	0.3	8.79	LPAIREQLQGINASFAAWARLHRLNASIA	12.17	112.49
Lama5_p2	1.13509	897	G	V	0.15	30.57	LEEATPEGHAVRFVFNPLEFENFSWRGY	6.42	9.59
Thbs1	1.00996	1008	D	Y	1	21.39	DPGLAVGYDEFNAVYFSGTFFINTERDDD	5185.15	1493.93
Pogz	0.876428	1086	A	S	0.6	11.43	ARRAVAHTLPKHVASNAGLFIEFVQRQIH	4764.27	2416.85
E2f8	0.866666	272	K	R	0.15	1.42	VNSRKDKSLRVMSQRFVMLFLVSTPQIVS	10.7	18.52
Slc9a9	0.838487	560	R	W	5.2	5.7	PLTTTTLPAWCGPVSWLLTSPQAYGEQLKE	2471.16	354.09
Cdk20	0.806392	87	D	Y	0.15	6.96	HGAGFVLAFEFMLSYLEVVRHAQRPLAP	66.33	1205.95

Slc20a2	0.743388	182	A	S	2.1	2.37	FILTKEDPVPNGLQSLPLFYAATIINVF	182.12	676.25
Htt	0.673869	2151	V	M	0.8	8.79	ANGQKSPLFEAARGMILNRVTSVVQQLPA	1565.24	151.92
Bcl9l	0.65359	950	K	M	1.45	3.47	PQVLSSSLGVRSPMTSPSRKSPSMAVPS	12106	4287.95
Prkd2	0.461632	484	G	V	0.85	6.53	IITANVTYFVGETPVGAPGGPSGQTEAV	1480.87	2255.95
Irx2	0.448434	100	S	I	0.85	2.37	SPYDTHTTGMTGAIYHPYGSAAYPYQLN	147.8	325.92
Shank3p1	0.420463	1083	G	C	7.5	5.91	LLLPSVPSALKPLVCGPSLGPSTFIHP	27002.91	42817.37
Dnajb5	0.410686	114	S	I	0.8	7.47	SFFGGSNPFDIFFAISRSTRPFSGDPDD	1458.54	2261.15
Sh3yl1	0.340289	100	D	Y	0.2	19.59	AGLGGGFEIGIEVSYLVIIILNYDRAVEAF	21.36	30.67
Clip4	0.309077	610	S	Y	0.7	13.2	FAKTKTTLRRSWSSYTTAGGLEGTVKLHE	32.29	198.66
Slc41a1	0.282134	281	K	M	0.6	4.82	ALLSGISWGLYLELMHWRYIYPLVCAFFV	361.83	180.35
Cep135_p1	0.256196	705	E	K	0.85	48.62	RGELESAQEIQMLKQKLENLSHRMTVQS	5274.9	3106.35
Arap1	1.23205	439	G	V	5.2	3.03	ARFRLSSASVLGVRVSEQPDRAGSLELRG	4803.92	2803.75
Ntn1	0.903181	179	T	A	0.7	8.09	MDYGRTWVPPFQFYSAQCRKMYNRPHRAPI	412.74	104.41
Ldlr	1.1279	844	Q	K	5.9	9.76	YQKTEDELHICRSKDGTYPSRQMVLSLE	16979.45	10217.16
Fasn	4.74407	2422	A	S	0.45	32.7	ELSFAAVSFYHKLRSADQYKPKAKYHGNV	496.47	95.96
Slc17a5	2.05384	287	K	R	0.5	17.14	QLSSQKVVPWGSILRSLPLWAIVVAHFSY	93.55	132.35
Anks3	0.835212	82	D	Y	0.6	21.29	GGWTALMYASYIGHYTIVHLLLEAGVSVN	299.99	3525.74
Eif3d	27.2546	124	K	M	0.6	10.71	DRRNMVQFNLQTLPMASAKQKERERIRLQK	5191.77	16119.44
Topors	2.72797	488	L	F	0.8	8.52	SSSDNCVIVGFVKPFAERTPELVELSSDS	273.29	280.61
Espl1	0.685533	608	L	V	1.05	9.13	TGQERFNIICDLLEVSPEETAAGAWARAT	16374.14	10153.6

Appendix B

Permission was obtained for the reproduction of figures in accordance with journal or publisher policies. Chapter 1 contains figures and excerpts from Swartz et al., 2017, *Translational Immunotherapy for Brain Tumors* and Swartz et al., 2014, *Immunotherapy* and Swartz et al., 2018, *Expert Opinion on Biological Therapy*. Chapter 2 contains figures and excerpts from Swartz et al., 2017, *Translational Immunotherapy for Brain Tumors*. Chapter 3 contains tables and excerpts from Swartz et al., 2018, *Expert Opinion on Biological Therapy* and Swartz et al., 2015, *J Neurooncol*. Chapter 4 contains tables and excerpts from Swartz et al., 2014, *Immunotherapy*. Chapter 5 contains figures and excerpts from Swartz et al., 2018, *J Immunol Methods*. Chapter 6 contains unpublished data by the candidate, for which a manuscript has been drafted and is ready for submission. Chapter 7 contains figures from Batich K, Swartz A, et al., 2016. *Methods Mol Biol* and unpublished data by the candidate, for which a manuscript is in progress. Chapter 8 contains excerpts from Swartz et al., 2018, *Expert Opinion on Biological Therapy*.

References

- Agnihotri, S., Burrell, K. E., Wolf, A., Jalali, S., Hawkins, C., Rutka, J. T., & Zadeh, G. (2013). Glioblastoma, a brief review of history, molecular genetics, animal models and novel therapeutic strategies. *Arch Immunol Ther Exp (Warsz)*, 61(1), 25-41. doi:10.1007/s00005-012-0203-0
- Agrawal, S., Anderson, P., Durbeej, M., van Rooijen, N., Ivars, F., Opdenakker, G., & Sorokin, L. M. (2006). Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. *J Exp Med*, 203(4), 1007-1019. doi:10.1084/jem.20051342
- Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., & Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol*, 10(5), 619-624. doi:10.1038/ncb1725
- Alexandrov, L. B., Nik-Zainal, S., Wedge, D. C., Aparicio, S. A., Behjati, S., Biankin, A. V., . . . Stratton, M. R. (2013). Signatures of mutational processes in human cancer. *Nature*, 500(7463), 415-421. doi:10.1038/nature12477
- Aliberti, J., Schulz, O., Pennington, D. J., Tsujimura, H., Reis e Sousa, C., Ozato, K., & Sher, A. (2003). Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. *Blood*, 101(1), 305-310. doi:10.1182/blood-2002-04-1088
- Anandappa, A. J., Keskin, D. B., Hu, Z., Sun, J., Tirosh, I., Mathewson, N. D., . . . Neuberg, D. (2018). Detecting neoepitope-specific intra-tumoral T cell responses in a glioblastoma patient treated with personal neoantigen vaccine: Am Assoc Immunol.
- Andtbacka, R. H., Kaufman, H. L., Collichio, F., Amatruda, T., Senzer, N., Chesney, J., . . . Coffin, R. S. (2015). Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol*, 33(25), 2780-2788. doi:10.1200/JCO.2014.58.3377
- Anichini, A., Maccalli, C., Mortarini, R., Salvi, S., Mazzocchi, A., Squarcina, P., . . . Parmiani, G. (1993). Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. *J Exp Med*, 177(4), 989-998.

- Ashley, D. M., Faiola, B., Nair, S., Hale, L. P., Bigner, D. D., & Gilboa, E. (1997). Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. *J Exp Med*, *186*(7), 1177-1182.
- Auffinger, B., Spencer, D., Pytel, P., Ahmed, A. U., & Lesniak, M. S. (2015). The role of glioma stem cells in chemotherapy resistance and glioblastoma multiforme recurrence. *Expert Rev Neurother*, *15*(7), 741-752. doi:10.1586/14737175.2015.1051968
- Bajana, S., Turner, S., Paul, J., Ainsua-Enrich, E., & Kovats, S. (2016). IRF4 and IRF8 Act in CD11c+ Cells To Regulate Terminal Differentiation of Lung Tissue Dendritic Cells. *J Immunol*, *196*(4), 1666-1677. doi:10.4049/jimmunol.1501870
- Ballesteros-Tato, A., Leon, B., Lund, F. E., & Randall, T. D. (2013). CD4+ T helper cells use CD154-CD40 interactions to counteract T reg cell-mediated suppression of CD8+ T cell responses to influenza. *J Exp Med*, *210*(8), 1591-1601. doi:10.1084/jem.20130097
- Bartholomaeus, I., Kawakami, N., Odoardi, F., Schlager, C., Miljkovic, D., Ellwart, J. W., . . . Flugel, A. (2009). Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature*, *462*(7269), 94-98. doi:10.1038/nature08478
- Batich, K. A., Reap, E. A., Archer, G. E., Sanchez-Perez, L., Nair, S. K., Schmittling, R. J., . . . Sampson, J. H. (2017). Long-term Survival in Glioblastoma with Cytomegalovirus pp65-Targeted Vaccination. *Clin Cancer Res*, *23*(8), 1898-1909. doi:10.1158/1078-0432.CCR-16-2057
- Batich, K. A., Swartz, A. M., & Sampson, J. H. (2016). Preconditioning Vaccine Sites for mRNA-Transfected Dendritic Cell Therapy and Antitumor Efficacy. *Methods Mol Biol*, *1403*, 819-838. doi:10.1007/978-1-4939-3387-7_47
- Batra, S. K., Castelino-Prabhu, S., Wikstrand, C. J., Zhu, X., Humphrey, P. A., Friedman, H. S., & Bigner, D. D. (1995). Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ*, *6*(10), 1251-1259.
- Baumgarten, P., Michaelis, M., Rothweiler, F., Starzetz, T., Rabenau, H. F., Berger, A., . . . Cinatl, J., Jr. (2014). Human cytomegalovirus infection in tumor cells of the

nervous system is not detectable with standardized pathologico-virological diagnostics. *Neuro Oncol*, 16(11), 1469-1477. doi:10.1093/neuonc/nou167

Belmans, J., Van Woensel, M., Creyns, B., Dejaegher, J., Bullens, D. M., & Van Gool, S. W. (2017). Immunotherapy with subcutaneous immunogenic autologous tumor lysate increases murine glioblastoma survival. *Sci Rep*, 7(1), 13902. doi:10.1038/s41598-017-12584-0

Blaylock, R. L. (2013). Immunology primer for neurosurgeons and neurologists part 2: Innate brain immunity. *Surg Neurol Int*, 4, 118. doi:10.4103/2152-7806.118349

Boockvar, J. A., Kapitonov, D., Kapoor, G., Schouten, J., Counelis, G. J., Bogler, O., . . . O'Rourke, D. M. (2003). Constitutive EGFR signaling confers a motile phenotype to neural stem cells. *Mol Cell Neurosci*, 24(4), 1116-1130.

Brahmer, J. R., Drake, C. G., Wollner, I., Powderly, J. D., Picus, J., Sharfman, W. H., . . . Topalian, S. L. (2010). Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol*, 28(19), 3167-3175. doi:10.1200/JCO.2009.26.7609

Brander, C., Corradin, G., Hasler, T., & Pichler, W. J. (1996). Peptide immunization in humans: a combined CD8+/CD4+ T cell-targeted vaccine restimulates the memory CD4 T cell response but fails to induce cytotoxic T lymphocytes (CTL). *Clin Exp Immunol*, 105(1), 18-25.

Brawand, P., Fitzpatrick, D. R., Greenfield, B. W., Brasel, K., Maliszewski, C. R., & De Smedt, T. (2002). Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol*, 169(12), 6711-6719.

Brem, H., Piantadosi, S., Burger, P. C., Walker, M., Selker, R., Vick, N. A., . . . et al. (1995). Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group. *Lancet*, 345(8956), 1008-1012.

Brennan, C. W., Verhaak, R. G., McKenna, A., Campos, B., Noushmehr, H., Salama, S. R., . . . Network, T. R. (2013). The somatic genomic landscape of glioblastoma. *Cell*, 155(2), 462-477. doi:10.1016/j.cell.2013.09.034

- Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., De Plaen, E., Lethe, B., . . . Boon, T. (1993). The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med*, 178(2), 489-495.
- Brown, M. C., Holl, E. K., Boczkowski, D., Dobrikova, E., Mosaheb, M., Chandramohan, V., . . . Nair, S. K. (2017). Cancer immunotherapy with recombinant poliovirus induces IFN-dominant activation of dendritic cells and tumor antigen-specific CTLs. *Sci Transl Med*, 9(408). doi:10.1126/scitranslmed.aan4220
- Brunet, J. F., Denizot, F., Luciani, M. F., Roux-Dosseto, M., Suzan, M., Mattei, M. G., & Golstein, P. (1987). A new member of the immunoglobulin superfamily--CTLA-4. *Nature*, 328(6127), 267-270. doi:10.1038/328267a0
- Bunse, L., Schumacher, T., Sahm, F., Pusch, S., Oezen, I., Rauschenbach, K., . . . Platten, M. (2015). Proximity ligation assay evaluates IDH1R132H presentation in gliomas. *J Clin Invest*, 125(2), 593-606. doi:10.1172/JCI77780
- Burnet, M. (1957). Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *Br Med J*, 1(5023), 841-847.
- Calarota, S. A., & Baldanti, F. (2013). Enumeration and characterization of human memory T cells by enzyme-linked immunospot assays. *Clin Dev Immunol*, 2013, 637649. doi:10.1155/2013/637649
- Calzascia, T., Masson, F., Di Bernardino-Besson, W., Contassot, E., Wilmotte, R., Aurrand-Lions, M., . . . Walker, P. R. (2005). Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. *Immunity*, 22(2), 175-184. doi:10.1016/j.immuni.2004.12.008
- Carrithers, M. D., Visintin, I., Kang, S. J., & Janeway, C. A., Jr. (2000). Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment. *Brain*, 123 (Pt 6), 1092-1101.
- Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D., & Ploix, C. C. (2006). CNS immune privilege: hiding in plain sight. *Immunol Rev*, 213, 48-65. doi:10.1111/j.1600-065X.2006.00441.x
- Carter, H., Chen, S., Isik, L., Tyekucheva, S., Velculescu, V. E., Kinzler, K. W., . . . Karchin, R. (2009). Cancer-specific high-throughput annotation of somatic

- mutations: computational prediction of driver missense mutations. *Cancer Res*, 69(16), 6660-6667. doi:10.1158/0008-5472.CAN-09-1133
- Castle, J. C., Kreiter, S., Diekmann, J., Lower, M., van de Roemer, N., de Graaf, J., . . . Sahin, U. (2012). Exploiting the mutanome for tumor vaccination. *Cancer Res*, 72(5), 1081-1091. doi:10.1158/0008-5472.CAN-11-3722
- Castro, M. G., Candolfi, M., Wilson, T. J., Calinescu, A., Paran, C., Kamran, N., . . . Lowenstein, P. R. (2014). Adenoviral vector-mediated gene therapy for gliomas: coming of age. *Expert Opin Biol Ther*, 14(9), 1241-1257. doi:10.1517/14712598.2014.915307
- Chamberlain, M. C. (2011). Bevacizumab for the treatment of recurrent glioblastoma. *Clin Med Insights Oncol*, 5, 117-129. doi:10.4137/CMO.S7232
- Chiocca, E. A. (2002). Oncolytic viruses. *Nat Rev Cancer*, 2(12), 938-950. doi:10.1038/nrc948
- Cho, H. I., Barrios, K., Lee, Y. R., Linowski, A. K., & Celis, E. (2013). BiVax: a peptide/poly-IC subunit vaccine that mimics an acute infection elicits vast and effective anti-tumor CD8 T-cell responses. *Cancer Immunol Immunother*, 62(4), 787-799. doi:10.1007/s00262-012-1382-6
- Cloughesy, T. F., Landolfi, J., Hogan, D. J., Bloomfield, S., Carter, B., Chen, C. C., . . . Vogelbaum, M. A. (2016). Phase 1 trial of vocimagene amiretrorepvec and 5-fluorocytosine for recurrent high-grade glioma. *Sci Transl Med*, 8(341), 341ra375. doi:10.1126/scitranslmed.aad9784
- Cloughesy, T. F., Landolfi, J., Vogelbaum, M. A., Ostertag, D., Elder, J. B., Bloomfield, S., . . . Walbert, T. (2018). Durable Complete Responses in Some Recurrent High Grade Glioma Patients Treated with Toca 511 & Toca FC. *Neuro Oncol*. doi:10.1093/neuonc/noy075
- Cobbs, C. S., Harkins, L., Samanta, M., Gillespie, G. Y., Bharara, S., King, P. H., . . . Britt, W. J. (2002). Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res*, 62(12), 3347-3350.
- Coley, W. B. (1910). The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). *Proc R Soc Med*, 3(Surg Sect), 1-48.

- Conrady, C. D., Drevets, D. A., & Carr, D. J. (2010). Herpes simplex type I (HSV-1) infection of the nervous system: is an immune response a good thing? *J Neuroimmunol*, 220(1-2), 1-9. doi:10.1016/j.jneuroim.2009.09.013
- Curtin, J. F., Liu, N., Candolfi, M., Xiong, W., Assi, H., Yagiz, K., . . . Castro, M. G. (2009). HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med*, 6(1), e10. doi:10.1371/journal.pmed.1000010
- Czerkinsky, C., Andersson, G., Ekre, H. P., Nilsson, L. A., Klareskog, L., & Ouchterlony, O. (1988). Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma-interferon-secreting cells. *J Immunol Methods*, 110(1), 29-36.
- Dandy, W. E. (1928). Removal of right cerebral hemisphere for certain tumors with hemiplegia: Preliminary report. *Journal of the American Medical Association*, 90(11), 823-825.
- Darmon, I., Morisse, M. C., Coutte, A., Blonski, M., Le Rhun, E., Taillandier, L., . . . Boone, M. (2017). Temozolomide and Bevacizumab Induction before Chemoradiotherapy in Patients with Bulky Glioblastoma and/or with Severe Neurological Impairment. *J Cancer*, 8(8), 1417-1424. doi:10.7150/jca.18339
- De Munck, J., Binks, A., McNeish, I. A., & Aerts, J. L. (2017). Oncolytic virus-induced cell death and immunity: a match made in heaven? *J Leukoc Biol*, 102(3), 631-643. doi:10.1189/jlb.5RU0117-040R
- Desch, A. N., Gibbings, S. L., Clambey, E. T., Janssen, W. J., Slansky, J. E., Kedl, R. M., . . . Jakubzick, C. (2014). Dendritic cell subsets require cis-activation for cytotoxic CD8 T-cell induction. *Nat Commun*, 5, 4674. doi:10.1038/ncomms5674
- Desjardins, A., Gromeier, M., Herndon, J. E., 2nd, Beaubier, N., Bolognesi, D. P., Friedman, A. H., . . . Bigner, D. D. (2018). Recurrent Glioblastoma Treated with Recombinant Poliovirus. *N Engl J Med*, 379(2), 150-161. doi:10.1056/NEJMoa1716435
- Dey, M., Chang, A. L., Miska, J., Wainwright, D. A., Ahmed, A. U., Balyasnikova, I. V., . . . Lesniak, M. S. (2015). Dendritic Cell-Based Vaccines that Utilize Myeloid Rather than Plasmacytoid Cells Offer a Superior Survival Advantage in Malignant Glioma. *J Immunol*, 195(1), 367-376. doi:10.4049/jimmunol.1401607

- Dhodapkar, M. V., Steinman, R. M., Krasovsky, J., Munz, C., & Bhardwaj, N. (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med*, 193(2), 233-238.
- Di Tomaso, T., Mazzoleni, S., Wang, E., Sovenia, G., Clavenna, D., Franzin, A., . . . Maccalli, C. (2010). Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. *Clin Cancer Res*, 16(3), 800-813. doi:10.1158/1078-0432.CCR-09-2730
- Dittrich, M., & Lehmann, P. V. (2012). Statistical analysis of ELISPOT assays. *Methods Mol Biol*, 792, 173-183. doi:10.1007/978-1-61779-325-7_14
- Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., . . . Mulligan, R. C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A*, 90(8), 3539-3543.
- Dudley, M. E., Wunderlich, J. R., Robbins, P. F., Yang, J. C., Hwu, P., Schwartzentruber, D. J., . . . Rosenberg, S. A. (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*, 298(5594), 850-854. doi:10.1126/science.1076514
- Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annu Rev Immunol*, 22, 329-360. doi:10.1146/annurev.immunol.22.012703.104803
- Ebrahimi-Nik, H., Corwin, W. L., Shcheglova, T., Das Mohapatra, A., Mandoiu, II, & Srivastava, P. K. (2018). CD11c(+) MHCII(lo) GM-CSF-bone marrow-derived dendritic cells act as antigen donor cells and as antigen presenting cells in neoepitope-elicited tumor immunity against a mouse fibrosarcoma. *Cancer Immunol Immunother*, 67(9), 1449-1459. doi:10.1007/s00262-018-2202-4
- Ekstrand, A. J., Longo, N., Hamid, M. L., Olson, J. J., Liu, L., Collins, V. P., & James, C. D. (1994). Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene*, 9(8), 2313-2320.
- Engelhardt, B., & Ransohoff, R. M. (2012). Capture, crawl, cross: the T cell code to breach the blood-brain barriers. *Trends Immunol*, 33(12), 579-589. doi:10.1016/j.it.2012.07.004

- Engelhardt, B., & Wolburg, H. (2004). Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol*, 34(11), 2955-2963. doi:10.1002/eji.200425327
- Evel-Kabler, K., & Chen, S. Y. (2006). Dendritic cell-based tumor vaccines and antigen presentation attenuators. *Mol Ther*, 13(5), 850-858. doi:10.1016/j.ymthe.2006.02.009
- Faiola, B., Doyle, C., Gilboa, E., & Nair, S. (2002). Influence of CD4 T cells and the source of major histocompatibility complex class II-restricted peptides on cytotoxic T-cell priming by dendritic cells. *Immunology*, 105(1), 47-55.
- Fenstermaker, R. A., Ciesielski, M. J., Qiu, J., Yang, N., Frank, C. L., Lee, K. P., . . . Hutson, A. D. (2016). Clinical study of a survivin long peptide vaccine (SurVaxM) in patients with recurrent malignant glioma. *Cancer Immunol Immunother*, 65(11), 1339-1352. doi:10.1007/s00262-016-1890-x
- Fiorentino, D. F., Bond, M. W., & Mosmann, T. R. (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*, 170(6), 2081-2095.
- Foreman, P. M., Friedman, G. K., Cassady, K. A., & Markert, J. M. (2017). Oncolytic Virotherapy for the Treatment of Malignant Glioma. *Neurotherapeutics*, 14(2), 333-344. doi:10.1007/s13311-017-0516-0
- Frederick, L., Eley, G., Wang, X. Y., & James, C. D. (2000). Analysis of genomic rearrangements associated with EGFRvIII expression suggests involvement of Alu repeat elements. *Neuro Oncol*, 2(3), 159-163.
- Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., . . . Honjo, T. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*, 192(7), 1027-1034.
- Friedman, A. H., Friedman, H. S., & McLendon, R. (2016). *The Duke Glioma Handbook*: Cambridge University Press.
- Friedman, H. S., Prados, M. D., Wen, P. Y., Mikkelsen, T., Schiff, D., Abrey, L. E., . . . Cloughesy, T. (2009). Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. *J Clin Oncol*, 27(28), 4733-4740. doi:10.1200/JCO.2008.19.8721

- Fueyo, J., Alemany, R., Gomez-Manzano, C., Fuller, G. N., Khan, A., Conrad, C. A., . . . Lang, F. F. (2003). Preclinical characterization of the antiglioma activity of a tropism-enhanced adenovirus targeted to the retinoblastoma pathway. *J Natl Cancer Inst*, 95(9), 652-660.
- Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P. S., McDonnell, T. J., Mitlianga, P., . . . Kyritsis, A. P. (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. *Oncogene*, 19(1), 2-12. doi:10.1038/sj.onc.1203251
- Fuge, O., Vasdev, N., Allchorne, P., & Green, J. S. (2015). Immunotherapy for bladder cancer. *Res Rep Urol*, 7, 65-79. doi:10.2147/RRU.S63447
- Fukumura, D., Xu, L., Chen, Y., Gohongi, T., Seed, B., & Jain, R. K. (2001). Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. *Cancer Res*, 61(16), 6020-6024.
- Gaugler, B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J. J., De Plaen, E., . . . Boon, T. (1994). Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med*, 179(3), 921-930.
- Gil-Gil, M. J., Mesia, C., Rey, M., & Bruna, J. (2013). Bevacizumab for the treatment of glioblastoma. *Clin Med Insights Oncol*, 7, 123-135. doi:10.4137/CMO.S8503
- Gilboa, E., Lyerly, H. K., Vieweg, J., & Saito, S. (1994). Immunotherapy of cancer using cytokine gene-modified tumor vaccines. *Semin Cancer Biol*, 5(6), 409-417.
- Giles, A. J., Hutchinson, M. N. D., Sonnemann, H. M., Jung, J., Fecci, P. E., Ratnam, N. M., . . . Gilbert, M. R. (2018). Dexamethasone-induced immunosuppression: mechanisms and implications for immunotherapy. *J Immunother Cancer*, 6(1), 51. doi:10.1186/s40425-018-0371-5
- Grandi, P., Peruzzi, P., Reinhart, B., Cohen, J. B., Chiocca, E. A., & Glorioso, J. C. (2009). Design and application of oncolytic HSV vectors for glioblastoma therapy. *Expert Rev Neurother*, 9(4), 505-517. doi:10.1586/ern.09.9
- Gromeier, M., Alexander, L., & Wimmer, E. (1996). Internal ribosomal entry site substitution eliminates neurovirulence in intergeneric poliovirus recombinants. *Proc Natl Acad Sci U S A*, 93(6), 2370-2375.

- Gubin, M. M., Zhang, X., Schuster, H., Caron, E., Ward, J. P., Noguchi, T., . . . Schreiber, R. D. (2014). Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature*, *515*(7528), 577-581. doi:10.1038/nature13988
- Guo, Z. S., Liu, Z., Kowalsky, S., Feist, M., Kalinski, P., Lu, B., . . . Bartlett, D. L. (2017). Oncolytic Immunotherapy: Conceptual Evolution, Current Strategies, and Future Perspectives. *Front Immunol*, *8*, 555. doi:10.3389/fimmu.2017.00555
- Han, J., Alvarez-Breckenridge, C. A., Wang, Q. E., & Yu, J. (2015). TGF-beta signaling and its targeting for glioma treatment. *Am J Cancer Res*, *5*(3), 945-955.
- Han, S., Zhang, C., Li, Q., Dong, J., Liu, Y., Huang, Y., . . . Wu, A. (2014). Tumour-infiltrating CD4(+) and CD8(+) lymphocytes as predictors of clinical outcome in glioma. *Br J Cancer*, *110*(10), 2560-2568. doi:10.1038/bjc.2014.162
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674. doi:10.1016/j.cell.2011.02.013
- Harris, M. G., Hulseberg, P., Ling, C., Karman, J., Clarkson, B. D., Harding, J. S., . . . Fabry, Z. (2014). Immune privilege of the CNS is not the consequence of limited antigen sampling. *Sci Rep*, *4*, 4422. doi:10.1038/srep04422
- Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., . . . Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med*, *194*(6), 769-779.
- Hegi, M. E., Diserens, A. C., Gorlia, T., Hamou, M. F., de Tribolet, N., Weller, M., . . . Stupp, R. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*, *352*(10), 997-1003. doi:10.1056/NEJMoa043331
- Heimberger, A. B., Archer, G. E., Crotty, L. E., McLendon, R. E., Friedman, A. H., Friedman, H. S., . . . Sampson, J. H. (2002). Dendritic cells pulsed with a tumor-specific peptide induce long-lasting immunity and are effective against murine intracerebral melanoma. *Neurosurgery*, *50*(1), 158-164; discussion 164-156.
- Heimberger, A. B., Crotty, L. E., Archer, G. E., Hess, K. R., Wikstrand, C. J., Friedman, A. H., . . . Sampson, J. H. (2003). Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin Cancer Res*, *9*(11), 4247-4254.

- Heimberger, A. B., & Sampson, J. H. (2011). Immunotherapy coming of age: what will it take to make it standard of care for glioblastoma? *Neuro Oncol*, *13*(1), 3-13. doi:10.1093/neuonc/noq169
- Heimberger, A. B., Suki, D., Yang, D., Shi, W., & Aldape, K. (2005). The natural history of EGFR and EGFRvIII in glioblastoma patients. *J Transl Med*, *3*, 38. doi:10.1186/1479-5876-3-38
- Hesse, M. D., Karulin, A. Y., Boehm, B. O., Lehmann, P. V., & Tary-Lehmann, M. (2001). A T cell clone's avidity is a function of its activation state. *J Immunol*, *167*(3), 1353-1361.
- Hickey, W. F. (1991). Migration of hematogenous cells through the blood-brain barrier and the initiation of CNS inflammation. *Brain Pathol*, *1*(2), 97-105.
- Hinrichs, C. S., Palmer, D. C., Rosenberg, S. A., & Restifo, N. P. (2005). Glucocorticoids do not inhibit antitumor activity of activated CD8+ T cells. *J Immunother*, *28*(6), 517-524.
- Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., . . . Urban, W. J. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*, *363*(8), 711-723. doi:10.1056/NEJMoa1003466
- Holm, G. H., Zurney, J., Tumilasci, V., Leveille, S., Danthi, P., Hiscott, J., . . . Dermody, T. S. (2007). Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3. *J Biol Chem*, *282*(30), 21953-21961. doi:10.1074/jbc.M702112200
- Hou, L. C., Veeravagu, A., Hsu, A. R., & Tse, V. C. (2006). Recurrent glioblastoma multiforme: a review of natural history and management options. *Neurosurg Focus*, *20*(4), E5.
- Huang, H. S., Nagane, M., Klingbeil, C. K., Lin, H., Nishikawa, R., Ji, X. D., . . . Cavenee, W. K. (1997). The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem*, *272*(5), 2927-2935.

- Ikehara, S., Pahwa, R. N., Fernandes, G., Hansen, C. T., & Good, R. A. (1984). Functional T cells in athymic nude mice. *Proc Natl Acad Sci U S A*, 81(3), 886-888.
- Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., . . . Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med*, 176(6), 1693-1702.
- Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J*, 11(11), 3887-3895.
- Janetzki, S., Price, L., Schroeder, H., Britten, C. M., Welters, M. J., & Hoos, A. (2015). Guidelines for the automated evaluation of Elispot assays. *Nat Protoc*, 10(7), 1098-1115. doi:10.1038/nprot.2015.068
- Jiang, H., Clise-Dwyer, K., Ruisaard, K. E., Fan, X., Tian, W., Gumin, J., . . . Fueyo, J. (2014). Delta-24-RGD oncolytic adenovirus elicits anti-glioma immunity in an immunocompetent mouse model. *PLoS One*, 9(5), e97407. doi:10.1371/journal.pone.0097407
- Jiang, H., Gomez-Manzano, C., Lang, F. F., Alemany, R., & Fueyo, J. (2009). Oncolytic adenovirus: preclinical and clinical studies in patients with human malignant gliomas. *Curr Gene Ther*, 9(5), 422-427.
- Johanns, T. M., Ward, J. P., Miller, C. A., Wilson, C., Kobayashi, D. K., Bender, D., . . . Dunn, G. P. (2016). Endogenous Neoantigen-Specific CD8 T Cells Identified in Two Glioblastoma Models Using a Cancer Immunogenomics Approach. *Cancer Immunol Res*, 4(12), 1007-1015. doi:10.1158/2326-6066.CIR-16-0156
- Johnson, B. E., Mazon, T., Hong, C., Barnes, M., Aihara, K., McLean, C. Y., . . . Costello, J. F. (2014). Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science*, 343(6167), 189-193. doi:10.1126/science.1239947
- Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., . . . Enk, A. H. (1997). Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol*, 27(12), 3135-3142. doi:10.1002/eji.1830271209

- Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., & Enk, A. H. (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med*, 192(9), 1213-1222.
- Jordan, J. T., Sun, W., Hussain, S. F., DeAngulo, G., Prabhu, S. S., & Heimberger, A. B. (2008). Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy. *Cancer Immunol Immunother*, 57(1), 123-131. doi:10.1007/s00262-007-0336-x
- Kajiwara, Y., Yamasaki, F., Hama, S., Yahara, K., Yoshioka, H., Sugiyama, K., . . . Kurisu, K. (2003). Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis. *Cancer*, 97(4), 1077-1083. doi:10.1002/cncr.11122
- Kammertoens, T., & Blankenstein, T. (2013). It's the peptide-MHC affinity, stupid. *Cancer Cell*, 23(4), 429-431. doi:10.1016/j.ccr.2013.04.004
- Kappler, J. W., Roehm, N., & Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell*, 49(2), 273-280.
- King, G. D., Muhammad, A. K., Curtin, J. F., Barcia, C., Puntel, M., Liu, C., . . . Castro, M. G. (2008). Flt3L and TK gene therapy eradicate multifocal glioma in a syngeneic glioblastoma model. *Neuro Oncol*, 10(1), 19-31. doi:10.1215/15228517-2007-045
- King, G. D., Muhammad, A. K., Larocque, D., Kelson, K. R., Xiong, W., Liu, C., . . . Lowenstein, P. R. (2011). Combined Flt3L/TK gene therapy induces immunological surveillance which mediates an immune response against a surrogate brain tumor neoantigen. *Mol Ther*, 19(10), 1793-1801. doi:10.1038/mt.2011.77
- Kranz, L. M., Diken, M., Haas, H., Kreiter, S., Loquai, C., Reuter, K. C., . . . Sahin, U. (2016). Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature*, 534(7607), 396-401. doi:10.1038/nature18300
- Kreer, C., Rauen, J., Zehner, M., & Burgdorf, S. (2011). Cross-presentation: how to get there - or how to get the ER. *Front Immunol*, 2, 87. doi:10.3389/fimmu.2011.00087
- Kreisl, T. N., Zhang, W., Odia, Y., Shih, J. H., Butman, J. A., Hammoud, D., . . . Fine, H. A. (2011). A phase II trial of single-agent bevacizumab in patients with recurrent anaplastic glioma. *Neuro Oncol*, 13(10), 1143-1150. doi:10.1093/neuonc/nor091

- Kreiter, S., Selmi, A., Diken, M., Sebastian, M., Osterloh, P., Schild, H., . . . Sahin, U. (2008). Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. *J Immunol*, *180*(1), 309-318.
- Kreiter, S., Vormehr, M., van de Roemer, N., Diken, M., Lower, M., Diekmann, J., . . . Sahin, U. (2015). Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature*, *520*(7549), 692-696. doi:10.1038/nature14426
- Kruys, V., Wathelet, M., Poupart, P., Contreras, R., Fiers, W., Content, J., & Huez, G. (1987). The 3' untranslated region of the human interferon-beta mRNA has an inhibitory effect on translation. *Proc Natl Acad Sci U S A*, *84*(17), 6030-6034.
- Kumar, A., Boyle, E. A., Tokita, M., Mikheev, A. M., Sanger, M. C., Girard, E., . . . Shendure, J. (2014). Deep sequencing of multiple regions of glial tumors reveals spatial heterogeneity for mutations in clinically relevant genes. *Genome Biol*, *15*(12), 530. doi:10.1186/s13059-014-0530-z
- Kushwah, R., & Hu, J. (2011). Role of dendritic cells in the induction of regulatory T cells. *Cell Biosci*, *1*(1), 20. doi:10.1186/2045-3701-1-20
- La Rosa, C., Longmate, J., Lacey, S. F., Kaltcheva, T., Sharan, R., Marsano, D., . . . Zaia, J. A. (2012). Clinical evaluation of safety and immunogenicity of PADRE-cytomegalovirus (CMV) and tetanus-CMV fusion peptide vaccines with or without PF03512676 adjuvant. *J Infect Dis*, *205*(8), 1294-1304. doi:10.1093/infdis/jis107
- Lafon, M., Megret, F., Lafage, M., & Prehaud, C. (2006). The innate immune facet of brain: human neurons express TLR-3 and sense viral dsRNA. *J Mol Neurosci*, *29*(3), 185-194. doi:10.1385/JMN:29:3:185
- Lai, R. K., Recht, L. D., Reardon, D. A., Paleologos, N., Groves, M., Rosenfeld, M. R., . . . Sampson, J. (2011). Long-term Follow-up of ACT III: A Phase II Trial of Rindopepimut (CDX-110) in Newly Diagnosed Glioblastoma. *Neuro-oncology*, *13*(Suppl 3), iii34–iii40.
- Lammering, G., Valerie, K., Lin, P. S., Hewit, T. H., & Schmidt-Ullrich, R. K. (2004). Radiation-induced activation of a common variant of EGFR confers enhanced radioresistance. *Radiother Oncol*, *72*(3), 267-273. doi:10.1016/j.radonc.2004.07.004

- Lampron, A., Elali, A., & Rivest, S. (2013). Innate immunity in the CNS: redefining the relationship between the CNS and Its environment. *Neuron*, 78(2), 214-232. doi:10.1016/j.neuron.2013.04.005
- Lang, F. F., Conrad, C., Gomez-Manzano, C., Yung, W. K. A., Sawaya, R., Weinberg, J. S., . . . Fueyo, J. (2018). Phase I Study of DNX-2401 (Delta-24-RGD) Oncolytic Adenovirus: Replication and Immunotherapeutic Effects in Recurrent Malignant Glioma. *J Clin Oncol*, 36(14), 1419-1427. doi:10.1200/JCO.2017.75.8219
- Latchman, Y., Wood, C. R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., . . . Freeman, G. J. (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*, 2(3), 261-268. doi:10.1038/85330
- Le, D. T., Uram, J. N., Wang, H., Bartlett, B. R., Kemberling, H., Eyring, A. D., . . . Diaz, L. A., Jr. (2015). PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med*, 372(26), 2509-2520. doi:10.1056/NEJMoa1500596
- Liau, L. M., Ashkan, K., Tran, D. D., Campian, J. L., Trusheim, J. E., Cobbs, C. S., . . . Bosch, M. L. (2018). First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *J Transl Med*, 16(1), 142. doi:10.1186/s12967-018-1507-6
- Litterman, A. J., Zellmer, D. M., Grinnen, K. L., Hunt, M. A., Dudek, A. Z., Salazar, A. M., & Ohlfest, J. R. (2013). Profound impairment of adaptive immune responses by alkylating chemotherapy. *J Immunol*, 190(12), 6259-6268. doi:10.4049/jimmunol.1203539
- Llopiz, D., Aranda, F., Diaz-Valdes, N., Ruiz, M., Infante, S., Belsue, V., . . . Sarobe, P. (2016). Vaccine-induced but not tumor-derived Interleukin-10 dictates the efficacy of Interleukin-10 blockade in therapeutic vaccination. *Oncoimmunology*, 5(2), e1075113. doi:10.1080/2162402X.2015.1075113
- Llopiz, D., Huarte, E., Ruiz, M., Bezunartea, J., Belsue, V., Zabaleta, A., . . . Sarobe, P. (2013). Helper cell-independent antitumor activity of potent CD8(+) T cell epitope peptide vaccines is dependent upon CD40L. *Oncoimmunology*, 2(12), e27009. doi:10.4161/onci.27009
- Louveau, A., Harris, T. H., & Kipnis, J. (2015). Revisiting the Mechanisms of CNS Immune Privilege. *Trends Immunol*, 36(10), 569-577. doi:10.1016/j.it.2015.08.006

- Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., . . . Kipnis, J. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature*, *523*(7560), 337-341. doi:10.1038/nature14432
- Lu, J., Higashimoto, Y., Appella, E., & Celis, E. (2004). Multiepitope Trojan antigen peptide vaccines for the induction of antitumor CTL and Th immune responses. *J Immunol*, *172*(7), 4575-4582.
- Mahnke, K., Qian, Y., Knop, J., & Enk, A. H. (2003). Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood*, *101*(12), 4862-4869. doi:10.1182/blood-2002-10-3229
- Mailliard, R. B., Wankowicz-Kalinska, A., Cai, Q., Wesa, A., Hilkens, C. M., Kapsenberg, M. L., . . . Kalinski, P. (2004). alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res*, *64*(17), 5934-5937. doi:10.1158/0008-5472.CAN-04-1261
- Maleckar, J. R., & Sherman, L. A. (1987). The composition of the T cell receptor repertoire in nude mice. *J Immunol*, *138*(11), 3873-3876.
- Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., & Coen, D. M. (1991). Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science*, *252*(5007), 854-856.
- Mason, D. W., Charlton, H. M., Jones, A. J., Lavy, C. B., Puklavec, M., & Simmonds, S. J. (1986). The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. *Neuroscience*, *19*(3), 685-694.
- Mbongue, J. C., Nicholas, D. A., Torrez, T. W., Kim, N. S., Firek, A. F., & Langridge, W. H. (2015). The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines (Basel)*, *3*(3), 703-729. doi:10.3390/vaccines3030703
- Medawar, P. B. (1948). Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol*, *29*(1), 58-69.
- Mitchell, D. A., Batich, K. A., Gunn, M. D., Huang, M. N., Sanchez-Perez, L., Nair, S. K., . . . Sampson, J. H. (2015). Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature*, *519*(7543), 366-369. doi:10.1038/nature14320

- Mitchell, D. A., Cui, X., Schmittling, R. J., Sanchez-Perez, L., Snyder, D. J., Congdon, K. L., . . . Sampson, J. H. (2011). Monoclonal antibody blockade of IL-2 receptor alpha during lymphopenia selectively depletes regulatory T cells in mice and humans. *Blood*, *118*(11), 3003-3012. doi:10.1182/blood-2011-02-334565
- Mitchell, D. A., Nair, S. K., & Gilboa, E. (1998). Dendritic cell/macrophage precursors capture exogenous antigen for MHC class I presentation by dendritic cells. *Eur J Immunol*, *28*(6), 1923-1933. doi:10.1002/(SICI)1521-4141(199806)28:06<1923::AID-IMMU1923>3.0.CO;2-9
- Mitchell, D. A., Xie, W., Schmittling, R., Learn, C., Friedman, A., McLendon, R. E., & Sampson, J. H. (2008). Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. *Neuro Oncol*, *10*(1), 10-18. doi:10.1215/15228517-2007-035
- Mitchell, L. A., Lopez Espinoza, F., Mendoza, D., Kato, Y., Inagaki, A., Hiraoka, K., . . . Robbins, J. M. (2017). Toca 511 gene transfer and treatment with the prodrug, 5-fluorocytosine, promotes durable antitumor immunity in a mouse glioma model. *Neuro Oncol*, *19*(7), 930-939. doi:10.1093/neuonc/nox037
- Mognol, G. P., Spreafico, R., Wong, V., Scott-Browne, J. P., Togher, S., Hoffmann, A., . . . Trifari, S. (2017). Exhaustion-associated regulatory regions in CD8(+) tumor-infiltrating T cells. *Proc Natl Acad Sci U S A*, *114*(13), E2776-E2785. doi:10.1073/pnas.1620498114
- Montgomery, R. B., Guzman, J., O'Rourke, D. M., & Stahl, W. L. (2000). Expression of oncogenic epidermal growth factor receptor family kinases induces paclitaxel resistance and alters beta-tubulin isotype expression. *J Biol Chem*, *275*(23), 17358-17363. doi:10.1074/jbc.M000966200
- Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., . . . Wong, A. J. (1995). Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res*, *55*(23), 5536-5539.
- Mulder, W. M., Koenen, H., van de Muysenberg, A. J., Bloemena, E., Wagstaff, J., & Scheper, R. J. (1994). Reduced expression of distinct T-cell CD molecules by collagenase/DNase treatment. *Cancer Immunol Immunother*, *38*(4), 253-258.
- Mumberg, D., Monach, P. A., Wanderling, S., Philip, M., Toledano, A. Y., Schreiber, R. D., & Schreiber, H. (1999). CD4(+) T cells eliminate MHC class II-negative cancer

cells in vivo by indirect effects of IFN-gamma. *Proc Natl Acad Sci U S A*, 96(15), 8633-8638.

Nagane, M., Coufal, F., Lin, H., Bogler, O., Cavenee, W. K., & Huang, H. J. (1996). A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res*, 56(21), 5079-5086.

Napoletano, C., Pinto, D., Bellati, F., Taurino, F., Rahimi, H., Tomao, F., . . . Nuti, M. (2007). A comparative analysis of serum and serum-free media for generation of clinical grade DCs. *J Immunother*, 30(5), 567-576. doi:10.1097/CJI.0b013e318046f396

Nduom, E. K., Wei, J., Yaghi, N. K., Huang, N., Kong, L. Y., Gabrusiewicz, K., . . . Heimberger, A. B. (2016). PD-L1 expression and prognostic impact in glioblastoma. *Neuro Oncol*, 18(2), 195-205. doi:10.1093/neuonc/nov172

Nduom, E. K., Weller, M., & Heimberger, A. B. (2015). Immunosuppressive mechanisms in glioblastoma. *Neuro Oncol*, 17 Suppl 7, vii9-vii14. doi:10.1093/neuonc/nov151

Nemazee, D. A., & Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature*, 337(6207), 562-566. doi:10.1038/337562a0

Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., & Huang, H. J. (1994). A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Natl Acad Sci U S A*, 91(16), 7727-7731.

Nobusawa, S., Watanabe, T., Kleihues, P., & Ohgaki, H. (2009). IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res*, 15(19), 6002-6007. doi:10.1158/1078-0432.CCR-09-0715

Ochiai, H., Campbell, S. A., Archer, G. E., Chewning, T. A., Dragunsky, E., Ivanov, A., . . . Sampson, J. H. (2006). Targeted therapy for glioblastoma multiforme neoplastic meningitis with intrathecal delivery of an oncolytic recombinant poliovirus. *Clin Cancer Res*, 12(4), 1349-1354. doi:10.1158/1078-0432.CCR-05-1595

Ohgaki, H., Dessen, P., Jourde, B., Horstmann, S., Nishikawa, T., Di Patre, P. L., . . . Kleihues, P. (2004). Genetic pathways to glioblastoma: a population-based study. *Cancer Res*, 64(19), 6892-6899. doi:10.1158/0008-5472.CAN-04-1337

- Osada, T., Chong, G., Tansik, R., Hong, T., Spector, N., Kumar, R., . . . Morse, M. A. (2008). The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. *Cancer Immunol Immunother*, 57(8), 1115-1124. doi:10.1007/s00262-007-0441-x
- Ostrom, Q. T., Gittleman, H., Liao, P., Vecchione-Koval, T., Wolinsky, Y., Kruchko, C., & Barnholtz-Sloan, J. S. (2017). CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. *Neuro Oncol*, 19(suppl_5), v1-v88. doi:10.1093/neuonc/nox158
- Ott, P. A., Hu, Z., Keskin, D. B., Shukla, S. A., Sun, J., Bozym, D. J., . . . Wu, C. J. (2017). An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature*, 547(7662), 217-221. doi:10.1038/nature22991
- Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S., Corradin, G., & Lanzavecchia, A. (1989). Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol*, 19(12), 2237-2242. doi:10.1002/eji.1830191209
- Parry, R. V., Chemnitz, J. M., Frauwirth, K. A., Lanfranco, A. R., Braunstein, I., Kobayashi, S. V., . . . Riley, J. L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol*, 25(21), 9543-9553. doi:10.1128/MCB.25.21.9543-9553.2005
- Patel, A. P., Tirosh, I., Trombetta, J. J., Shalek, A. K., Gillespie, S. M., Wakimoto, H., . . . Bernstein, B. E. (2014). Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science*, 344(6190), 1396-1401. doi:10.1126/science.1254257
- Pearl, R. (1929). Cancer and Tuberculosis. *American Journal of Hygiene*, 9, 97-159.
- Pellegatta, S., Eoli, M., Cuccarini, V., Anghileri, E., Pollo, B., Pessina, S., . . . Finocchiaro, G. (2018). Survival gain in glioblastoma patients treated with dendritic cell immunotherapy is associated with increased NK but not CD8(+) T cell activation in the presence of adjuvant temozolomide. *Oncoimmunology*, 7(4), e1412901. doi:10.1080/2162402X.2017.1412901
- Perez-Diez, A., Joncker, N. T., Choi, K., Chan, W. F., Anderson, C. C., Lantz, O., & Matzinger, P. (2007). CD4 cells can be more efficient at tumor rejection than CD8 cells. *Blood*, 109(12), 5346-5354. doi:10.1182/blood-2006-10-051318

- Perez, O. D., Logg, C. R., Hiraoka, K., Diago, O., Burnett, R., Inagaki, A., . . . Jolly, D. J. (2012). Design and selection of Toca 511 for clinical use: modified retroviral replicating vector with improved stability and gene expression. *Mol Ther*, 20(9), 1689-1698. doi:10.1038/mt.2012.83
- Pessina, S., Cantini, G., Kapetis, D., Cazzato, E., Di Ianni, N., Finocchiaro, G., & Pellegatta, S. (2016). The multidrug-resistance transporter Abcc3 protects NK cells from chemotherapy in a murine model of malignant glioma. *Oncoimmunology*, 5(5), e1108513. doi:10.1080/2162402X.2015.1108513
- Phetsouphanh, C., Zaunders, J. J., & Kelleher, A. D. (2015). Detecting Antigen-Specific T Cell Responses: From Bulk Populations to Single Cells. *Int J Mol Sci*, 16(8), 18878-18893. doi:10.3390/ijms160818878
- Phuphanich, S., Wheeler, C. J., Rudnick, J. D., Mazer, M., Wang, H., Nuno, M. A., . . . Yu, J. S. (2013). Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. *Cancer Immunol Immunother*, 62(1), 125-135. doi:10.1007/s00262-012-1319-0
- Pickart, C. M., Kaspersek, E. M., Beal, R., & Kim, A. (1994). Substrate properties of site-specific mutant ubiquitin protein (G76A) reveal unexpected mechanistic features of ubiquitin-activating enzyme (E1). *J Biol Chem*, 269(10), 7115-7123.
- Platten, M., Bunse, L., Riehl, D., Bunse, T., Ochs, K., & Wick, W. (2018). Vaccine Strategies in Gliomas. *Curr Treat Options Neurol*, 20(5), 11. doi:10.1007/s11940-018-0498-1
- Polyzoidis, S., & Ashkan, K. (2014). DCVax(R)-L--developed by Northwest Biotherapeutics. *Hum Vaccin Immunother*, 10(11), 3139-3145. doi:10.4161/hv.29276
- Porgador, A., Snyder, D., & Gilboa, E. (1996). Induction of antitumor immunity using bone marrow-generated dendritic cells. *J Immunol*, 156(8), 2918-2926.
- Prestwich, R. J., Errington, F., Ilett, E. J., Morgan, R. S., Scott, K. J., Kottke, T., . . . Melcher, A. A. (2008). Tumor infection by oncolytic reovirus primes adaptive antitumor immunity. *Clin Cancer Res*, 14(22), 7358-7366. doi:10.1158/1078-0432.CCR-08-0831

- Prestwich, R. J., Harrington, K. J., Pandha, H. S., Vile, R. G., Melcher, A. A., & Errington, F. (2008). Oncolytic viruses: a novel form of immunotherapy. *Expert Rev Anticancer Ther*, 8(10), 1581-1588. doi:10.1586/14737140.8.10.1581
- Prevost-Blondel, A., Zimmermann, C., Stemmer, C., Kulmburg, P., Rosenthal, F. M., & Pircher, H. (1998). Tumor-infiltrating lymphocytes exhibiting high ex vivo cytolytic activity fail to prevent murine melanoma tumor growth in vivo. *J Immunol*, 161(5), 2187-2194.
- Prins, R. M., Wang, X., Soto, H., Young, E., Lisiero, D. N., Fong, B., . . . Liau, L. M. (2013). Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients. *J Immunother*, 36(2), 152-157. doi:10.1097/CJI.0b013e3182811ae4
- Purcell, A. W., McCluskey, J., & Rossjohn, J. (2007). More than one reason to rethink the use of peptides in vaccine design. *Nat Rev Drug Discov*, 6(5), 404-414. doi:10.1038/nrd2224
- Rampling, R., Peoples, S., Mulholland, P. J., James, A., Al-Salihi, O., Twelves, C. J., . . . Ritchie, J. W. (2016). A Cancer Research UK First Time in Human Phase I Trial of IMA950 (Novel Multipptide Therapeutic Vaccine) in Patients with Newly Diagnosed Glioblastoma. *Clin Cancer Res*, 22(19), 4776-4785. doi:10.1158/1078-0432.CCR-16-0506
- Reap, E. A., Suryadevara, C. M., Batich, K. A., Sanchez-Perez, L., Archer, G. E., Schmittling, R. J., . . . Sampson, J. H. (2018). Dendritic Cells Enhance Polyfunctionality of Adoptively Transferred T Cells That Target Cytomegalovirus in Glioblastoma. *Cancer Res*, 78(1), 256-264. doi:10.1158/0008-5472.CAN-17-0469
- Reardon, D., Omuro, A., Brandes, A., Rieger, J., Wick, A., Sepulveda, J., . . . Lim, M. (2017). OS10. 3 Randomized phase 3 study evaluating the efficacy and safety of nivolumab vs bevacizumab in patients with recurrent glioblastoma: CheckMate 143. *Neuro-oncology*, 19(suppl_3), iii21-iii21.
- Reardon, D. A., Desjardins, A., Schuster, J., Tran, D. D., Fink, K. L., Nabors, L. B., . . . Ashby, L. S. (2015). IMCT-08 ReACT: long-term survival from a randomized phase II study of rindopepimut (CDX-110) plus bevacizumab in relapsed glioblastoma. *Neuro-oncology*, 17(suppl_5), v109-v109.

- Reid, D. W., Shenolikar, S., & Nicchitta, C. V. (2015). Simple and inexpensive ribosome profiling analysis of mRNA translation. *Methods*, *91*, 69-74. doi:10.1016/j.ymeth.2015.07.003
- Ricupito, A., Grioni, M., Calcinotto, A., Hess Michelini, R., Longhi, R., Mondino, A., & Bellone, M. (2013). Booster vaccinations against cancer are critical in prophylactic but detrimental in therapeutic settings. *Cancer Res*, *73*(12), 3545-3554. doi:10.1158/0008-5472.CAN-12-2449
- Ridge, J. P., Di Rosa, F., & Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*, *393*(6684), 474-478. doi:10.1038/30989
- Rojas, J. M., Avia, M., Martin, V., & Sevilla, N. (2017). IL-10: A Multifunctional Cytokine in Viral Infections. *J Immunol Res*, *2017*, 6104054. doi:10.1155/2017/6104054
- Rosalia, R. A., Quakkelaar, E. D., Redeker, A., Khan, S., Camps, M., Drijfhout, J. W., . . . Melief, C. J. (2013). Dendritic cells process synthetic long peptides better than whole protein, improving antigen presentation and T-cell activation. *Eur J Immunol*, *43*(10), 2554-2565. doi:10.1002/eji.201343324
- Rosenberg, S. A., Yang, J. C., & Restifo, N. P. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nat Med*, *10*(9), 909-915. doi:10.1038/nm1100
- Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P., & Yamaguchi, T. (2009). Regulatory T cells: how do they suppress immune responses? *International Immunology*, *21*(10), 1105-1111. doi:10.1093/intimm/dxp095
- Sampson, J. H., Aldape, K. D., Archer, G. E., Coan, A., Desjardins, A., Friedman, A. H., . . . Heimberger, A. B. (2011). Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro Oncol*, *13*(3), 324-333. doi:10.1093/neuonc/noq157
- Sampson, J. H., Archer, G. E., Ashley, D. M., Fuchs, H. E., Hale, L. P., Dranoff, G., & Bigner, D. D. (1996). Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8+ cell-mediated immunity against tumors located in the "immunologically privileged" central nervous system. *Proc Natl Acad Sci U S A*, *93*(19), 10399-10404.

- Sampson, J. H., Archer, G. E., Mitchell, D. A., Heimberger, A. B., Herndon, J. E., 2nd, Lally-Goss, D., . . . Bigner, D. D. (2009). An epidermal growth factor receptor variant III-targeted vaccine is safe and immunogenic in patients with glioblastoma multiforme. *Mol Cancer Ther*, *8*(10), 2773-2779. doi:10.1158/1535-7163.MCT-09-0124
- Sampson, J. H., Ashley, D. M., Archer, G. E., Fuchs, H. E., Dranoff, G., Hale, L. P., & Bigner, D. D. (1997). Characterization of a spontaneous murine astrocytoma and abrogation of its tumorigenicity by cytokine secretion. *Neurosurgery*, *41*(6), 1365-1372; discussion 1372-1363.
- Sampson, J. H., Crotty, L. E., Lee, S., Archer, G. E., Ashley, D. M., Wikstrand, C. J., . . . Bigner, D. D. (2000). Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc Natl Acad Sci U S A*, *97*(13), 7503-7508. doi:10.1073/pnas.130166597
- Sampson, J. H., Heimberger, A. B., Archer, G. E., Aldape, K. D., Friedman, A. H., Friedman, H. S., . . . Bigner, D. D. (2010). Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J Clin Oncol*, *28*(31), 4722-4729. doi:10.1200/JCO.2010.28.6963
- Samson, A., Scott, K. J., Taggart, D., West, E. J., Wilson, E., Nuovo, G. J., . . . Melcher, A. A. (2018). Intravenous delivery of oncolytic reovirus to brain tumor patients immunologically primes for subsequent checkpoint blockade. *Sci Transl Med*, *10*(422). doi:10.1126/scitranslmed.aam7577
- Sanchez-Perez, L., Choi, B. D., Reap, E. A., Sayour, E. J., Norberg, P., Schmittling, R. J., . . . Sampson, J. H. (2013). BlyS levels correlate with vaccine-induced antibody titers in patients with glioblastoma lymphodepleted by therapeutic temozolomide. *Cancer Immunol Immunother*, *62*(6), 983-987. doi:10.1007/s00262-013-1405-y
- Sanchez-Perez, L., Suryadevara, C. M., Choi, B. D., Reap, E. A., & Sampson, J. H. (2014). Leveraging chemotherapy-induced lymphopenia to potentiate cancer immunotherapy. *Oncoimmunology*, *3*(7), e944054. doi:10.4161/21624011.2014.944054

- Sanchez-Perez, L. A., Choi, B. D., Archer, G. E., Cui, X., Flores, C., Johnson, L. A., . . . Sampson, J. H. (2013). Myeloablative temozolomide enhances CD8(+) T-cell responses to vaccine and is required for efficacy against brain tumors in mice. *PLoS One*, *8*(3), e59082. doi:10.1371/journal.pone.0059082
- Saunders, N. R., Dreifuss, J. J., Dziegielewska, K. M., Johansson, P. A., Habgood, M. D., Mollgard, K., & Bauer, H. C. (2014). The rights and wrongs of blood-brain barrier permeability studies: a walk through 100 years of history. *Front Neurosci*, *8*, 404. doi:10.3389/fnins.2014.00404
- Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., & Melief, C. J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*, *393*(6684), 480-483. doi:10.1038/31002
- Schumacher, T., Bunse, L., Pusch, S., Sahn, F., Wiestler, B., Quandt, J., . . . Platten, M. (2014). A vaccine targeting mutant IDH1 induces antitumour immunity. *Nature*, *512*(7514), 324-327. doi:10.1038/nature13387
- Shirota, H., Petrenko, L., Hong, C., & Klinman, D. M. (2007). Potential of transfected muscle cells to contribute to DNA vaccine immunogenicity. *J Immunol*, *179*(1), 329-336.
- Simpson, T. R., Li, F., Montalvo-Ortiz, W., Sepulveda, M. A., Bergerhoff, K., Arce, F., . . . Quezada, S. A. (2013). Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med*, *210*(9), 1695-1710. doi:10.1084/jem.20130579
- Soeda, A., Hara, A., Kunisada, T., Yoshimura, S., Iwama, T., & Park, D. M. (2015). The evidence of glioblastoma heterogeneity. *Sci Rep*, *5*, 7979. doi:10.1038/srep07979
- Sotomayor, E. M., Borrello, I., Tubb, E., Allison, J. P., & Levitsky, H. I. (1999). In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. *Proc Natl Acad Sci U S A*, *96*(20), 11476-11481.
- Sottoriva, A., Spiteri, I., Piccirillo, S. G., Touloumis, A., Collins, V. P., Marioni, J. C., . . . Tavaré, S. (2013). Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A*, *110*(10), 4009-4014. doi:10.1073/pnas.1219747110

- Spranger, S., Bao, R., & Gajewski, T. F. (2015). Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature*, 523(7559), 231-235. doi:10.1038/nature14404
- Spranger, S., Dai, D., Horton, B., & Gajewski, T. F. (2017). Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell*, 31(5), 711-723 e714. doi:10.1016/j.ccell.2017.04.003
- Strebhardt, K., & Ullrich, A. (2008). Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer*, 8(6), 473-480. doi:10.1038/nrc2394
- Street, S. E., Cretney, E., & Smyth, M. J. (2001). Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood*, 97(1), 192-197.
- Street, S. E., Trapani, J. A., MacGregor, D., & Smyth, M. J. (2002). Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *J Exp Med*, 196(1), 129-134.
- Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., . . . National Cancer Institute of Canada Clinical Trials, G. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol*, 10(5), 459-466. doi:10.1016/S1470-2045(09)70025-7
- Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., . . . National Cancer Institute of Canada Clinical Trials, G. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*, 352(10), 987-996. doi:10.1056/NEJMoa043330
- Stutman, O. (1974). Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science*, 183(4124), 534-536.
- Swartz, A. M., Batich, K. A., Fecci, P. E., & Sampson, J. H. (2015). Peptide vaccines for the treatment of glioblastoma. *J Neurooncol*, 123(3), 433-440. doi:10.1007/s11060-014-1676-y

- Swartz, A. M., Li, Q. J., & Sampson, J. H. (2014). Rindopepimut: a promising immunotherapeutic for the treatment of glioblastoma multiforme. *Immunotherapy*, 6(6), 679-690. doi:10.2217/imt.14.21
- Swartz, A. M., Reap, E., Norberg, P., Schmittling, R., Janetzki, S., Sanchez-Perez, L., & Sampson, J. H. (2018). A simple and enzyme-free method for processing infiltrating lymphocytes from small mouse tumors for ELISpot analysis. *J Immunol Methods*, 459, 90-93. doi:10.1016/j.jim.2018.05.015
- Swartz, A. M., Schaller, T. H., & Sampson, J. H. (2017). Introduction to translational immunotherapy for brain tumors. In S. JH (Ed.), *Translational Immunotherapy for Brain Tumors* (pp. xiii-xxix). London: Elsevier.
- Swartz, A. M., Shen, S. H., Salgado, M. A., Congdon, K. L., & Sanchez-Perez, L. (2018). Promising vaccines for treating glioblastoma. *Expert Opinion on Biological Therapy*, null-null. doi:10.1080/14712598.2018.1531846
- Thomas, A. A., Fisher, J. L., Hampton, T. H., Christensen, B. C., Tsongalis, G. J., Rahme, G. J., . . . Fadul, C. E. (2017). Immune modulation associated with vascular endothelial growth factor (VEGF) blockade in patients with glioblastoma. *Cancer Immunol Immunother*, 66(3), 379-389. doi:10.1007/s00262-016-1941-3
- Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., . . . Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol*, 28(5), 511-515. doi:10.1038/nbt.1621
- Turcan, S., Rohle, D., Goenka, A., Walsh, L. A., Fang, F., Yilmaz, E., . . . Chan, T. A. (2012). IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature*, 483(7390), 479-483. doi:10.1038/nature10866
- Umlauf, S. W., Beverly, B., Lantz, O., & Schwartz, R. H. (1995). Regulation of interleukin 2 gene expression by CD28 costimulation in mouse T-cell clones: both nuclear and cytoplasmic RNAs are regulated with complex kinetics. *Mol Cell Biol*, 15(6), 3197-3205.
- Vajkoczy, P., Laschinger, M., & Engelhardt, B. (2001). Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J Clin Invest*, 108(4), 557-565. doi:10.1172/JCI12440

- Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., . . . Cancer Genome Atlas Research, N. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*, *17*(1), 98-110. doi:10.1016/j.ccr.2009.12.020
- Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., Jr., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science*, *339*(6127), 1546-1558. doi:10.1126/science.1235122
- Vogt, N., Lefevre, S. H., Apiou, F., Dutrillaux, A. M., Cor, A., Leuraud, P., . . . Malfoy, B. (2004). Molecular structure of double-minute chromosomes bearing amplified copies of the epidermal growth factor receptor gene in gliomas. *Proc Natl Acad Sci U S A*, *101*(31), 11368-11373. doi:10.1073/pnas.0402979101
- Wang, M., Larsen, M. V., Nielsen, M., Harndahl, M., Justesen, S., Dziegiel, M. H., . . . Claesson, M. H. (2010). HLA class I binding 9mer peptides from influenza A virus induce CD4 T cell responses. *PLoS One*, *5*(5), e10533. doi:10.1371/journal.pone.0010533
- Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., . . . Verhaak, R. G. W. (2018). Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. *Cancer Cell*, *33*(1), 152. doi:10.1016/j.ccell.2017.12.012
- Warncke, M., Doderer, A., Dierbach, H., Follo, M., & Veelken, H. (2006). Murine dendritic cells generated under serum-free conditions have a mature phenotype and efficiently induce primary immune responses. *J Immunol Methods*, *310*(1-2), 1-11. doi:10.1016/j.jim.2005.09.012
- Warshakoon, H. J., Hood, J. D., Kimbrell, M. R., Malladi, S., Wu, W. Y., Shukla, N. M., . . . David, S. A. (2009). Potential adjuvant properties of innate immune stimuli. *Hum Vaccin*, *5*(6), 381-394.
- Wasiuk, A., Testa, J., Weidlick, J., Sisson, C., Vitale, L., Widger, J., . . . He, L. Z. (2017). CD27-Mediated Regulatory T Cell Depletion and Effector T Cell Costimulation Both Contribute to Antitumor Efficacy. *J Immunol*, *199*(12), 4110-4123. doi:10.4049/jimmunol.1700606

- Watkins, S., Robel, S., Kimbrough, I. F., Robert, S. M., Ellis-Davies, G., & Sontheimer, H. (2014). Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells. *Nat Commun*, *5*, 4196. doi:10.1038/ncomms5196
- Watowich, S. S., & Liu, Y. J. (2010). Mechanisms regulating dendritic cell specification and development. *Immunol Rev*, *238*(1), 76-92. doi:10.1111/j.1600-065X.2010.00949.x
- Weller, M., Butowski, N., Tran, D. D., Recht, L. D., Lim, M., Hirte, H., . . . investigators, A. I. t. (2017). Rindopepimut with temozolomide for patients with newly diagnosed, EGFRvIII-expressing glioblastoma (ACT IV): a randomised, double-blind, international phase 3 trial. *Lancet Oncol*, *18*(10), 1373-1385. doi:10.1016/S1470-2045(17)30517-X
- Weller, M., & Yung, W. K. (2013). Angiogenesis inhibition for glioblastoma at the edge: beyond AVAGlio and RTOG 0825. *Neuro Oncol*, *15*(8), 971. doi:10.1093/neuonc/not106
- Wen, P., Reardon, D., Phuphanich, S., Aiken, R., Landolfi, J., Curry, W., . . . Markert, J. (2014). AT-60A RANDOMIZED DOUBLE BLIND PLACEBO-CONTROLLED PHASE 2 TRIAL OF DENDRITIC CELL (DC) VACCINE ICT-107 FOLLOWING STANDARD TREATMENT IN NEWLY DIAGNOSED PATIENTS WITH GBM. *Neuro-oncology*, *16*(Suppl 5), v22.
- Westphal, M., Hilt, D. C., Bortey, E., Delavault, P., Olivares, R., Warnke, P. C., . . . Ram, Z. (2003). A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma. *Neuro Oncol*, *5*(2), 79-88. doi:10.1215/S1522-8517-02-00023-6
- Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., . . . et al. (1995). Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res*, *55*(14), 3140-3148.
- Wikstrand, C. J., Reist, C. J., Archer, G. E., Zalutsky, M. R., & Bigner, D. D. (1998). The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *J Neurovirol*, *4*(2), 148-158. doi:10.3109/13550289809114515

- Wilcox, M. E., Yang, W., Senger, D., Rewcastle, N. B., Morris, D. G., Brasher, P. M., . . . Forsyth, P. A. (2001). Reovirus as an oncolytic agent against experimental human malignant gliomas. *J Natl Cancer Inst*, 93(12), 903-912.
- Williams, J. W., Tjota, M. Y., Clay, B. S., Vander Lugt, B., Bandukwala, H. S., Hrusch, C. L., . . . Sperlberg, A. I. (2013). Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun*, 4, 2990. doi:10.1038/ncomms3990
- Wollmann, G., Ozduman, K., & van den Pol, A. N. (2012). Oncolytic virus therapy for glioblastoma multiforme: concepts and candidates. *Cancer J*, 18(1), 69-81. doi:10.1097/PPO.0b013e31824671c9
- Wong, A. J., Bigner, S. H., Bigner, D. D., Kinzler, K. W., Hamilton, S. R., & Vogelstein, B. (1987). Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc Natl Acad Sci U S A*, 84(19), 6899-6903.
- Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., & Vogelstein, B. (1992). Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A*, 89(7), 2965-2969.
- Wooldridge, L., Lissina, A., Cole, D. K., van den Berg, H. A., Price, D. A., & Sewell, A. K. (2009). Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology*, 126(2), 147-164. doi:10.1111/j.1365-2567.2008.02848.x
- Woroniccka, K., Chongsathidkiet, P., Rhodin, K., Kemeny, H., Dechant, C., Farber, S. H., . . . Fecci, P. E. (2018a). T-Cell Exhaustion Signatures Vary with Tumor Type and Are Severe in Glioblastoma. *Clin Cancer Res*, 24(17), 4175-4186. doi:10.1158/1078-0432.CCR-17-1846
- Woroniccka, K., Chongsathidkiet, P., Rhodin, K. E., Kemeny, H. R., Dechant, C. A., Farber, S. H., . . . Fecci, P. E. (2018b). T Cell Exhaustion Signatures Vary with Tumor Type and are Severe in Glioblastoma. *Clin Cancer Res*. doi:10.1158/1078-0432.CCR-17-1846
- Xu, L. W., Chow, K. K., Lim, M., & Li, G. (2014). Current vaccine trials in glioblastoma: a review. *J Immunol Res*, 2014, 796856. doi:10.1155/2014/796856

- Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H., & Kershaw, M. H. (2007). Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol*, 179(11), 7577-7584.
- Yan, H., Parsons, D. W., Jin, G., McLendon, R., Rasheed, B. A., Yuan, W., . . . Bigner, D. D. (2009). IDH1 and IDH2 mutations in gliomas. *N Engl J Med*, 360(8), 765-773. doi:10.1056/NEJMoa0808710
- Yarchoan, M., Hopkins, A., & Jaffee, E. M. (2017). Tumor Mutational Burden and Response Rate to PD-1 Inhibition. *N Engl J Med*, 377(25), 2500-2501. doi:10.1056/NEJMc1713444
- Yeh, S., Karne, N. K., Kerkar, S. P., Heller, C. K., Palmer, D. C., Johnson, L. A., . . . Nussenblatt, R. B. (2009). Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology*, 116(5), 981-989 e981. doi:10.1016/j.ophtha.2008.12.004
- Yu, W., Jiang, N., Ebert, P. J., Kidd, B. A., Muller, S., Lund, P. J., . . . Davis, M. M. (2015). Clonal Deletion Prunes but Does Not Eliminate Self-Specific alpha beta CD8(+) T Lymphocytes. *Immunity*, 42(5), 929-941. doi:10.1016/j.immuni.2015.05.001
- Zhang, X., Huang, H., Yuan, J., Sun, D., Hou, W. S., Gordon, J., & Xiang, J. (2005). CD4-8-dendritic cells prime CD4+ T regulatory 1 cells to suppress antitumor immunity. *J Immunol*, 175(5), 2931-2937.

Biography

Adam Michael Swartz graduated from East Pennsboro High School in Enola, Pennsylvania in 2003. After spending 1 year at Harrisburg Area Community College, he transferred to Pennsylvania State University, where he earned a Bachelor of Science degree in Life Sciences in 2008. During his undergraduate career, he took a strong interest in biological research while working in the lab of Dr. Robert E. Farrell. Upon graduating, he joined the ORISE research program at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) at Aberdeen Proving Ground, Maryland. Under the patronage of Drs. Denise Milhorn and Patrick M. McNutt, he further developed his research skills working on countermeasures for chemical and biological warfare agents, including sulfur mustard and botulinum neurotoxin. Concurrently, he earned a Master of Science in Biotechnology from Johns Hopkins University in 2011.

Adam entered Duke University in Durham, North Carolina through the Cell and Molecular Biology umbrella program in 2012. In the lab of Dr. John H. Sampson, he performed his thesis research developing and evaluating cancer vaccines targeting astrocytoma-specific missense mutations. Additionally, he was a fellow in the inaugural Duke Office of Licensing and Ventures fellowship program, where he assessed the commercial potential of various inventions. His publications are as follows:

- Swartz, A. M., Congdon, C. L., Nair, S., Li, Q. J., Herndon, J. E. II, Suryadevara, C. M., Riccione, K., Archer, G. E., Norberg, P., Skora, A. D., Sanchez-Perez, L., & Sampson, J. H. (2018). The rational design of synthetic long peptides using a universal helper epitope can improve the therapeutic effects of neoantigen vaccines. In progress.
- Swartz, A. M., Shen, S. H., Salgado, M. A., Congdon, K. L., & Sanchez-Perez, L. (2018). Promising vaccines for treating glioblastoma. *Expert Opinion on Biological Therapy*, null-null. doi:10.1080/14712598.2018.1531846
- Swartz, A. M., Reap, E., Norberg, P., Schmittling, R., Janetzki, S., Sanchez-Perez, L., & Sampson, J. H. (2018). A simple and enzyme-free method for processing infiltrating lymphocytes from small mouse tumors for ELISpot analysis. *J Immunol Methods*, 459, 90-93. doi:10.1016/j.jim.2018.05.015
- Huang, M., Nicholson, L. T., Batich, K. A., Swartz, A. M., Kopin, D., Prabhakar, V., Lipes, B. D., Nair, S. K., Sampson, J. H., Gunn, M. D. (2018). Monocyte-mediated antigen transfer via gap junctions to CD8+ dendritic cells triggers therapeutic cellular immunity against cancer. In progress.
- Reap, E. A., Suryadevara, C. M., Batich, K. A., Sanchez-Perez, L., Archer, G. E., Schmittling, R. J., Norberg, P. K., Herndon, J. E. 2nd, Healy, P., Congdon, K. L., Gedeon, P. C., Campbell, O. C., Swartz, A. M., . . . Sampson, J. H. (2018). Dendritic Cells Enhance Polyfunctionality of Adoptively Transferred T Cells That Target Cytomegalovirus in Glioblastoma. *Cancer Res*, 78(1), 256-264. doi:10.1158/0008-5472.CAN-17-0469
- Riccione, K., He, L., Norberg, P., Suryadevara, C. M., Swartz, A. M., Healy, P., Reap, E., Keler, T., Li, Q., Sanchez-Perez, L., Sampson, J. H. (2018). CD27 stimulation unveils the efficacy of linked class I/II peptide vaccines in poorly immunogenic tumors by orchestrating a coordinated CD4/CD8 T cell response. *Oncoimmunology*. 1-14.
- Suryadevara, C. M., Desai, R., Farber, S., Choi, B., Swartz, A. M., Shen, S., Gedeon, P., Snyder, D., Herndon, II J., Healy, P., Reap, E., Archer, G., Fecci, P., Sampson, J. H., Sanchez-Perez L. (2018). Preventing Lck activation in CAR T cells confers Treg resistance but requires 4-1BB signaling for them to persist and treat solid tumors in non-lymphodepleted hosts. *Clin. Cancer Res*. Submitted

- Swartz, A. M., Schaller, T. H., & Sampson, J. H. (2017). Introduction to translational immunotherapy for brain tumors. In S. JH (Ed.), *Translational Immunotherapy for Brain Tumors* (pp. xiii-xxix). London: Elsevier.
- Batich, K. A., Swartz, A. M., & Sampson, J. H. (2016). Preconditioning Vaccine Sites for mRNA-Transfected Dendritic Cell Therapy and Antitumor Efficacy. *Methods Mol Biol*, 1403, 819-838. doi:10.1007/978-1-4939-3387-7_47
- Suryadevara, C. M., Desai, R., Farber, S. H., Gedeon, P. C., Swartz, A., Snyder, D., . . . Fecci, P. E. (2016). 369 Chimeric antigen receptors deficient in Lck signaling require 4-1BB costimulation to expand in vivo, resist regulatory T-cell suppression, and treat solid tumors in immune-intact hosts. *Neurosurgery*, 63(CN_suppl_1), 209-210.
- Swartz, A. M., Batich, K. A., Fecci, P. E., & Sampson, J. H. (2015). Peptide vaccines for the treatment of glioblastoma. *J Neurooncol*, 123(3), 433-440. doi:10.1007/s11060-014-1676-y
- Batich, K. A., Swartz, A. M., & Sampson, J. H. (2015). Enhancing dendritic cell-based vaccination for highly aggressive glioblastoma. *Expert Opin Biol Ther*, 15(1), 79-94. doi:10.1517/14712598.2015.972361
- Swartz, A. M., Li, Q. J., & Sampson, J. H. (2014). Rindopepimut: a promising immunotherapeutic for the treatment of glioblastoma multiforme. *Immunotherapy*, 6(6), 679-690. doi:10.2217/imt.14.21