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Modulating Antitumor T Cell Responses using Cytomegalovirus-based Vaccines

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Cytomegalovirus (CMV) is a ubiquitous herpes virus that generates a unique T cell response, characterized by the maintenance of a high frequency of virus-specific T cells over the lifetime of the host. This prolonged T cell response makes CMV an attractive vaccine platform. Our lab has previously generated a recombinant murine CMV (MCMV) expressing a modified melanoma antigen, MCMVgp100KGP, which protects mice from tumor challenge. In Chapter 3 of this current study, we hypothesized that CMV vectors expressing multiple tumor antigens would be more effective vaccines in the treatment of melanoma. However, our data show that novel MCMV vectors expressing two melanoma antigens do not delay tumor growth compared to MCMVgp100KGP, highlighting the difficulty in targeting shared tumor antigens.

In Chapter 4, we sought to improve the efficacy of MCMVgp100KGP by combination immunotherapy. Herein, we show that adoptive cell therapy enhances the antitumor effects of MCMV-based vaccines. MCMVgp100KGP maintains adoptively transferred cells at higher frequencies than an acute viral vector, but fails to eradicate established tumors. In response to vaccination with MCMVgp100KGP, several immunosuppressive molecules including PD-L1, Qa-1b, and IDO1 are upregulated within the tumor microenvironment, suggesting several
mechanisms of tumor resistance. Surprisingly, blockade of these molecules did not improve the antitumor activity of MCMVgp100KGP vaccination. This data further calls into question the reliability of PD-L1, Qa-1b, and IDO1 expression as predictive markers for response to therapies targeting these pathways. In the context of vaccination, these molecules may serve as indicators of effective vaccination rather than predictive biomarkers for combination immunotherapy.

Lastly, this study also identifies a novel population of CD169+ tumor associated macrophages (TAMs). In Chapter 5, we characterize a population of CD169+ TAMs found within the melanoma tumor tissue. This population expresses higher levels of MHCII and CD80, suggesting a potential to prime antitumor T cells. Preliminary data also suggests that CD169+ TAMs may preferentially phagocytose tumor cell fragments directly within the tumor bed. Future work will determine the role of these TAMs in priming antitumor T cells and regulating antitumor immunity.
Modulating Antitumor T Cell Responses using Cytomegalovirus-based Vaccines

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B.A.&Sc., McGill University, Montréal, Canada, 2011

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Doctor of Philosophy Dissertation

Modulating Antitumor T Cell Responses using Cytomegalovirus-based Vaccines

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Chapter 1: Introduction

1. Evidence of Tumor-Immune Interactions

Immunotherapy of cancer has been a long-sought goal of scientists and oncologists which until recently, had been largely discredited as a viable treatment modality for cancers unassociated with infectious agents. Some of the earliest clinical studies utilizing a form of immunotherapy for the treatment of cancer were performed by Dr. William Coley in the late 1800’s. Inspired by a case of spontaneously regressing sarcoma following post-operative bacterial infection, Dr. Coley attempted to cure his own patients, with limited success, using different bacterial toxins to induce tumor regression.\(^1\) This early work hinted that manipulating the immune system could have profound effects on cancer progression.

The importance of the immune system in cancer has also been highlighted by clinical observations that immunocompromised patients have differing incidences of certain malignancies.\(^2,3\) Following these observations, many studies have shown that malignant tissue is often infiltrated by diverse classes of immune cells, including innate immune cells like macrophages and neutrophils as well as adaptive immune cells like B lymphocytes and T lymphocytes. Importantly, recent studies have shown that T cell infiltration is associated with a better prognosis for several cancers, whereas myeloid cell infiltration tends to be associated with poor prognosis.\(^4-7\) As will be discussed below, the most convincing data regarding the importance of the immune system in the control of human cancer comes from clinical trials using therapies designed to specifically modulate antitumor T cell responses.

Substantial preclinical data using animal models has shown the immune system influences cancer progression. Pioneering work in the 1950’s showed that inbred mice could be immunized
against a chemically-induced tumor and confer protection against challenge by the same tumor.\textsuperscript{8,9} Using several immunodeficient mouse lines, studies in the last two decades have elucidated some of the machinery involved in tumor rejection, showing that lymphocytes, perforin, and IFN\(\gamma\) are critically important for tumor immunity.\textsuperscript{10-12}

2. Melanoma

2.1 Disease prevalence

Melanoma is a malignancy of melanocytes. It has been estimated that greater than 80,000 patients in the United States will be diagnosed with melanoma and over 9,000 patients will die from this disease in 2017 alone.\textsuperscript{13} While most primary tumors occur in the skin, metastases often seed the lungs, liver, bones, and brain of patients, making treatment much more difficult.\textsuperscript{14} Melanomas often arise from an accumulation of mutations induced by UV radiation from sun exposure. Common mutations include activating mutations in BRAF (often \textit{BRAF}\textsuperscript{V600E}) and \textit{KIT}.\textsuperscript{15,16} These oncogenes can be targeted with molecular inhibitors with some clinical success.\textsuperscript{17,18} Recently, antibodies targeting the T cell checkpoint molecules CTLA-4 and PD-1 have been approved for metastatic melanoma, marking the beginning of a new era in the treatment of melanoma.\textsuperscript{19} These therapies will be discussed in greater detail below.

2.2 Melanoma antigens

Tumor antigens are peptides expressed in cancerous cells that can be recognized by the immune system. They can be divided into two broad categories: the first category includes antigens shared by both malignant cells and healthy tissue, and the second consists of antigens produced as a result of nonsynonymous mutations in the cancer genome. Shared tumor antigens are peptides found in both malignant and healthy tissues that are either overexpressed in transformed
cells or have limited expression in normal cells such as proteins expressed during embryonic development that may be re-expressed in tumor tissue. Melanoma differentiation antigens (MDA) like gp100, tyrosinase, Trp1, and Trp2 are proteins involved in melanosome function whose expression are restricted to normal melanocytes and melanoma. In some patients with melanoma, T cell responses to MDAs develop spontaneously. Because of this observation, MDAs have been the target for many immunotherapies against melanoma, including vaccines and adoptive cell therapy.

A second class of tumor antigen has garnered much enthusiasm in recent years. Neoepitopes are non-self-peptides formed as a result of nonsynonymous mutations within the cancer genome. Because these epitopes are produced as a result of mutations occurring only in the malignant cell, they are theoretically highly specific to transformed tissue, making them intriguing targets for immunotherapy. In theory, immune responses against neoepitopes should show limited side effects due to reduced targeting of healthy tissue. Evidence for immune recognition of neoepitopes (or tumor-specific immune recognition) has existed for many years. However, until recently, technological limitations made it extremely difficult to definitely identify these mutated peptides. With the great advances in sequencing technologies, significant effort has been expended to map and predict immunogenic neoepitopes. While predicting which mutations give rise to an immunogenic neoepitope remains a difficult task, clinic evidence for the importance neoepitopes in immunotherapy exists. As will be discussed below, several studies have suggested that the profound clinical results seen in some patients following checkpoint blockade therapy or adoptive cell therapy may rely on enhancing the endogenous T cell response to neoepitopes.
3. Immunotherapy of Melanoma

3.1 Vaccines

Several decades have passed since the identification of T cell epitopes in melanoma patients. This discovery energized scientists and clinicians to attempt to generate or boost the T cell responses against melanoma using vaccination. Early work focused on targeting MDAs like gp100 and MART-1 in peptide vaccine formulations. While it was possible to detect MDA-reactive T cells following vaccination, clinical responses were modest at best. These studies and our own preclinical data highlight the difficulty in generating effective antitumor immune responses against shared antigens. Several preclinical studies show that vaccination against neoepitopes is possible and can delay tumor growth. Two clinical trials are currently underway to determine if vaccination against neoepitopes might generate more dramatic clinical responses in patients with melanoma (NCT01970358; NCT02035956).

3.2 Adoptive cell therapy

Pioneering work by Dr. Steven Rosenberg at the NCI has developed a form of immunotherapy involving the transfer of large numbers of tumor-reactive lymphocytes into patients. This modality of immunotherapy consists of two major methods. The first to be developed involves culturing tumor infiltrating lymphocytes (TIL) obtained directly from tumor tissue to greatly expand the TILs. The patient then receives a lymphodepleting regimen, consisting of combination chemotherapy, before transfer of large numbers of cultured TIL to aid in the expansion and persistence of transferred cells, which has been correlated with improved responses. Patients then receive several doses of system IL-2 to aid the expansion of transferred TIL. This therapy has shown up to 55% objective response rate in patients with
metastatic melanoma, illustrating the potential power of this treatment modality and again providing perhaps the most convincing clinical data that T cells can control tumor growth.\textsuperscript{49} Subsequent studies have shown that adoptively transferred TIL can recognize neoepitopes.\textsuperscript{37,38} This recognition likely explains the success of this form of immunotherapy because transfer of autologous T cells enriched for reactivity to MDAs caused autoimmunity and had little clinical benefit, while transfer of autologous T cells enriched for reactivity against neoepitopes shows promising clinical activity.\textsuperscript{50,51}

A second class of adoptive cell therapy involves engineering receptors in cells to redirect T cell specificity prior to transfer into the patients. Several studies have tried to redirect patient T cells to attack melanoma by transducing the expression of a traditional T cell receptor (TCR) recognizing different MDAs. Morgan et al. engineered patient T cells to express a TCR recognizing MART-1. Following lymphodepletion and cell transfer, two patients experienced tumor regression.\textsuperscript{52} In a larger study in which T cells were engineered to express a TCR recognizing MART-1 or gp100, objective tumor regression was seen in 30\% and 19\% of patients, respectively. However, patients also experienced dermatitis, uveitis, and hearing loss due to destruction of normal melanocytes.\textsuperscript{25} These results make engineered T cells targeting shared antigens much less attractive.

An exception to this pessimism is seen in results from engineered T cells targeting the B cell surface antigen, CD19. Studies targeting this antigen have utilized T cells engineered to express a chimeric antigen receptor (CAR). CARs are artificial receptors consisting of the extracellular variable regions of a tumor-antigen specific antibody and intracellular signaling domains consisting of CD3ζ and costimulatory signaling domains. This artificial receptor allows recognition of tumor antigen without the need for MHC presentation.\textsuperscript{53} CAR T cells targeting
CD19 have shown impressive results in patients with B Cell Acute Lymphoblastic Leukemia.\textsuperscript{54-57} Current research is focused on improving the persistence and trafficking of CAR T cells while increasing their resistance to tumor microenvironment immunosuppression.\textsuperscript{53} Extending these technologies to target other tumor types is also a major focus of research. However, finding relevant targets on other solid tumors will likely be difficult because as discussed before, major toxicities are likely when targeting shared antigens.

3.3 Checkpoint Inhibitors

With the recent FDA approval of several T cell checkpoint inhibitors, cancer therapy has entered a new era in which immunotherapy joins surgery, radiation, and chemotherapy as pillars of clinical oncology. Immune checkpoint inhibitors are thought to work by blocking the interaction of inhibitory receptors on the surface of antitumor T cells with their respective ligands, thereby “releasing the brake” on antitumor T cells and allowing tumor destruction.

Ipilimumab, an antibody targeting CTLA-4, was the first checkpoint inhibitor approved by the FDA after it was shown to improve overall survival in patients with metastatic melanoma.\textsuperscript{58,59} CTLA-4 (Cytotoxic T Lymphocyte Associated Protein 4) is an inhibitory receptor expressed on the surface of T cells during activation. It recognizes CD80 and CD86 on antigen presenting cells during activation, and it binds with them with higher affinity than the costimulatory receptor CD28 which is also expressed on the surface of T cells.\textsuperscript{60-62} CTLA-4 is therefore believed to limit the availability of CD80 and CD86 for CD28 costimulation during T cell activation. Based on these observations, Leach et al. first showed the antitumor effects of CTLA-4 blockade in murine tumor models.\textsuperscript{63} It has since been discovered that CTLA-4 is also highly expressed on Foxp3\(^{+}\) regulatory T cells (Treg), and CTLA-4 signaling in Tregs can
increase their immunosuppressive effects. Subsequent studies have suggested that the clinical responses to CTLA-4 blockade are likely due to effects on both effector T cells and Tregs.

Shortly after the approval of ipilimumab, several antibodies targeting the PD-1/PD-L1 axis were also shown to significantly improve survival in patients with metastatic melanoma. PD-1 (Programmed Cell Death Protein 1) is a surface receptor expressed by activated T cells, B cells, and some myeloid cells. PD-1 signaling dampens T cell proliferation and cytokine secretion primarily by reducing costimulatory signals through CD28. PD-1 has two major ligands, PD-L1 and PD-L2. PD-L1 is expressed on a diverse set of cells including immune cells, endothelial cells, and malignant cells. PD-L2 expression, on the other hand, is limited mainly to myeloid cells. PD-L1 is upregulated in tumor tissue in response to T cell infiltration and IFNγ release, and the interaction between PD-1 and PD-L1 protects tumors from T cell-mediated destruction.

Unfortunately, not all patients respond checkpoint inhibitors. In the case of PD-1 blockade, several studies have suggested that PD-L1 expression within the tumor can be predictive of clinical responses. In responders, PD-L1 is likely expressed as a consequence of T cell infiltration while also negatively regulating T cells within the tumor microenvironment. If so, what are the target antigens for these T cells? Several studies have shown that clinical response to PD-1 or CTLA-4 blockade correlates with mutational burden and predicted neoepitope frequency. Thus, it is likely that tumor regression following checkpoint inhibition requires a T cell response against neoepitopes in most cases.
4. T Cell Response to Cytomegalovirus

4.1 Cytomegalovirus is a prevalent herpesvirus

Cytomegalovirus (CMV) is a DNA, enveloped β-herpes virus that infects more than 60% of Americans over the age of 50. Like other members of the herpes virus family, CMV is never cleared from the infected host. Following an asymptomatic acute infection, CMV enters a state of latency in several organs including lungs, liver, spleen, and brain. During this period, few infectious viral particles are produced yet viral transcription is detectable. For most immunocompetent adults, CMV never produces symptomatic disease, instead relying on periods of asymptomatic reactivation and shedding for spread to new hosts. However, CMV can produce severe pathology in neonates and immunocompromised adults due to unrestrained viral spread. More recent work has also suggested that CMV can undergo subclinical reactivation in critically ill immunocompetent hosts, and this reactivation is associated with worse outcomes. However, the exact mechanism for this association has not been elucidated.

4.2 T cell memory inflation

The immune response to CMV has become an increasingly popular research topic in recent years. Given the persistent nature of the virus, the host immune system answers the infection with a unique T cell response termed “memory inflation” in which a high frequency of virus-specific lymphocytes persists in blood, lymphoid tissue, and non-lymphoid tissue over the lifetime of the host. This phenomenon has been best characterized with CD8+ T cells in humans, non-human primates, and rodents (Figure 1-1). In healthy CMV-seropositive humans, CMV-specific CD8+ T cells can represent up to 20-30% of circulating CD8+ T cells, and these cells display an effector memory or terminally differentiated effector phenotype characterized as
CD45RA⁻CCR7⁻ or CD45RA⁺CCR7⁺, respectively.⁹⁵⁻⁹⁷ A similar accumulation of CMV-specific CD4⁺ T cells seems to occur in humans albeit at lower frequencies.⁹⁵,⁹⁷,⁹⁸ It has been postulated that having such a large proportion of the immune system responding to CMV may cause premature aging of the immune system and limit the repertoire of non-CMV-specific T cells.⁹⁹⁻¹⁰¹ More work is needed to fully elucidate the role of CMV in aging.

Murine Cytomegalovirus (MCMV) is a well-established model for human CMV infection with similar cellular tropisms and a similar host immune response.⁸³,⁹⁴,¹⁰² As such, it has been an invaluable tool for learning more about the inflationary response to CMV viruses. Similar to human CMV, following an acute infection, MCMV spreads to several tissues including lungs, salivary glands, spleen, and liver, among others.¹⁰²,¹⁰³ Within two weeks, viral load decreases significantly in most tissues except salivary gland where the viral load kinetics are delayed.¹⁰³ True latency defined by absence of detectable viral transcripts is established within 4 months of infection in Balb/c mice and probably earlier in C57BL/6 mice which are more resistant to MCMV.⁹¹,¹⁰⁴ Like human CMV, MCMV elicits an inflationary CD8⁺ T cell response characterized by large frequencies of virus-specific T cells that are maintained over the lifetime of the host.⁹⁴,¹⁰⁵,¹⁰⁶ These cells display an effector memory phenotype characterized by high expression of KLRG1 and low expression of the IL-7 Receptor α, CD127.¹⁰⁵ In contrast to other persistent viral infections that maintain high frequencies of virus-specific T cells, MCMV inflationary T cells do not become “exhausted”, as they display low expression of PD-1 and retain the capacity to produce IFNγ and TNFα following peptide stimulation.¹⁰⁵,¹⁰⁷ The reason for this is likely the rapid turnover of individual cells within inflationary cell populations. While the frequency of inflationary T cells may remain relatively constant over time, individual cells within this
population do not persist over the course of the infection.\textsuperscript{105} The current model suggests that the majority of inflationary T cells displaying an effector memory phenotype are not long-lived but are constantly replaced by proliferating naïve cells or traditional memory cells that have recently encountered viral antigen.\textsuperscript{94,105} A recent study by Quinn et al. suggests that within the inflationary pool, a long-lived population of KLRG1\textsuperscript{−}CD27\textsuperscript{+} “memory-like” cells can be repeatedly stimulated to produce progeny with an effector memory phenotype.\textsuperscript{108} Thus, it is likely that the vast majority of inflationary T cells are short-lived cells constantly replaced by proliferating memory or naïve cells. Because CMV is only sporadically transcriptionally active during latency, inflationary CMV-specific T cells likely only rarely encounter viral antigen.\textsuperscript{94,109} Sporadic stimulation by antigen may explain why MCMV inflationary T cells do not become exhausted while T cells seen in other chronic infections do.

Importantly, not all MCMV-specific T cells undergo inflationary expansion. In C57BL/6 mice, CD8\textsuperscript{+} T cells recognizing the peptides m38, m139, and ie3 undergo inflationary expansion.\textsuperscript{94,105,106} Other CD8\textsuperscript{+} T cells recognizing the peptides m45 and m57 show more traditional CD8\textsuperscript{+} T cell kinetics characterized by rapid expansion in the first week of infection followed by dramatic contraction and maintenance of a low-frequency of central memory cells in lymphoid tissues.\textsuperscript{94,105,106} The exact mechanisms determining whether an epitope generates an inflationary or non-inflationary T cell response are still incomplete. However, several studies have attempted to address this issue. It is now clear that the promoter regulating the expression of the peptide is crucial for determining what kind of T cell response will form. Dekhtiareenko et al. showed that a recombinant MCMV engineered to express an HSV-1 epitope fused to ie2, a peptide that is expressed early during MCMV reactivation, generated an inflationary T cell response to the HSV-1 epitope, whereas a virus engineered with the HSV-1 epitope fused to the
m45 peptide, which elicits a traditional T cell response, generated a non-inflationary T cell response. This data supports the model that inflationary T cells are stimulated by viral peptides that are expressed in latency or early during reactivation. Thus, a promoter expressed during latency or early reactivation is likely required to elicit an inflationary T cell response.

4.3 Cytomegalovirus as a vaccine vector

Historically, vaccines have largely focused on generating long-lived humoral immunity by exposing vaccinated individuals to short bursts of target peptide along with adjuvant. While neutralizing antibodies may be effective against some extracellular pathogens, they are clearly ineffective for other microbes that establish intracellular reservoirs. For this reason, vaccine formulations aimed at generating long-lived T cell responses have been a major focus of research.

Cytomegalovirus has several properties making it an attractive vaccine platform. 1) As discussed above, CMV generates a lifelong T cell response characterized by persistence of a large population of functional T cells in both lymphoid and non-lymphoid tissue. Thus, engineering a CMV vector generating a similar T cell response against another antigen of interest could potentially provide lifelong immunity without the need for multiple vaccinations. 2) CMV utilizes many immunoevasive mechanisms to avoid elimination from infected hosts. These mechanisms also prevent neutralizing immunity, allowing reinfection of individuals with multiple strains of CMV. Thus, unlike vaccine vectors against other microbes, CMV-based vaccines could be used in CMV-seronegative and –seropositive patients alike, and vaccination with one CMV-based vaccine should not diminish the response generated by a second CMV-based vaccine. 3) Another critical and intriguing characteristic of CMV-based vaccines is that a fully functional virus is not required to generate inflationary T cell responses. As discussed
above, CMV is a human pathogen that causes severe disease in immunocompromised patients and may impact critically-ill immunocompetent patients as well.\textsuperscript{84-87,89,90} Vaccinating patients with a live virus therefore increases risks for some patients. However, a critical study in mice showed that a recombinant MCMV virus incapable of spreading from cell-to-cell after the initial infection still produced an inflationary T cell response.\textsuperscript{116,117} Thus, CMV-based vaccines can be engineered to dramatically reduce the chances of CMV pathology due to viral reactivation, while also maintaining an effective inflationary T cell response. These properties make CMV an attractive and relatively safe platform for vaccine development.

Several groups have generated recombinant Cytomegalovirus-based vaccines against different infectious agents. Tsuda et al. showed that a recombinant MCMV expressing an epitope from Ebolavirus could generate an inflationary CD8\textsuperscript{+} T cell response against the Ebola epitope and vaccination with this recombinant MCMV protected mice from challenge with Ebolavirus.\textsuperscript{118,119} Similarly, other groups have shown that MCMV-based vaccines for Influenza and Respiratory Syncytial Virus can produce inflationary T cell responses and protect mice from challenge with these pathogens.\textsuperscript{120,121} Another study showed that an MCMV-based vaccine can protect mice against challenge with Mycobacterium Tuberculosis even though the vaccine generates a modest inflationary T cell response.\textsuperscript{122} Perhaps the most promising studies using CMV-based vaccines have shown that recombinant rhesus Cytomegalovirus expressing epitopes from Simian Immunodeficiency Virus (SIV) can elicit impressive protection against challenge with SIV.\textsuperscript{123,124} In this case, the efficacy of the vaccine may rely on non-traditional CD8\textsuperscript{+} T cell responses that recognize both MHC-I and MHC-II restricted epitopes.\textsuperscript{125} Future studies will undoubtedly continue to develop CMV-based vaccines for infectious disease, particularly for HIV.
Given the properties of CMV-based vaccines described above, our group and others have been interesting in developing CMV-based vaccines against cancer. The first study to test this idea was published by Klyushnenkova et al. in 2012. In this study, mice vaccinated with a MCMV vector expressing an epitope from Prostate Specific Antigen (PSA) developed inflationary CD8+ T cell responses against PSA and showed delayed tumor growth in a model of prostate cancer. \(^{126}\) Interestingly, the same vector expressing full-length PSA induced a weaker PSA-specific CD8+ T cell response which correlated with reduced tumor control compared to the vector expressing the CD8 epitope alone. The authors speculate that the full length peptide may generate regulatory T cells through CD4 epitope expression; however, the exact reason for the discordant results is unknown. \(^{126}\)

Another study by Xu et al. in 2013 generated a CMV-based vaccine for melanoma. In this study, a MCMV vector expressing the melanoma differentiation antigen, Trp2, did not elicit a detectable T cell response to Trp2. Instead, mice vaccinated with this vector developed an antibody response to Trp2 which had modest effects on B16 melanoma tumor growth. \(^{127}\) A more recent study by the same group showed that a MCMV vector expressing a modified CD8 epitope for the melanoma antigen, gp100, could elicit a gp100-reactive CD8+ T cell response, but this had only modest effects on tumor growth following intraperitoneal vaccination. Intratumoral vaccination, however, completely eradicated some B16 melanomas, but surprisingly, this effect was less dependent on viral expression of the tumor antigen, as MCMV expressing gp100 had similar effects as wild type MCMV injected directly into the tumor. \(^{128}\)

Our lab has also previously generated a MCMV-based vaccine for melanoma. This MCMV vector (MCMVgp100KGP) contains the full-length gp100 peptide sequence which has been mutated at 3 amino acids within the well-characterized CD8+ T cell epitope gp100\(^{25-33}\) (EGSRNQDWL=>KGPRNQDWL). MCMVgp100KGP induces a CD8+ T cell response against
the native gp100\textsubscript{25-33} epitope in vaccinated mice and significantly delays tumor growth in a lung metastatic model of B16F10 melanoma.\textsuperscript{129,130} The major focus of my thesis has been to explore methods for improving the antitumor efficacy of MCMV-based vaccines by creating novel vectors expressing multiple tumor antigens and combining MCMV-based vaccines with other immunotherapies targeting immunosuppressive pathways found in the tumor microenvironment (Figure 1-2).

**Figures:**

![Graph showing the accumulation of CD8\(^+\) T cells recognizing MCMV antigens over the lifetime of the host.](image)

**Figure 1-1: Inflationary CD8\(^+\) T cells recognizing MCMV antigens accumulate over the lifetime of the host.** C57BL/6 mice were infected with \(10^5\) MCMV Smith strain. CD8\(^+\) T cells recognizing the inflationary epitope, m38, are maintained at high frequencies over the lifetime of the host, while T cells recognizing a non-inflationary epitope, m45, display a traditional T cell response. Frequencies of epitope-specific T cells were determined by staining peripheral blood leukocytes with m38- and m45-peptide tetramers.
Figure 1-2: Rationale for combining CMV-based tumor vaccines with other immunotherapies. 1) CMV-based vaccines allow persistent presentation of tumor antigen in peripheral tissues to allow continuous stimulation of tumor-specific T cells. 2) Activated tumor-specific T cells migrate into tumor microenvironment. 3) Tumors utilize many strategies to limit antitumor T cell activity in the tumor microenvironment including accumulation of immunosuppressive immune cells, expression of inhibitory ligands, local tryptophan depletion, and tissue hypoxia, among others. Targeting these immunosuppressive pathways may enhance the efficacy of CMV-based vaccines.
Chapter 2: Materials and Methods

2.1 Mice

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River (Frederick, MD). All mice used in these studies were between 6 and 12 weeks of age at the start of the experiment. Breeding pairs of PMEL mice (B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J) were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in house. Mice were housed at the University of Connecticut Health Center in a pathogen-free facility, and all experiments were performed with approval by the University of Connecticut Health Center Institutional Animal Care and Use Committee.

2.2 Cell Lines and Viruses

B16F10 and B16ova were provided by Dr. Leo Lefrançois (University of Connecticut Health Center). B16F10-RFP was purchased from AntiCancer, Inc. (San Diego, CA). B16F10 cells were cultured in DMEM media (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 1mM sodium pyruvate (Life Technologies), 1% non-essential amino acids (Life Technologies), and 1% Penicillin/Streptomycin (Life Technologies). B16ova cells were cultured in B16F10 culture media supplemented with 500 µg/mL G418 (Life Technologies). B16F10-RFP were cultured according to supplier’s instructions in RPMI 1640 media (Life Technologies) supplemented with 10% fetal bovine serum, 2mM L-Glutamine (Life Technologies), 1% Penicillin/Streptomycin, and 400µg/mL G418. Mouse embryonic fibroblasts (MEFs) were cultured in DMEM media supplemented with 5% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 2mM L-Glutamine, and 1% Penicillin/Streptomycin. M2-10B4 (a kind gift from Dr. Christopher Snyder; Thomas Jefferson University) were cultured
in RPMI-1640 media supplemented with 10% fetal bovine serum, 2mM L-Glutamine, and 1% Penicillin/Streptomycin.

MCMVova was provided by Dr. Carol Wu (University of Connecticut Health Center). Wild type MCMV, MCMVgp100, and MCMVgp100KGP were previously generated in the lab by Dr. Zhijuan Qiu using the MCMV BAC pSM3fr-MCK-2fl provided by Dr. Barbara Adler (Ludwig-Maximilians-University Munich, Germany). Briefly, the full-length murine gp100 coding sequence or the altered sequence (gp100KGP) was inserted into the MCMV ie2 locus under the control of the HCMV ie1 promoter. Previously generated viruses (WT MCMV, MCMVgp100, and MCMVgp100KGP) were expanded using murine embryonic fibroblasts or the bone marrow stromal cell line, M2-10B4, as previously described.

2.3 Generating MCMVgp100KGP-Trp2 and MCMVgp100KGP-Trp2-2M

Recombinant MCMV viruses expressing gp100KGP and the melanoma antigen Trp2 were generated by BAC recombineering using galK positive/negative selection. The MCMV BAC pSM3fr-MCK-2fl was provided by Dr. Barbara Adler (Ludwig-Maximilians-University Munich, Germany). pSM3fr-MCK-2fl-gp100KGP was generated as previously described. To generate pSM3fr-MCK-2fl-gp100KGP-Trp2 and pSM3fr-MCK-2fl-gp100KGP-Trp2-2M, a two-step galK selection in the SW102 bacterial strain (provided by Biological Resources Branch, National Cancer Institute Preclinical Repository) was used to insert the Trp2 or Trp2-2M coding sequence along with an Internal Ribosomal Entry Sequence (IRES) just downstream of the gp100KGP coding sequence. To begin, a galK targeting cassette was generated by amplifying pgalK (provided by Biological Resources Branch, National Cancer Institute Preclinical Repository) using primers galK-targeting-F 5’-ACAGCCCGCTCCTCAGTGGACAGCAGGT CTGAGTCG ACGGTACCGCGGGCCCTGTTGACAATTAATCATCGGCA-3’ and galK-targeting-R 5’-
AAAGCAAGTAAAACCTCTACAAATGTGGTGTTGCTCCTTTTATGATCA GTTATCA
GCACTGTCTGTCTCCTT-3’. Underlined sequences are homologous to pgalK and non-underlined sequences are homologous to pSM3fr-MCK-2fl-gp100KGP. A first recombineering step using galK positive selection was used to generate pSM3fr-MCK-2fl-gp100KGP-galK. The Trp2 coding sequence was amplified from a plasmid containing the Trp2 gene (Origene) with primers containing the restriction sites for NcoI and SalI, Trp2-F 5’-
CCATGGGCCACCATGGGCCTTGTGGGATGGG-3’ and Trp2-R 5’- GTGCACCTAGGCT TCCTCCGTGTATC-3’. Sequences in italics correspond to restriction sites for NcoI and SalI, respectively, and underlined sequences are homologous to Trp2 gene encoding plasmid. Trp2 was cloned into pMigR just downstream of the EMCV IRES sequence generating pMigR-Trp2. To produce pMigR-Trp2-2M, site-directed mutagenesis was performed on pMigR-Trp2-2M using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and primers Trp2-2M-F 5’- ACACAAAAAGTCATACATGCTGCAGTTGGCGATCTG-3’ and Trp2-2M-R 5’- CAGATCGCCAACCTGCAGCATGTATGACTTTTTTGTTGAT-3’. Mutation of the Trp2 CD8 T cell epitope was confirmed by sequencing. Finally, to generate pSM3fr-MCK-2fl-gp100KGP-Trp2 and pSM3fr-MCK-2fl-gp100KGP-Trp2-2M, a second recombineering step using galK negative selection was performed to replace the galK sequence with our target construct. To do this, targeting cassettes containing IRES-Trp2 or IRES-Trp2-2M were amplified using primers IRES-trp2-targeting-F 5’-
ACAGCCCGCTCTCCAGTGGACAGCAGTGCTCTGAGTCGACGGTACC
GCGGGCCTCTCGAGGTTAAGAAATCCGCCCCCCCCC-3’ and IRES-trp2-targeting-R 5’-
AAAGCAAGTAAAACCTCTCTACAAATGTGGTGATTGCTGATTATGATCAGTTACTAGGCT
TCCTCCGTGTATCTCTTGCTGCT-3’. Underlined sequences are homologous to pSM3fr-
MCK-2fl-gp100KGP and the non-underlined sequences are homologous for pMigR-Trp2. To increase the efficiency of recombineering, the homologous arms of the targeting cassettes IRES-Trp2 and IRES-Trp2-2M were extended using a second amplification step containing 5’ and 3’ overlapping homologous arms. These overlapping homologous arms were generated by amplifying segments from pSM3fr-MCK-2fl-gp100KGP using the primers homologyarm1-F 5’-TTGAGCTGACTGTGTCCTGC-3’ and homologyarm1-R 5’-CAGACCTGCTGTTCCACTGAG-3’ for one reaction and homologyarm2-F 5’-AGCCATACCACATTCTGAGAGGT-3’ and homologyarm2-R 5’-GGGCTTCTAATTACGTGACGC-3’ for the second reaction. The products of these two PCR reactions were then used to amplify the targeting cassettes IRES-Trp2 or IRES-Trp2-2M, generating the targeting cassettes with roughly 400-500bp 5’ and 3’ homology to pSM3fr-MCK-2fl-gp100KGP. This extended targeting construct was then used in the final recombineering step to replace the galK construct within pSM3fr-MCK-2fl-gp100KGP-galK, generating pSM3fr-MCK-2fl-gp100KGP-Trp2 and pSM3fr-MCK-2fl-gp100KGP-Trp2-2M. Correct insertion was verified by restriction enzyme digest and by sequencing of the target insertion sequence. To produce live virus, MEFs were transfected with pSM3fr-MCK-2fl-gp100KGP-Trp2 or pSM3fr-MCK-2fl-gp100KGP-Trp2-2M using X-tremeGENE 9 DNA Transfection Reagent (Roche). Each recombinant virus was passaged in vitro at least three times before use in vivo.

2.4 Western blot

MEFs were infected with WT MCMV, MCMVgp100KGP-Trp2, or MCMVgp100KGP-Trp2-2M. Two days later, cells were harvested and lysed RIPA lysis buffer. Uninfected MEFs and B16F10 cells were also harvested as controls. Total protein content was quantified using Pierce BCA Protein Assay Kit (ThermoScientific). 15µg protein was mixed with 4X Laemmli Sample
Buffer (Bio-Rad), boiled for 5 minutes, and run on 10% Mini-PROTEAN TGX Precast Gel (Bio-Rad). Protein was transferred to nitrocellulose membrane overnight by wet electrotransfer. The membrane was blocked for 1 hour in 5% Milk in TBS-T (0.1% Tween 20 in Tris-buffered saline (Bio-Rad)) followed by overnight incubation with anti-Trp2 (Abcam ab740473). After washing with TBS-T, membrane was incubated with anti-Rabbit-HRP for 1 hour. Secondary was washed away with TBS-T and bands were detected by chemiluminescence (ThermoScientific). The same membrane was then stripped with Restore Western Blot Stripping Buffer (ThermoScientific) and reprobed with anti-gp100 (Abcam ab137078) and anti-β tubulin (Cell Signaling 2128).

2.5 Ex vivo peptide stimulation

Splenocytes were stimulated with 1µg/mL murine gp100 peptide or 1µg/mL PMA/Ionomycin (BD Biosciences) for 5 hours at 37°C in the presence of Brefeldin A (BD Biosciences) followed by intracellular staining for IFNγ.

2.6 In vitro culture of PMEL cells

Splenocytes from PMEL mice were cultured in RPMI containing 10% fetal bovine serum, 1% sodium pyruvate, 2% HEPES, 1% L-Glutamine, 100µM non-essential amino acids, 0.05mM 2-mercaptoethanol, and 1% penicillin/streptomycin supplemented with 1µg/mL human gp100 peptide and 30IU/mL recombinant human IL-2. Cells were split after daily after two days and harvested on day 7 of culture for adoptive transfer in tumor-bearing mice.

2.7 B16-specific ELISA

96-well plates were coated overnight with B16F10 lysate. After blocking with 5% BSA for 1 hour, dilutions of sera from unvaccinated mice or mice vaccinated with recombinant MCMV
were added to individual wells for 2 hours. Wells were then incubated with HRP-conjugated anti-mouse IgG for 1 hour. 3,3’,5,5’-tetramethylbenzidine was then added to each well and plate was read at 492 nm in colorimetric plate reader.

2.8 Tumor challenge experiments

Female C57Bl/6 mice received an intradermal or intravenous injection of $10^5$ B16F10 or B16RFP or $3 \times 10^5$ B16ova. Mice were then vaccinated with WT or recombinant MCMV depending on the experiment. For intravenous tumor inoculation studies, mice were euthanized 2-3 weeks after challenge, lungs excised, and tumor nodules were counted manually under a dissecting microscope. In some experiments, splenocytes corresponding to $10^5$ CD8$^+$ PMEL cells from naïve PMEL mice or $10^5$ CD8$^+$ OT-I cells from OT-I/Rag$^{-}$ mice were transferred into naïve WT tumor-bearing mice two hours prior to vaccination with WT or recombinant MCMV. Intradermal tumor growth was monitored every 2-3 days by measuring length and width of the tumor using calipers and multiplying to calculate surface area. Mice were euthanized when tumors reached >100 mm$^2$ or ulcerated. In some experiments, tumor-bearing mice also received i.p. injections of anti-PD1 antibody (RMP1-14; BioXcell), anti-Qa-1$^b$ (4C2.4A7.5H11; BioXcell), anti-CTLA4 (UC10-4F10-11; BioXcell) or isotype controls. For IDO inhibition experiments, the IDO inhibitor 1-methyl-D-tryptophan (1-MT; Sigma) was dissolved in 1M NaOH, diluted to 2mg/mL in drinking water, and pH was adjusted to 9 using 1M HCl. Control mice received water adjusted to pH 9 without 1-MT. Water was replaced every 4-5 days.

2.9 Flow cytometry

Tumor tissue was mechanically dissociated and digested in 0.7mg/mL Collagenase D (Roche) and 3mg/mL DNase I (Roche) for 30-45 minutes to obtain single cell suspension. For
experiments looking at tumor infiltrating leukocytes, TIL were isolated using Percoll gradient prior to staining. For experiments looking at B16RFP\(^+\) tumor cells, cells were stained immediately after digestion for 20 minutes. Cells were blocked with anti-CD16/32 (clone 93; Biolegend) prior to surface staining. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) or FoxP3 Staining Kit (eBioscience) prior to staining for intracellular antigens. Antibodies against the following antigens were used: CD11a (clone M17/4; ThermoFisher), CD8a (clone 53-6.7; BD Biosciences or Biolegend or eBioscience), CD45 (clone 30-F11; Invitrogen or eBioscience), CD45.1 (clone A20; Biolegend), CD3 (clone eBio500A2; eBioscience), CD45.2 (clone 104; eBioscience), CD90.1 (clone OX-7; BD Biosciences), CD127 (clone A7R34; eBioscience or clone SB/199; Biolegend), KLRG1 (clone 2F1/KLRG1; Biolegend or eBioscience), CD4 (clone RM4-5; eBioscience or clone GK1.5; Biolegend), PD-1 (clone RMP1-30; eBioscience), CTLA-4 (clone UC10-4F10-11; BD Biosciences), NKG2A/C/E (clone 20d5; eBioscience), LAG3 (clone C9B7W; Biolegend), 2B4 (clone m2B4(B6)458.1; Biolegend), CD44 (clone IM7; eBioscience or Biolegend or BD Biosciences), Gr-1 (clone RB6-8C5; Biolegend), Ly6C (clone HK1.4; Biolegend), Ly6G (clone 1A8; Biolegend), CD11b (M1/70; eBioscience or Biolegend), CD11c (clone N418; Biolegend or eBioscience), CD169 (clone SER-4; eBioscience), CD115 (clone AFS98; eBioscience), CD64 (clone X54-5/7.1; Biolegend), CCR2 (clone 475301; R&D Systems), CD80 (clone 16-10A1; Biolegend), MHCII (clone AF6-120.1), F4/80 (clone BM8; Biolegend), Qa-1\(^b\) (clone 6A8.6F10.1A6; Miltenyi Biotec), PD-L1 (10F.9G2; Biolegend), IDO1 (clone 2E2/IDO1; Biolegend), IFN\(\gamma\) (clone XMG1.2; Biolegend).
2.10 Confocal Imaging

Tumor tissue was fixed and processed as previously described. Briefly, tumor tissue was fixed overnight in 0.05M phosphate buffer, 0.1M L-Lysine, 2mg/mL NaIO₄, and 10mg/mL paraformaldehyde. Tissue was dehydrated in 30% sucrose then frozen in OCT (Tissue-Tek). Tumors were cut into 20μm sections, blocked for two hours in 2% fetal bovine serum, 2% goat serum, and 0.5% Fc Block before staining for CD169 or F4/80 for 1 hour. Images were acquired on Zeiss LSM 780 FCS/NLO (Carl Zeiss) or Zeiss LSM 880. Images were analyzed using Imaris software (Bitplane, Inc.).

2.11 Statistical analysis

Statistical tests were performed in Prism (Graphpad). For tumor growth experiments, tumor growth curves were compared using two-way repeated measures ANOVA. For other experiments, a student t-test was used when comparing two groups, and a one-way ANOVA was used when comparing more than two groups. A paired t-test was used to compare inhibitory receptor expression in blood and TIL from the same mouse. Survival curves were analyzed using Logrank test in Prism.
Chapter 3: Generating MCMV-based Vectors Expressing Multiple Melanoma Antigens

Abstract:

Tumor antigen-specific CD8+ T lymphocytes can have potent antitumor effects, and evidence from studies using adoptive cell therapy have shown that sustained antitumor T cell responses confer the greatest clinical benefit. Thus, generating robust and sustained T cell responses against tumor antigens is a critical focus of cancer immunotherapy research. Cytomegalovirus is a herpesvirus that induces dramatic T cell responses against certain viral epitopes that are sustained at high frequencies over the lifetime of the host, a phenomenon termed “memory inflation”. Impressively, vaccination with a recombinant murine Cytomegalovirus (MCMV) expressing an altered melanoma antigen induces a memory inflation T cell response to the antigen, and this protects mice against a metastatic model of melanoma. In this study, we set out to engineer a novel recombinant MCMV expressing multiple tumor antigens with the aim of inducing memory inflation responses against multiple tumor antigens and improving the clinical efficacy of vaccination. Herein, we show that altering the melanoma antigen Trp2 at one amino acid reduces the expression of the antigen by recombinant MCMV vectors. We also show, in contrast to a previously published work, that MCMV expression of the native Trp2 protein does not delay tumor progression.

Introduction:

With the FDA approval of ipilimumab and nivolumab, cancer immunotherapy has entered mainstream clinical oncology.58,66 The potency of these therapies in some patients has shown the immense power of T cells in the fight against cancer. Unfortunately, not all patients respond to this therapy. T cell infiltration prior to therapy has been correlated with response to checkpoint
blockade and is a possible marker for stratifying potential responders prior to therapy.\textsuperscript{134} However, not all patients exhibit this spontaneous antitumor T cell response and thus require other forms of therapy.

Cancer vaccines have long been a focus of research within the tumor immunology field, but few vaccines have been effective in human trials.\textsuperscript{24,45} Our lab previously hypothesized that a persistent vaccine vector generating a prolonged antitumor T cell response would provide optimal tumor protection.\textsuperscript{129,130} Cytomegalovirus generates a unique CD8↑ T cell response termed memory inflation, characterized by the maintenance of a high-frequency of activated, virus-specific T cells for the lifetime of the host.\textsuperscript{95} Previously, we showed that a recombinant murine Cytomegalovirus (MCMV) expressing an altered melanoma antigen, gp100KGP, could induce a memory inflation CD8↑ T cell response against the native gp100 peptide, and vaccination with this recombinant virus delayed melanoma growth in a metastatic tumor model.\textsuperscript{129} Here, we engineer this same vaccine to express a second melanoma antigen, Trp2, and show that altering Trp2 at one amino acid reduces the expression of the protein in infected cells \textit{in vitro}. Surprisingly, vaccination with MCMV vectors expressing both gp100 and Trp2 antigens did not significantly delay tumor growth compared to the MCMV vector expressing the altered gp100 alone.

\textbf{Results:}

\textit{Altering Trp2 limits expression by MCMV-based vectors}

Our group has previously generated recombinant MCMV vectors expressing the melanoma antigen, gp100, or a mutated form of the antigen, gp100KGP. Highlighting the difficulty in vaccinating against self-peptides, we showed that mice immunized with MCMVgp100 did not
develop a CD8⁺ T cell response to gp100. However, mice vaccinated with MCMVgp100KGP developed a memory inflation T cell response against the native gp100 peptide and were protected against tumor challenge in a metastatic model of B16F10 melanoma. We hypothesized that the addition of a second melanoma antigen could enhance the efficacy of our MCMV-based vaccine. To this end, we sought to develop an MCMV vaccine expressing gp100KGP and a shared melanoma antigen, Trp2, or the mutated protein Trp2-2M. Trp2-2M is mutated within the CD8⁺ T cell epitope Trp2₁₈₀₋₁₈₈ (SVYDFVFVL => SMYDFVFVL) and has been shown to generate cross-reactive T cell responses upon peptide vaccination. Given the importance of promoter activity in regulating memory inflation responses and given the effective antitumor responses seen with MCMVgp100KGP, we aimed to have Trp2 expression controlled by the same promoter as gp100KGP. To do this, we inserted the native or altered murine Trp2 coding sequence downstream of the gp100KGP coding sequence, separated by an Internal Ribosomal Entry Site (IRES). This design enables the gp100KGP and the Trp2 genes to be transcribed into one mRNA transcript under the same promoter. The IRES then allows translation of Trp2 independent of gp100KGP translation (Figure 3-1). Using a two-step galK positive/negative selection recombineering protocol, we generated two recombinant MCMV vectors. MCMVgp100KGP-Trp2 was engineered to express the full-length murine Trp2 protein. MCMVgp100KGP-Trp2-2M was engineered to express a mutated Trp2 protein consisting of a single amino acid substitution within the CD8⁺ T cell epitope Trp2₁₈₀₋₁₈₈ (SVYDFVFVL => SMYDFVFVL).

We confirmed correct insertion of the IRES-Trp2 construct by sequencing and restriction mapping of the final bacterial artificial chromosomes (BAC) containing the recombinant MCMV genomes (Fig. 3-2). We next sought to confirm expression of the melanoma antigens by
performing Western blots on lysates from infected murine embryonic fibroblasts (MEFs) (Figure 3-3). Trp2 and gp100KGP proteins were not detected in lysates from MEFs infected with WT MCMV, whereas gp100KGP expression was confirmed in lysates from cells infected with either recombinant virus. Trp2 was easily detected in lysates from MEFs infected with MCMVgp100KGP-Trp2; however, there was minimal expression of Trp2-2M in infected MEFs, suggesting that the 2M mutation may have influenced Trp2 expression or protein stability.

*Trp2-specific T cells or antibodies are not detected following vaccination with recombinant MCMV vectors.*

Though it is difficult, several studies have shown it is possible to vaccinate mice with native Trp2 peptides and elicit a T cell response.\textsuperscript{137,138} Thus, we decided to test the ability of both recombinant MCMV viruses to induce CD8\textsuperscript{+} T cell responses to the native Trp2\textsubscript{180-188} epitope. To this end, mice were vaccinated with 10\textsuperscript{5} PFU WT MCMV, MCMVgp100KGP-Trp2, or MCMVgp100KGP-Trp2-2M. Seven days after vaccination, splenocytes were stimulated with native gp100\textsubscript{25-33} or native Trp2\textsubscript{180-188} peptide for 5 hours and stained for IFN\textgamma expression (Figure 3-4). None of the mice tested showed any reactivity to Trp2, again highlighting the difficulty of vaccinating against self-antigens. Recently, Xu et al. constructed a MCMV vector expressing native Trp2 and also could not detect a Trp2-specific T cell response following vaccination. However, this group showed that vaccination with MCMV expressing Trp2 could generate an antibody response against Trp2 and slightly delay tumor growth.\textsuperscript{127} We therefore asked if MCMVgp100KGP-Trp2 or MCMVgp100KGP-Trp2-2M could also generate tumor-specific antibodies. To test this, mice were again vaccinated with WT MCMV or recombinant MCMV and serum was collected 7 days later. An ELISA assay targeting B16F10 tumor lysate was performed to detect B16-specific antibodies in vaccinated mice. Serum from mice vaccinated
with WT MCMV showed an increase in reactivity compared to unvaccinated mice, likely a result of non-specific binding due to increased virus-specific IgG. Serum from mice vaccinated with either recombinant virus showed similar B16-reactivity compared to WT MCMV, suggesting a lack of B16-specific IgG in these mice (Figure 3-5). Thus, in contrast to the report by Xu et al., we were unable to detect an increase in B16-specific antibodies following vaccination with our recombinant viruses.

*Addition of Trp2 to MCMVgp100KGP does not enhance antitumor response*

While we did not detect adaptive immune responses to Trp2 in our previous assays, it was possible that the addition of Trp2 to MCMVgp100KGP would still yield beneficial antitumor effects as we were merely failing to detect these cellular responses in our specific assays. We therefore performed a final set of experiments to determine if addition of Trp2 to MCMVgp100KGP had any effects on tumor growth. Our previous study utilized a pulmonary metastatic model of melanoma in which mice were inoculated with B16F10 via the tail vein. In this model, the melanoma cells seed the lungs, forming individual nodules which can be quantified. To test the antitumor efficacy of our recombinant MCMV vectors, mice were inoculated with $10^5$ B16F10 cells via tail vein injection. Three days later, mice were vaccinated with $10^5$ PFU MCMVgp100KGP, MCMVgp100KGP-Trp2, or MCMVgp100KGP-Trp2-2M. On day 22, mice were euthanized and pulmonary tumor nodules were counted (Figure 3-6A). As shown in our previous study, vaccination with MCMVgp100KGP significantly decreased the tumor burden compared to untreated mice. Similarly, vaccination with MCMVgp100KGP-Trp2 or MCMVgp100KGP-Trp2-2M also significantly reduced the tumor burden compared to untreated mice. However, tumor burden between vaccinated groups was not significantly different. Lastly, we confirmed this result in a solid tumor model of melanoma in which B16F10
cells are injected intradermally. In this model, a solid tumor mass forms in the skin which can be measured over time using calipers. To try to increase the sensitivity of detecting differences between vaccinated groups, mice were vaccinated seven days prior to inoculation with B16 tumors. As in the metastatic model, vaccination with recombinant MCMV vectors delayed tumor growth when given prophylactically, but the addition of Trp2 did not enhance the efficacy of MCMVgp100KGP (Figure 3-6B).

Discussion:

The clinical development of vaccines targeting tumor antigens has been largely disappointing, reducing the optimism of many scientists for this approach to cancer therapy. However, the recent clinical successes of checkpoint inhibitors and adoptive cell therapy have shown the immense potential of antitumor T cell responses in fighting cancer. Unfortunately, only a subset of patients respond to these therapies. The effectiveness of these therapies likely depends on reinvigorating a pre-existing antitumor T cell response, meaning patients without this pre-existing response are unlikely to respond. Therefore, novel methods for generating antitumor T cell responses in these patients is urgently needed. Vaccination may be one of several effect means of producing these antitumor T cells, which can then be reinvigorated by checkpoint inhibition.

With this in mind, our lab has investigated the preclinical utility of Cytomegalovirus-based vaccines for use in melanoma. CMV infection elicits a unique CD8+ T cell response termed “memory inflation” which is characterized by the lifelong accumulation of activated virus-specific cells in lymphoid as well as non-lymphoid tissues. Impressively, this memory inflation response is still observed following vaccination with a non-replicative viral vector, suggesting that vectors with limited virulence could still be used for vaccination. A vaccine
that could generate such a sustained T cell response against a target antigen could be highly effective in different settings. Based on this idea, several groups have already tested recombinant MCMV viruses expressing epitopes from infectious agents like Influenza and Ebola. In these studies, recombinant MCMV vectors elicit memory inflation CD8+ T cell responses against the target epitope and provide long-term protection against challenge.\textsuperscript{119,139} This platform has also shown impressive preclinical results protecting rhesus macaques from highly pathogenic Simian Immunodeficiency Virus.\textsuperscript{123-125}

Our lab has previously applied this technology to create a novel MCMV-based vaccine expressing a modified melanoma antigen. By mutating the self-antigen, gp100, within a known CD8+ T cell epitope, we were able to generate tumor-reactive T cells following vaccination that delayed tumor progression in a metastatic model of melanoma. However, vaccination was not curative.\textsuperscript{129} Antigen loss is a potential escape mechanism for tumors under immune pressure.\textsuperscript{140,141} One way to minimize this escape mechanism is to target several antigens. At the same time, polyclonal T cell responses are likely to be more effective in initial therapy as well. For these reasons, we sought to develop an MCMV-based vaccine expressing multiple melanoma antigens.

In this study, we engineered our original MCMV melanoma vaccine (MCMVgp100KGP) to express a second melanoma antigen, Trp2, generating MCMVgp100KGP-Trp2. Because it is difficult to vaccinate against self-proteins, we also developed a second virus expressing a modified Trp2, MCMVgp100KGP-Trp2-2M. We hypothesized that this second modification would break self-tolerance and stimulate CD8+ T cells recognizing the Trp2\textsuperscript{180-188} epitope.\textsuperscript{135} While MCMVgp100KGP-Trp2 expressed high levels of gp100 and Trp2 \textit{in vitro}, MCMVgp100KGP-Trp2-2M showed dramatically reduced expression of Trp2 at the protein
level. The previous study using Trp2-2M vaccination utilized synthesized peptides and therefore did not investigate the effects of the 2M alteration on Trp2 protein expression.\textsuperscript{135} MCMVgp100KGP-Trp2 and MCMVgp100KGP-Trp2-2M differ only at one amino acid within the protein coding region. Thus, the regulatory units influences Trp2 transcription should remain functional between our two vectors. It is more likely that the 2M modification influences either the translation or protein stability of Trp2. These results highlight the potential problems in engineering altered epitopes into full-length proteins within expression vectors. Engineering MCMV vectors expressing shorter peptides containing the epitopes of interest may improve epitope expression by reducing potential misfolding.

Previous reports have shown that potent vaccine formulations can induce T cell responses against the native Trp2 peptide.\textsuperscript{137,138} We therefore decided to test our novel MCMV vectors \textit{in vivo}. We were unable to detect Trp2-specific CD8\textsuperscript{+} T cell responses following vaccination with either recombinant MCMV, illustrating the difficulty of vaccinating against self-proteins. A previous study by Xu et al. similarly showed that a MCMV vector expressing the native Trp2 was unable to induce a Trp2-specific T cell response following vaccination. However, this study showed that vaccination with this vector slightly protected mice from tumor challenge by eliciting a Trp2-specific antibodies.\textsuperscript{127} We were unable to detect tumor-specific IgG following vaccination with MCMVgp100KGP-Trp2, and vaccination with either MCMVgp100KGP-Trp2 or MCMVgp100KGP-Trp2-2M did not delay tumor growth compared to MCMVgp100KGP in a solid tumor or metastatic model of B16 melanoma.

Our results stand in stark contrast to the study by Xu et al. While neither study could generate Trp2-reactive T cells, we were unable to show any clinical benefit of Trp2 expression by an MCMV vector. Several differences in our studies may explain this, the discrepancy. First, the
regulatory elements controlling Trp2 were different in the two studies. Xu et al. inserted the complete Trp2 coding sequence under the control of the human CMV major immediate early promoter and enhancer element, whereas, in our study, Trp2 transcription was controlled by the human CMV immediate early promoter without enhancer element. As described above, this ensured that in our study Trp2 was transcribed along with gp100KGP but was translated independent of gp100KGP through an internal ribosomal entry site. Our data show that Trp2 was successfully expressed in MEFs infected with MCMVgp100KGP-Trp2. Nonetheless, the different regulatory elements involved in these two studies may influence the overall expression levels of Trp2 or the cell-specific expression of Trp2. Differences in the overall expression of Trp2 or cell-specific expression may account for the differences observed in the two studies.

Second, discrepancies in inoculation dose may contribute to our conflicting results. Xu et al. vaccinated mice with $4 \times 10^6$ PFU MCMV-TRP2 which is a large inoculation dose for MCMV. In our current study, mice were vaccinated with $10^5$ PFU MCMVgp100KGP-Trp2. Inoculation dose is known to impact the magnitude of antibody responses to MCMV antigens. While this lower dose was effective enough to delay tumor growth compared to untreated mice, we did not observe an IgG response to B16 or enhanced tumor protection compared to MCMVgp100KGP vaccination.

In conclusion, in this study we set out to generate a recombinant MCMV vaccine expressing multiple melanoma antigens with the aim of generating multiple CD8$^+$ T cell memory inflation responses against melanoma. Herein, we show that the addition of native Trp2 to MCMV vectors is unable to break self-tolerance. We were unable to detect T cell or antibody responses to Trp2 following vaccination with MCMVgp100KGP-Trp2. In order to break self-tolerance against Trp2, we created a second MCMV vector expressing an altered Trp2. However,
mutating Trp2 within the CD8$^+$ T cell epitope Trp2$_{180-188}$ significantly reduced the expression of the full-length protein in infected MEFs. This study highlights the difficulty in vaccinating against shared tumor antigens. Overcoming tolerance mechanisms is a problematic step in vaccinating against shared tumor antigens. Future studies generating MCMV-based vaccines against tumor-specific epitopes, so called neo-epitopes, may enhance the efficacy of this vaccine platform in tumor immunotherapy.

**Figures:**

**Figure 3-1: Expression of gp100KGP and Trp2 in MCMV vectors.** In order to keep Trp2 under an effective memory inflation promoter, the Trp2 coding sequence was inserted downstream of gp100KGP before the poly(A) signal sequence and separated by an internal ribosomal entry site (IRES). This allows gp100KGP and Trp2 transcription under the HCMV promoter into a single mRNA transcript. Translation of Trp2 will occur independent of gp100KGP due to IRES recruitment of ribosomal machinery.
Figure 3-2: Restriction map of bacterial artificial chromosomes containing recombinant MCMV genomes. BAC DNA encoding (A) MCMVgp100KGP, (B) MCMVgp100KGP-Trp2, or (C) MCMVgp100-Trp2-2M were purified and digested with HindIII for 4 hours and run on 0.6% agarose gel for 24 hours. Arrows show the expected changes in restriction bands following correct insertion of the IRES-Trp2 sequences.
Figure 3-3: Expression of melanoma antigens in fibroblasts infected with recombinant MCMV vectors. Mouse embryonic fibroblasts (MEFs) were infected with recombinant MCMV vectors and lysates were analyzed by Western blot for protein expression of gp100 or Trp2. Lysates from 1) uninfected MEFs or 2) MEFs infected with WT MCMV showed no Trp2 or gp100 expression. Lysates from 3) MEFs infected with MCMVgp100KGP-Trp2 showed clear bands for Trp2 and gp100, whereas lysates from 4) MEFs infected with MCMVgp100KGP-Trp2-2M showed clear gp100 expression but minimal Trp2 expression. Band specificity was confirmed using lysates from 5) B16F10 cells in culture.
Figure 3-4: Trp2-specific T cells are not detected following vaccination with recombinant MCMV vectors. C57BL/6 mice were vaccinated with $10^5$ PFU WT MCMV, MCMVgp100KGP, MCMVgp100KGP-Trp2, or MCMVgp100KGP-Trp2-2M i.p. Seven days later, splenocytes were stimulated with m38 peptide (SSPPMFRV), gp100 peptide (EGSRNQDWL), Trp2 peptide (SVYDFFVWL), or PMA/I for 5 hours followed by intracellular cytokine staining. Representative plots of 3 mice/group gated on CD45+CD3+CD8+ cells.
Figure 3-5: B16-specific antibodies are not detected following vaccination with recombinant MCMV vectors. Serum was collected from mice that were previously vaccinated 7 days prior with WT or recombinant MCMV. Serum was serially diluted and incubated on plates pre-coated with B16 tumor lysate. Plates were washed and stained with anti-mouse IgG-HRP and incubated with TMB substrate prior to absorbance reading at 492 nm. Serum from mice infected with WT MCMV or recombinant MCMV all showed increased absorption compared to serum from uninfected mice. However, there was no significant difference in absorption between the different MCMV viruses. n=3 mice/group.
Figure 3-6: Addition of Trp2 to MCMVgp100KGP does not improve antitumor response vs. MCMVgp100KGP. A) Mice were inoculated with $10^5$ B16F10 via tail vein injection and vaccinated with $10^5$ PFU recombinant MCMV three days later. On Day 22, mice were sacrificed and lung tumor nodules were counted. B) Mice were vaccinated with $10^5$ PFU recombinant MCMV seven days prior to intradermal inoculation with $10^5$ B16F10. Tumor surface area was monitored over time using manual calipers. n=7-8 mice/group for A) and 10-16 mice/group for B). *p<0.05; **p<0.01.
Chapter 4: Combination Immunotherapy with MCMV-based Vaccines

Abstract:

With the exception of cancers associated with infectious agents, cancer vaccines have largely failed to impact disease progression. The reasons for this are multifactorial, including suboptimal vaccine formulations, poor choice of target antigens, and a highly immunosuppressive tumor microenvironment. In this study, we sought to increase the antitumor effects of a MCMV-based tumor vaccines by combining vaccination with other clinically-relevant immunotherapies to enhance antitumor T cell responses within the tumor microenvironment. Herein, we show that vaccination against a foreign tumor antigen can significantly delay tumor growth, and increasing the magnitude of this response through adoptive cell therapy cures mice with established tumors. In contrast, vaccination against a shared tumor antigen is insufficient to delay growth of an established solid tumor. Increasing the frequency of the antitumor T cell response through adoptive cell therapy significantly delays tumor growth, but vaccination also induces a robust counter-regulatory response marked by elevated PD-L1, Qa-1b, IDO expression, and recruitment of monocytic MDSCs. Surprisingly, combination immunotherapy targeting several of these pathways did not impact survival of tumor-bearing mice. Thus, this study highlights several potential barriers to effective tumor vaccination when targeting a shared tumor antigen and questions the accuracy of inhibitory pathway expression as a biomarker for responses to therapy.
Introduction:

After decades of research, immunotherapy has finally joined surgery, radiotherapy, and chemotherapy as a standard treatment modality in clinical oncology. Unfortunately, these therapies seem to only work in patients who have a pre-existing immune response against the tumor, so called “inflamed” tumors, leaving little to no benefit for patients with “non-inflamed” tumors. Methods for converting “non-inflamed” tumor into “inflamed” ones are therefore needed to treat this subset of patients. Vaccination is one potential method to generate an adaptive immune response against tumor cells. Vaccination against tumor antigen has been a highly active area of research for decades that has yielded little clinical benefit as monotherapy. Reasons for the failures likely include choice target antigen and an immunosuppressive tumor microenvironment.

Recent studies have suggested that the clinical responses seen in some patients following T cell checkpoint blockade or adoptive cell therapy are likely due to T cell responses against mutated tumor peptides termed neo-epitopes. Because they target non-self-peptides, high affinity neo-epitope-specific T cells do not get deleted during development in the thymus and are likely more effective in recognizing tumor cells. Several groups have shown in preclinical models that it is possible to vaccinate against neo-epitopes and delay tumor growth, and clinical trials vaccinating patients against these antigens are currently underway (NCT01970358; NCT02035956).

The majority of tumor vaccines tested thus far have targeted shared antigens, i.e. antigens expressed by malignant and healthy tissue alike. Vaccination against this class of tumor antigen is difficult because high affinity T cells recognizing these antigens are largely deleted in
the thymus during development or are rendered unresponsive due to peripheral tolerance. However, it is possible to stimulate T cells against these antigens in certain cases.43

Studies focused on a particular form of immunotherapy called adoptive cell therapy have suggested that prolonged T cell responses against tumor antigen inversely correlate with clinical progression.48 Our lab has previously hypothesized that a persistent vaccine stimulating lifelong T cell responses against tumor antigen would be an effective cancer immunotherapy.129,130 To test this, our group developed a vaccine based on Murine Cytomegalovirus targeting a shared melanoma antigen, gp100. By mutating gp100 within a CD8+ T cell epitope, we were able to generate gp100-reactive T cells which delayed metastatic tumor progression following vaccination with MCMVgp100KGP. However, vaccinated mice eventually succumb to disease.129 In the current study, we tested whether MCMV-based vaccines were effective in a solid tumor model (as opposed to the pulmonary metastatic model previously described). In this way, we could examine the influence of an established tumor microenvironment on vaccine-stimulated T cells. Our results show that an MCMV-based vaccine targeting a model neo-epitope is highly effective in treating melanoma-expressing the same neo-epitope, and combining this vaccination with adoptive cell therapy completely cures mice of disease. We also show that prophylactic vaccination with MCMVgp100KGP delays tumor growth, but therapeutic vaccination has no effect on growth of established tumors. Combining MCMVgp100KGP vaccination with adoptive cell therapy delays growth of established tumors and also induces several counter-regulatory mechanisms, including expression of inhibitory receptor ligands and recruitment of immunoregulatory cells. Surprisingly, combination immunotherapy targeting several of these counter-regulatory pathways did not enhance the efficacy of vaccination. This study highlights the difficulty in treating a highly immunosuppressive tumor with a vaccine
targeting a shared antigen and suggests that expression of immunosuppressive molecules within the tumor microenvironment does not always predict response to some modes of immunotherapy.

**Results:**

*Therapeutic vaccination with MCMVgp100KGP does not delay tumor growth in an intradermal solid tumor model.*

Our group has previously shown that MCMVgp100KGP vaccination reduces tumor burden in a pulmonary metastatic model of B16 melanoma.\textsuperscript{129} In order to more closely examine the impact of the tumor microenvironment on vaccine efficacy, we switched to an intradermal solid tumor model in which a single solid tumor forms rather than multiple pulmonary nodules, allowing us to track tumor growth over time and easily isolate tumor infiltrating leukocytes (TIL). To test the efficacy of vaccination in this model, female C57BL/6 mice were vaccinated with $10^5$ PFU MCMVgp100KGP or mock vaccinated seven days prior to intradermal challenge with $10^5$ B16F10 melanoma cells. As expected, vaccinated mice were protected from tumor challenge, showing significantly delayed tumor growth (Figure 4-1A). In contrast, therapeutic vaccination three days following tumor challenge had minimal effect on tumor growth (Figure 4-1B). This suggested that an established tumor microenvironment may severely limit the efficacy of vaccine-stimulated T cells. There are several mechanisms that may explain this limitation, one of which is that vaccine-stimulated T cells fail to migrate into the tumor parenchyma. To test this idea, mice were inoculated with B16F10 i.d. and vaccinated three days later. Tumors were harvested and TIL isolated for flow cytometry. Vaccinated mice had significantly more CD8\textsuperscript{+} TIL than unvaccinated mice, and CD8\textsuperscript{+} TIL expressed higher levels of the activation marker, CD11a in vaccinated mice vs. mock vaccinated (Figure 4-2A&B). Though some of the CD8\textsuperscript{+}
TIL in vaccinated mice are likely MCMV-specific and not gp100-specific, this data nonetheless shows that MCMV-stimulated T cells can infiltrate a solid tumor, suggesting lack of T cell migration is unlikely to account for therapeutic inefficiency.

*PD-1 and CTLA-4 blockade do not improve antitumor response of therapeutic MCMV-gp100KGP*

Immune checkpoint inhibitors against PD-1 and CTLA-4 have recently been approved for use in metastatic melanoma. PD-1 and CTLA-4 are inhibitory receptors expressed on the surface of activated T cells that limit T cell activation. We asked whether TIL from MCMVgp100KGP vaccinated mice expressed these inhibitory receptors. Around 50% of CD8+ TIL expressed PD-1, regardless of whether mice received vaccination, while only 5% of CD8+ TIL expressed CTLA-4 (Figure 4-2C-F). A previous study showed that PD-1 or CTLA-4 blockade could synergize with vaccination to B16 melanoma when given early in tumor progression. We therefore asked if checkpoint blockade could synergize with MCMVgp100KGP vaccination. To this end, mice were inoculated with B16F10 i.d. and vaccinated with MCMVgp100KGP or mock vaccinated. Mice either received anti-PD-1 on days 6, 9, and 12 (Figure 4-3B) or anti-CTLA-4 on days 3, 6, and 9 (Figure 4-3C). Neither therapy significantly delayed tumor growth compared to unvaccinated mice.

*Adoptive cell therapy targeting model neo-epitope enhances MCMVova vaccination*

Adoptive cell therapy is a form of immunotherapy that involves transferring high frequencies of *ex vivo* cultured tumor-reactive T cells into patients. These tumor-reactive T cells are either isolated directly from tumor tissue and expanded in culture before transfer, or peripheral blood lymphocytes are genetically engineered to express a tumor-specific receptor. Importantly,
clinical response has been correlated with persistence of transferred cells in the patient, sparking a wave of interest in extending transferred cell survival.\(^{48}\) Several groups have tried to take advantage of the persistent nature of the T cell response to CMV to extend the survival of tumor-reactive T cells. Most of these efforts have focused on redirecting CMV-specific inflationary T cells to recognize tumor antigen.\(^{145-147}\) In a similar way, we hypothesized that CMV-based vaccines would promote persistence of adoptively transferred tumor-reactive T cells. To test this, we first utilized a recombinant MCMV expressing the model antigen, ovalbumin (MCMVova). MCMVova infection induces a robust inflationary T cell response that protects mice from challenge with B16 tumor cells expressing ova.\(^{129}\) We first asked if MCMVova could promote long-term persistence of adoptively transferred T cells. C56BL/6 mice received \(10^5\) OT-I T cells i.v., which express a transgenic T cell receptor recognizing SIINFEKL epitope within ovalbumin, followed by vaccination with \(10^5\) PFU WT MCMV or MCMVova. As expected, OT-I cells expanded dramatically in response to MCMVova vaccination but were undetectable in mice vaccinated with WT MCMV (Figure 4-4A). OT-I expansion peaked seven days after transfer and displayed a typical effector memory phenotype characterized as KLRG1\(^+\) CD127\(^{lo}\) (Figure 4-4B). OT-I frequency progressively declined in 8/9 mice, while in one mouse, OT-I cells were maintained at high frequency over four months after transfer (Figure 4-4C).

Our lab has previously shown that MCMVova delays tumor growth and improves survival in a metastatic model of melanoma.\(^{129}\) We hypothesized that adoptive cell therapy could synergize with MCMV-based vaccines to delay tumor growth. Mice were inoculated with B16ova i.d. and eight days later received \(10^5\) OT-I cells i.v. followed by vaccination with WT MCMV or MCMVova. Like in the metastatic model, MCMVova significantly delayed growth of an intradermal solid tumor, yet mice eventually succumbed to disease. Adoptive cell transfer and
MCMVova vaccination significantly improved therapeutic efficacy and cured several mice (Figure 4-5). This data shows that adoptive cell therapy can synergize with a persistent vaccine vector to cure mice, even if transferred cells do not persist long-term. This may be a result of the dramatic expansion of OT-I cells early followed by the persistent endogenous OVA-specific response generated by MCMVova or the continued presence of endogenous OVA-specific T cells stimulated by MCMVova.

*MCMVgp100KGP is a more potent stimulatory of adoptively transferred gp100-specific T cells than VSVgp100KGP*

Given the dramatic results seen when combining adoptive cell therapy and MCMV vaccines targeting foreign antigens, we next asked whether this combination therapy would be as effective when targeting a shared tumor antigen. For this, we utilized transgenic T cells (PMEL) expressing a T cell receptor recognizing the CD8+ T cell epitope gp10025-33. We began by asking whether MCMVgp100KGP could stimulate adoptively transferred PMEL cells. C57BL/6 mice received 10^5 CD8+ PMEL cells i.v. followed by vaccination with PBS, WT MCMV, MCMV expressing native murine gp100 (MCMVgp100), or MCMVgp100KGP. Five days later, only mice vaccinated with MCMVgp100KGP showed expansion of transferred PMELs (Figure 4-6A). Similar to the OT-I and MCMVova response, PMEL cells were not maintained long-term in the blood of mice vaccinated with MCMVgp100KGP (Figure 4-6B). We next asked how MCMVgp100KGP stimulation compared to an acute viral vector, Vesicular Stomatitis Virus expressing gp100KGP (VSVgp100KGP). Mice received 10^5 PMEL cells i.v. followed by vaccination with PBS, MCMVgp100KGP, or VSVgp100KGP. Five days later, mice vaccinated with MCMVgp100KGP displayed a higher frequency of PMEL cells in blood compared to mice vaccinated with VSVgp100KGP. This difference was also seen in peripheral blood and lungs.
five (blood) and eight (lungs) months after vaccination (Figure 4-6C & Figure 4-7). Thus, while MCMVgp100KGP did not sustain a very high frequency of PMEL cells after transfer, it generates more potent T cell responses compared to an acute viral vector.

Adoptive cell therapy and MCMVgp100KGP vaccination significantly delays growth of established solid tumors

As discussed above, prophylactic vaccination with MCMVgp100KGP significantly delays B16 tumor growth, but therapeutic vaccination does not influence tumor growth. In contrast, we found that vaccination with MCMVova potently delayed B16ova tumor growth. This discrepancy may be partly attributed to the nature of the target antigen. Because ova is a foreign antigen, high affinity T cells recognizing ova should not be hindered by central and peripheral tolerance mechanisms. However, gp100 is a shared tumor antigen expressed in B16 melanoma as well as normal melanocytes. Because of this, high affinity T cells recognizing gp100\textsubscript{25-33} are deleted in the thymus during development. Thus, any vaccine targeting gp100 is severely limited by a low precursor frequency. We therefore hypothesized that increasing the precursor frequency of gp100-reactive T cells would enhance the effect of MCMVgp100KGP vaccination. As expected, combining MCMVgp100KGP vaccination with adoptive transfer of PMEL cells significantly delayed growth of established tumors, while either monotherapy had no effect on tumor growth (Figure 4-8). Thus, like our results using MCMVova, adoptive cell therapy greatly enhances the efficacy of MCMV-based vaccines even though adoptively transferred cells do not persist at high frequency.
The tumor microenvironment induces the expression of several inhibitory receptors on the surface of MCMVgp100KGP-stimulated T cells

Though tumor growth was delayed with adoptive cell therapy and vaccination, tumors continued to progress even with therapy. We asked what factors may limit the efficacy of this therapy. The tumor microenvironment can greatly influence immune cell function.\textsuperscript{149,150} We first asked whether MCMVgp100KGP-stimulated T cells were influenced by the tumor microenvironment. To test this, tumor-bearing mice were treated with PMEL transfer and MCMVgp100KGP vaccination, and six days later, peripheral blood and tumor tissue were harvested for analysis by flow cytometry. At this time point, PMEL cells made up 5-10\% of CD8\textsuperscript{+} T cells from peripheral blood but constituted up to 50\% of CD8\textsuperscript{+} T cells in tumor tissue, suggesting a preferential accumulation of these cells within the tumor tissue (data not shown). PMEL cells isolated from tumor tissue expressed significantly more PD-1, LAG-3, 2B4, and NKG2A/C/E compared to PMEL cells isolated from peripheral blood (Figure 4-9). PD-1, LAG3, and 2B4 are inhibitory receptors upregulated on exhausted T cells. Expression of several of these receptors on T cells is associated with reduced effector function in exhausted T cells.\textsuperscript{151} Thus, our data suggests that the tumor microenvironment alters the functionality of PMEL cells and may limit the efficacy of therapy.

Inhibitory ligands are upregulated on tumor cells and tumor infiltrating leukocytes after vaccination with MCMVgp100KGP

PD-1 binds its cognate ligand PD-L1 within the tumor microenvironment to limit T cell function.\textsuperscript{77} PD-L1 is upregulated in response to IFN\textgamma, but it can also be constitutively expressed in some cancers.\textsuperscript{152} PD-L1 expression has also been postulated as a predictive marker for response to anti-PD-1 therapy.\textsuperscript{76,152} NKG2A is an inhibitory receptor expressed on the surface of
NK cells and CD8+ T cells. NKG2A binding its cognate ligand, HLA-E in humans and Qa-1b in mice, limits CD8+ T cell function during infection and protects tumor cells from cytolysis.153,154 Because PMEL cells within the tumor tissue expressed high levels of PD-1 and NKG2A, we next asked if PD-L1 and Qa-1b are also expressed in the tumor microenvironment. To this end, mice were inoculated with B16F10 expressing red fluorescent protein (B16RFP) and left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. Six days later, tumor tissue was harvested for flow cytometry. Only 2% of B16RFP cells from culture expressed PD-L1 and less than 2% expressed Qa-1b (Figure 4-10A-C). 20-50% of RFP+ cells from untreated tumors and control treated tumors were PD-L1+ and about 5-20% were PD-L1+ and Qa-1b+. In contrast, PMEL transfer and MCMVgp100KGP induced significant upregulation of PD-L1 and Qa-1b on RFP+ tumor cells (Figure 4-10A-C). Essentially all tumor cells expressed PD-L1 and 50-60% of tumor cells expressed both PD-L1 and Qa-1b. Similarly, both inhibitory ligands were also upregulated on CD45+CD8- tumor infiltrating leukocytes following MCMVgp100KGP vaccination (Figure 4-10D-F). This data highlights the highly dynamic nature of inhibitory ligand expression within the tumor microenvironment and shows that infiltration by tumor non-specific T cells (i.e. virus specific T cells following WT MCMV) does not induce a counter-regulatory response.

Combination checkpoint blockade does not extend survival following MCMVgp100KGP vaccination

Antibodies targeting PD-1 and PD-L1 are now approved therapies for metastatic melanoma.66 Blocking antibodies against NKG2A are also in development for the treatment of cancer.155 Given the high expression of these inhibitory pathways following MCMVgp100KGP vaccination, we hypothesized that these counter-regulatory pathways limit T cell function within
the tumor microenvironment. We therefore asked if blockade of PD-1 and Qa-1\(^b\) could synergize with MCMVgp100KGP vaccination to delay tumor growth. Mice were inoculated with B16F10 and five days later were left untreated or received PMEL transfer and MCMVgp100KGP vaccination. On days 10, 12, and 14, mice were treated with anti-PD-1 and/or anti-Qa-1\(^b\) and tumor growth measured every two to three days. Anti-PD-1 therapy had a minor effect on survival, but neither anti-PD1, anti-Qa-1\(^b\), nor combination blockade significantly prolonged survival of vaccinated mice (Figure 4-11).

**MCMVgp100KGP vaccination induces accumulation of monocytic MDSCs and upregulation of IDO1**

Several studies have reported the induction of other immunosuppressive mechanisms in response to antitumor T cell activity.\(^{72,156}\) We therefore asked if MCMVgp100KGP vaccination increased the recruitment of other immunosuppressive cells to the tumor microenvironment. To this end, mice were inoculated with B16F10 i.d. and left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. As expected, Thy1.1+ PMEL cells accumulated in tumor tissue in mice vaccinated with MCMVgp100KGP but not WT MCMV (Figure 4-12A). In response to MCMVgp100KGP vaccination, we noted an impressive accumulation of Gr-1\(^+\)CD11b\(^+\) myeloid cells which was not noted by PMEL transfer and vaccination with control viruses WT MCMV or MCMVgp100 (Figure 4-12B). Gr-1\(^+\) cells within the tumor microenvironment are generally characterized as Myeloid Derived Suppressor Cells (MDSCs). The Gr-1 antibody recognizes both Ly6G and Ly6C on MDSCs, and therefore, labels two populations of MDSCs: monocytic-MDSCs characterized by Ly6C expression and granulocytic-MDSCs characterized by Ly6G expression.\(^{157}\) B16F10 tumors contained few Ly6G\(^+\) cells regardless of vaccination status.
In contrast, Ly6C$^+$ myeloid cells greatly outnumbered Ly6G$^+$ cells in all tumors and significantly increased in number following MCMVgp100KGP vaccination (Figure 4-12C). A previous study showed that adoptive transfer of \textit{in vitro} activated PMEL cells induced a counter-regulatory recruitment of Ly6C$^+$ MDSCs that potently inhibited T cell responses.\textsuperscript{156} We hypothesize that our results show an accumulation of these same previously described MDSCs.

Indoleamine 2,3-dioxygenase 1 (IDO) is a cytosolic enzyme responsible for the degradation of tryptophan, and its expression is regulated by IFN$\gamma$.\textsuperscript{72,158} IDO is expressed in myeloid cells as well as tumor cells and has been shown to limit the response to checkpoint blockade.\textsuperscript{159-161} A recent study also showed that IDO expression by B16 melanoma resulted in the recruitment of monocytic-MDSCs within the tumor microenvironment.\textsuperscript{162} Given the significant accumulation of Ly6C$^+$ myeloid cells in response to MCMVgp100KGP and the responsiveness of IDO to IFN$\gamma$, we hypothesized that MCMVgp100KGP would also upregulate IDO expression within the tumor and this upregulation may be partly responsible for the accumulation of Ly6C$^+$ cells following MCMVgp100KGP vaccination. As before, mice were inoculated with B16RFP i.d. and left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. Six days after treatment, IDO expression was trending upwards in RFP$^+$ tumor cells from mice treated with MCMVgp100KGP vaccination but failed to reach statistical significance (Figure 4-13). Thus, in addition to PD-L1, tumor cells also appeared to upregulate IDO (albeit at low levels) in response to antitumor T cell attack following MCMVgp100KGP vaccination.
Combination IDO inhibition and checkpoint blockade does not improve response to MCMVgp100KGP vaccination

As stated above, IDO has been shown to inhibit antitumor T cell responses and diminish therapeutic responses to checkpoint blockade.\textsuperscript{161,163} Several inhibitors of IDO1 are entering clinical trials, including 1-methyl-tryptophan (1-MT).\textsuperscript{164} Given the enhanced expression of both PD-L1 and IDO in response to MCMVgp100KGP vaccination, we hypothesized that inhibition of these pathways would improve therapeutic responses to MCMVgp100KGP. To test this, mice were inoculated with B16F10 i.d. and treated with PMEL transfer and MCMVgp100KGP vaccination five days later. Starting on day 7, mice received 1-MT in their drinking water or control water. Mice were also treated with anti-PD-1 blocking antibody; however, combination therapy did not influence tumor growth and did not extend survival of mice following vaccination (Figure 4-14).

Combination chemo-immunotherapy delays tumor growth and prolongs survival of mice bearing established tumors

Studies have shown that host preconditioning with chemotherapy or radiation can significantly enhance the effects of immunotherapy.\textsuperscript{165} Cyclophosphamide (CTX) is a commonly used alkylating agent that can synergize with immunotherapy. At low doses, CTX can reduce numbers of immunosuppressive Tregs, and at high doses, it can modulate type I IFN signaling to enhance vaccination.\textsuperscript{166-168} We therefore asked if MCMVgp100KGP vaccination in combination with cyclophosphamide preconditioning could treat established tumors. To test this, mice were challenged with B16F10 and six days later received a single 4mg dose of CTX. The following day mice were treated with $10^5$ in vitro activated PMEL cells, vaccination with MCMVgp100KGP, or both (Figure 4-15A). CTX alone significantly delayed tumor growth.
compared to untreated mice (Figure 4-15B). MCMVgp100KGP vaccination alone, PMEL transfer alone, or PMEL transfer and WT MCMV vaccination did not delay tumor growth compared to CTX alone. However, PMEL transfer and MCMVgp100KGP vaccination significantly delayed tumor growth compared to all other treatment groups. Combination therapy also significantly prolonged survival, suggesting that combination therapy combining several therapeutic modalities may be most beneficial in delaying tumor growth (Figure 4-15C).

Discussion:

With the FDA approval of ipilimumab and nivolumab, cancer immunotherapy has finally become a standard treatment modality for some cancers. Unfortunately, not all patients respond to these therapies. Increasing evidence suggests that response is often seen in patients with tumors containing a high frequency of nonsynonymous mutations and high levels of T cell infiltration.41,42,73,77,134 These observations have led scientists and clinicians to distinguish tumors as “inflamed” i.e. containing many T cells or “non-inflamed” in which tumors-specific T cells are lacking.134,143 “Inflamed” tumors contain spontaneous T cell responses recognizing tumor antigen, yet these T cells are prevented from destroying malignant cells. Patients with these kinds of tumors are more likely to benefit from checkpoint blockade than patients with “non-inflamed” tumors. Therefore, novel methods for converting “non-inflamed” tumors into “inflamed” tumors are highly sought. A potential method to do this is through vaccination against tumor antigen.

Cytomegalovirus is a β-herpes virus that infects most individuals over the age of 40.80 This persistent infection induces a substantial expansion of CD8+ T cells recognizing viral antigens, and these T cells are maintained at high frequency over the lifetime of the host.95 Our lab has previously shown that a persistent vaccine vector based on murine Cytomegalovirus generates
prolonged T cell responses against the melanoma antigen, gp100 and protects mice from a model of metastatic melanoma. However, mice eventually succumb to disease.

In this study, we sought to determine if our vaccine MCMVgp100KGP would be effective against a solid intradermal tumor. As hypothesized, prophylactic MCMVgp100KGP vaccination protected mice from an intradermal challenge of B16F10 melanoma. However, therapeutic vaccination had no effect on tumor growth. This inefficiency was not due to T cell exclusion from the tumor microenvironment as MCMVgp100KGP vaccination significantly increased the number of CD8$^+$ T cells within the tumor.

Adoptive cell therapy is an effective form of immunotherapy involving the transfer of large numbers of \textit{ex vivo} cultured tumor-reactive T cells into patients. One version of this therapy involves genetically engineering T cells to express tumor-specific receptors. Because most self-reactive T cells are deleted in the thymus during development, this form of therapy avoids the problem of vaccinating against self-antigens. In this study, we asked whether MCMVgp100KGP was limited by the low precursor frequency of gp100-specific T cells. Our previous results showed that about 2\% of CD8$^+$ T cells recognized gp100 following MCMVgp100KGP vaccination. Here, we show that following MCMVgp100KGP vaccination, PMEL cells made up 10-20\% of circulating CD8$^+$ T cells. This increase in magnitude significantly delayed tumor growth, confirming our hypothesis that MCMVgp100KGP vaccination is limited by precursor frequency.

Work within the field of adoptive cell therapy has shown that persistence of transferred cells correlates with clinical efficacy. Therefore, significant research is focused on identifying methods to improve the persistence of adoptively transferred cells. Several groups have tried to utilize the persistent and ubiquitous nature of Cytomegalovirus to maintain adoptively
transferred cells.\textsuperscript{146,147} These studies have genetically engineered CMV-specific T cells to express a tumor-specific receptor with the thought that continuous stimulation through the endogenous CMV-specific TCR would maintain transferred cells long-term. In this same line of thought, we hypothesized that a CMV-based vaccine could maintain adoptively transferred T cells long-term by continuously stimulating the expansion of these cells. We tested this in two models. MCMVova vaccination induced a massive expansion of adoptively transferred OT-I cells, but these cells were not maintained long-term. Only one of nine mice showed a substantial maintenance of OT-I’s in peripheral blood several months after vaccination. This is in contrast to a previous study by Turula et al. showing that infection with MCMVova maintained transferred OT-I’s at high frequency over time.\textsuperscript{169} This report also noted immune rejection of transferred OT-I’s in some mice due to minor allelic changes between mouse strains. Though unlikely, this same rejection may account for the reduced persistence of OT-I’s in our study as well. Another possible reason for this discrepancy is the number of transferred cells. In the study by Turula et al., mice received 600 OT-I cells prior to infection, whereas our study involved the transfer of $10^5$ OT-I cells.\textsuperscript{169} This difference in starting cell number may influence persistence of cells through clonal competition or reduction in viral load, which is known to influence MCMV T cell inflation.\textsuperscript{170} In a second model, we show that MCMVgp100KGP maintains transferred PMEL cells at higher frequencies than VSVgp100KGP, albeit still only at 1\% of total CD8$^+$ T cells in peripheral blood. PMEL cells were found at slightly higher frequencies in lungs of mice vaccinated with MCMVgp100KGP 263 days prior. Thus, similar to MCMVova, MCMVgp100KGP maintains transferred antitumor T cells but at low frequency. Impressively, despite the decline in frequency of transferred cells over time, OT-I transfer and MCMVova vaccination cured several mice of B16ova, and PMEL transfer and
MCMVgp100KGP vaccination significantly delayed growth of B16F10 tumors. These results highlight the potential of MCMV-based vaccines in combination with adoptive cell transfer and emphasize the difficulty in targeting a non-mutated shared tumor antigen.

While adoptive transfer and MCMVgp100KGP vaccination delayed B16 tumor growth, mice eventually succumb to disease. Many mechanisms exist that allow malignant cells to evade host immunity, and several of these pathways are highly upregulated in response to T cell attack, a process termed adaptive resistance. In response to T cell attack, “inflamed” tumors will upregulate counter-regulatory mechanisms like PD-L1 expression, IDO1 expression, and recruitment of regulatory immune cells. As stated above, patients who respond to current immunotherapies tend to show increased T cell infiltration within the tumor tissue and expression of these counter-regulatory pathways, suggesting an active immune response.

B16F10 is notoriously difficult to treat with single agent immunotherapy and is characterized by low levels of immune cell infiltration relative to other murine tumors. We would therefore classify B16F10 as a “non-inflamed” tumor. As discussed above, “non-inflamed” tumors are thought to be unresponsive to single agent immunotherapy due to lack of T cell infiltration within the tumor tissue. In this study, we show that following PMEL transfer and vaccination with MCMVgp100KGP PMEL cells accumulate within the tumor tissue at high frequency, suggestive of an “inflamed” tumor microenvironment. We also show upregulation of several inhibitory pathways (PD-L1, Qa-1b, IDO1, and mo-MDSCs recruitment) following T cell infiltration. Thus, at least by these parameters, our data suggests that vaccination against a shared tumor antigen can produce an “inflamed” tumor phenotype.

Surprisingly, we were unable to enhance the efficacy of vaccination by checkpoint blockade or inhibition of IDO. Expression of PD-L1 has been investigated as a predictive biomarker for
response to antibodies blocking PD-1 or PD-L1.\textsuperscript{152} A previous study in murine pancreatic cancer showed that vaccination induced expression of PD-L1 and synergized with PD-1 blockade; however, this same study showed that tumors were also responsive to PD-1 blockade without vaccination.\textsuperscript{174} Our results suggest that even in the presence of high levels of PD-L1, established B16F10 tumors are still resistant to PD-1 blockade. Previous studies have shown that PD-1/PD-L1 blockade can synergize with anti-CTLA-4 and IDO inhibitors.\textsuperscript{161,163} However, these studies began combination therapy early after tumor challenge or in tumors expressing a foreign model antigen. Our studies with B16ova show that presence of a foreign antigen can greatly impact the efficacy of immunotherapy, but the relevance of these models to human disease is unclear. In our studies, we show that the parental B16F10 model is much more resistant to immunotherapy. To better model clinical disease, we also initiated therapy after tumors were visible and the tumor microenvironment was established. These differences in experimental design may explain the discrepancy in response to anti-PD-1 therapy in this tumor.

NKG2A is an inhibitory receptor expressed on the surface of NK cells and activated T cells.\textsuperscript{153,155} The expression of its ligand, HLA-E, in human tumor cells prevents T cell-mediated cytotoxicity through NKG2A engagement.\textsuperscript{154} Like MHC Class I molecules and PD-L1, HLA-E and its murine homolog, Qa-1\textsuperscript{b}, are upregulated in response to IFN\textgreek{y}.\textsuperscript{175,176} Our results show that Qa-1\textsuperscript{b} is highly upregulated in response to vaccination with MCMVgp100KGP. We also noted a high percentage of PMEL cells expressing NKG2A within the tumor microenvironment, suggesting a potential mechanism for immunoevasion. However, blockade of Qa-1\textsuperscript{b} had no effect on tumor growth following vaccination. A potential reason for this may be redundant inhibitory checkpoint signaling through other receptors. In our studies, PMEL cells also express
high levels of LAG3 and 2B4 within the tumor microenvironment. These pathways may also restrict the efficacy of vaccine-stimulated T cells.

Several other counter-regulatory mechanisms may account for the resistance of B16F10 tumors to vaccination. As we have shown, MCMVgp100KGP vaccination results in the recruitment of Ly6C⁺ MDSCs to the tumor microenvironment. A previous study by Hosoi et al. showed that adoptive transfer of tumor-specific T cells results in the recruitment of Ly6C⁺ MDSCs that limit T cell function.¹⁵⁶ We also noted a slight, but insignificant upregulation of IDO1 in tumor cells following vaccination. IDO1 expression in tumor cells is known to induce the recruitment of MDSCs in B16 tumors and increase resistance to immunotherapy.¹⁶² IDO inhibitors have been evaluated in several preclinical tumor models and are currently being tested in combination with checkpoint inhibitors in patients with metastatic melanoma (NCT02073123; NCT02752074).¹⁶¹,¹⁶³ Unfortunately, our initial experiments suggest that an IDO inhibitor with or without anti-PD1 therapy does not prolong of mice vaccinated with MCMVgp100KGP.

In summary, this study shows that MCMV-based vaccines can significantly impact tumor growth particularly when combined with adoptive cell transfer. Vaccines targeting foreign antigens were more effective in tumor rejection, suggesting that MCMV-based vaccines targeting neo-epitopes may be a more effective strategy to eliminate immunotherapy resistant tumors. Here, we also show that several immunosuppressive pathways are upregulated in response to MCMVgp100KGP vaccination, including PD-L1, Qa-1ᵇ, IDO1, and expansion of regulatory immune cells. These pathways highlight an ongoing immune response within the tumor environment and also suggested potential points of intervention for combination immunotherapy. Unfortunately, we were unable to improve antitumor activity of MCMVgp100KGP vaccination by inhibiting several of these pathways, suggesting that other pathways may be responsible for
tumor relapse. We have not yet tested the effects of targeting MDSCs with MCMVgp100KGP vaccination, but several studies have shown that targeting these cells can improve responses to combination therapy.\textsuperscript{177,178} Tumor necrosis and hypoxia have also been shown to limit the activity of antitumor T cells.\textsuperscript{179,180} In summary, this study shows that a persistent vaccine vector can dramatically transform the phenotype of a “non-inflamed” tumor; however, expression of several clinically-relevant immunosuppressive pathways following vaccination does not correlate with increased sensitivity to inhibitors of these pathways. Future work looking at the role of recruited MDSCs will investigate another potential role of resistance. Our data also suggests that CMV-based vaccines targeting neo-epitopes may be a highly effective method for fighting melanoma.

**Figures:**

![Figure 4-1: Prophylactic but not therapeutic MCMVgp100KGP delays intradermal solid tumor growth.](image)

A) Prophylactic vaccination with MCMVgp100KGP 7 days prior to challenge with B16F10 cells significantly delays tumor growth. B) Therapeutic vaccination with MCMVgp100KGP 3 days after tumor challenge does not significantly delay tumor growth. n=5 mice/group for A) and 6-7 mice/group for B); n.s. Not significant; p>0.05; *p<0.05.

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Figure 4-2: Vaccination increases number and activation status of CD8⁺ TIL but does not influence inhibitory receptor expression. Mice were treated with MCMVgp100KGP 3 days after tumor challenge and TIL were harvested 15 days later for flow cytometry. Vaccination increases A) total number of CD8⁺ TIL and B) expression of CD11a on CD8⁺ TIL. Vaccination does not change C) percentage of CTLA-4⁺ CD8⁺ TIL, D) CTLA-4 MFI on CD8⁺ TIL, E) percentage of PD-1⁺ CD8⁺ TIL, and E) PD-1 MFI on CD8⁺ TIL. n=5-6/group; **p<0.01.
Figure 4-3: Checkpoint blockade does not synergize with therapeutic MCMVgp100KGP vaccination. A) Experimental schema for B) and C). Three days after tumor challenge, mice were vaccinated with MCMVgp100KGP or mock vaccinated and treated with B) anti-PD-1 on days 6, 9, and 12 or C) anti-CTLA-4 on days 3, 6, and 9. Control mice received isotype control antibodies.
Figure 4-4: MCMVova stimulates adoptively transferred OT-I cells but does not maintain them long-term. C57BL/6 mice received $10^5$ CD45.1$^+$ OT-I CD8$^+$ T cells i.v. followed by vaccination with $10^5$ PFU WT MCMV or MCMVova i.p. A) Seven days after transfer, OT-I cells are found at high frequency in peripheral blood of mice vaccinated with MCMVova but not WT MCMV. B) OT-I cells display an effector memory phenotype (KLRG1$^+$CD127$^{lo}$) seven days after transfer. C) Frequency of OT-I cells in peripheral blood of MCMVova vaccinated mice from two separate experiments. One of nine mice showed a high frequency of OT-I cells four months after vaccination. Results are representative of two independent experiments.
Figure 4-5: Adoptive cell therapy and MCMVova cure mice with established B16ova tumors. Mice bearing B16ova tumors were treated with $10^5$ naïve OT-I cells, vaccination with MCMVova, OT-I transfer and WT MCMV vaccination, or OT-I transfer and MCMVova vaccination. A) Tumor growth curves of individual mice in each treatment group. B) Survival curves of treated mice. Results are representative of 3 independent experiments with 5 mice/group. ***p<0.001
Figure 4-6: MCMVgp100KGP stimulates adoptively transferred PMEL cells. A) C57BL/6 mice received $10^5$ CD8$^+$ Thy1.1$^+$ PMEL cells followed by vaccination with PBS, WT MCMV, MCMVgp100, or MCMVgp100KGP. Frequency of Thy1.1$^+$ cells in peripheral blood five days after vaccination. B) Frequency of Thy1.1$^+$ cells in peripheral blood of mice vaccinated with MCMVgp100KGP. C) Frequency of Thy1.1$^+$ cells in peripheral blood of mice vaccinated with MCMVgp100KGP or VSVgp100KGP 5 or 160 days after vaccination. n=3 mice/group; **p<0.01
Figure 4-7: MCMVgp100KGP maintains PMEL cells in lungs long-term. Transferred PMEL cells are detected in lungs of mice vaccinated with MCMVgp100KGP but not VSVgp100KGP 263 days following transfer. A) Flow cytometry plots from individual mice. B) Representative plot showing effector memory phenotype of PMEL cells in lungs of mice vaccinated with MCMVgp100KGP 263 days prior. n=3 mice/group.
Figure 4-8: Adoptive cell therapy and MCMVgp100KGP vaccination delays growth of established B16 tumors. Mice were challenged with B16F10 5 days prior to transfer of $10^5$ CD8$^+$ PMEL cells and vaccination with WT MCMV or MCMVgp100KGP. A) Individual tumor growth curves. B) Combined tumor growth curves. Statistical differences are noted as: PMEL+MCMVgp100KGP vs. Mock Treated *; PMEL+MCMVgp100KGP vs. PMEL *; PMEL+MCMVgp100KGP vs. PMEL+WT MCMV *; PMEL+MCMVgp100KGP vs. MCMVgp100KGP *. C) Survival curves. Data was combined from two independent experiments with 5 mice/group. One mouse from PMEL+MCMVgp100KGP group did not grow a tumor over the course of the experiment and was excluded from the analysis. *p<0.05; **p<0.01
Figure 4-9: MCMVgp100KGP stimulated T cells upregulate inhibitory receptors in tumor microenvironment. Tumor bearing mice were treated with PMEL transfer and MCMVgp100KGP vaccination. Six days later, blood and tumor tissue were harvested for flow cytometry. A). Representative plots showing expression of inhibitory receptors on PMEL cells from blood or tumor in the same mouse. B) MFI of inhibitory receptors on PMEL cells from blood compared to MFI on PMEL cells isolated from tumors in the same mouse. n=4 mice.

Data representative of at least 2 independent experiments. *p<0.05; ***p<0.001
Figure 4-10: Combination immunotherapy induces expression of inhibitory ligands in vivo.

Five days after challenge with B16RFP, mice were left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. Six days later, tumor cells were harvested for flow cytometry. A) Representative flow plots of RFP+ cells. B) Percentage of RFP+ cells expressing C) PD-L1 and D) both PD-L1 and Qa-1b. D) Representative plots of CD45+CD8-RFP- cells. Percentage of CD45+CD8-RFP- cells expressing A) PD-L1 and B) both PD-L1 and Qa-1b. Representative of two independent experiments with n=4-5 mice/group. *p<0.05; ***p<0.001
Figure 4-11: PD-1 and Qa-1b blockade do not enhance antitumor effects of MCMVgp100KGP immunotherapy. Mice were challenged with B16F10 and five days later treated with PMEL transfer and MCMVgp100KGP vaccination. Mice received anti-PD-1, anti-Qa-1b, or both on day 10, 12, and 14. Untreated mice or mice receiving monotherapy also received isotype control antibodies. A) Individual tumor growth curves and B) survival curves of mice receiving combination immunotherapies. Data combined from two independent experiments. n=10 mice/group.
**Figure 4-12:** MCMVgp100KGP vaccination induces accumulation of monocytic MDSCs. Mice bearing B16F10 tumors were left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. Six days after treatment, tumors were processed for flow cytometry. Numbers of A) Thy1.1+ PMEL cells, B) Gr-1+CD11b+ cells, C) Ly6C+CD11b+ cells, and D) Ly6G+CD11b+ cells were normalized to initial tumor weight. A, C, and D are representative of three independent experiments with n=4-5 mice/group. B is representative of one experiment. *p<0.05; **p<0.01; ***p<0.001
Figure 4-13: MCMVgp100KGP vaccination slightly upregulates expression of IDO in tumor cells. Mice were inoculated with B16RFP tumors via intradermal injection and 5 days later left untreated, treated with PMEL transfer and WT MCMV vaccination, or treated with PMEL transfer and MCMVgp100KGP vaccination. Six days later tumors were processed for flow cytometry. A) Percentage of RFP+ tumor cells expressing IDO. B) MFI of IDO in RFP+ tumor cells relative to isotype control. C) Representative figure of IDO expression in RFP+ tumor cells. Data for A) and B) were compiled from two independent experiments with 9-10 mice/group.
Figure 4-14: IDO inhibitor does not enhance response to combination immunotherapy.

Mice were challenged with B16F10 tumors and were treated with PMEL transfer and MCMVgp100KGP vaccination five days later. On day 10, mice were started on 2mg/mL 1-methyltryptophan (1-MT) in the drinking water or control water. Mice were also treated with anti-PD1 or isotype control on days 10, 13, and 18. n=5 mice/group.
Figure 4-13: Combination chemo-immunotherapy significantly delays tumor growth. A) Schematic of experimental design. Mice were challenged with B16F10 six days prior to receiving one dose of cyclophosphamide. On day 7, mice received $10^5$ in vitro activated PMEL cells and vaccination with WT MCMV or MCMVgp100KGP. B) Tumor growth curves and C) survival curves show combination chemo-immunotherapy significantly delays growth of established tumors. Data is representative of 3 independent experiments with n=5 mice/group.

***p<0.001
Chapter 5: Identification of Novel CD169+ Macrophage Population within Melanoma Tumors

Abstract:

Malignant tissue is often inundated with myeloid cells of diverse phenotype. Tumor associated macrophages make up a large proportion of tumor myeloid cells and generally are thought to promote tumor progression. Macrophages expressing the marker CD169 have critical roles in filtering lymph in lymph nodes and blood in the spleen and are thought to be critical to initiating the adaptive immune response. Here we show that CD169+ macrophages are present in high frequency in murine melanoma and these macrophages are phenotypically distinct from skin resident macrophages, and preferentially ingest tumor particles. Future work will determine the effect of CD169+ macrophages in tumor growth.

Introduction:

Cancerous tissue is often infiltrated by high frequencies of myeloid cells. Tumor associated macrophages (TAMs) are a diverse group of cells making up a large percentage of tumor infiltrating leukocytes.\textsuperscript{181,182} In many cancers, increasing frequencies of TAMs are associated with reduced overall survival.\textsuperscript{183} This association has also been confirmed in patients with melanoma, leading to the predominate hypothesis that TAMs are generally protumorigenic.\textsuperscript{184} The plasticity of macrophages under diverse environmental conditions often makes the study of their functions difficult, particularly \textit{in vivo}.\textsuperscript{185} However, studies have shown that TAMs can influence tumor growth through a variety of mechanisms, including increased tumor angiogenesis, promotion of metastasis and invasive characteristics of tumor cells, and suppression of antitumor T cell responses.\textsuperscript{186}
CD169+ macrophages represent a subset of myeloid cells largely studied within lymphoid tissues. Within the spleen and lymph nodes, CD169+ macrophages act as filters to capture microbes and exposed antigens in blood or lymph, initiating the activation of lymphocytes within these tissues.\textsuperscript{187} A handful of studies have begun to investigate the role of these cells in tumor immunity. A study by Asano et al. showed that CD169+ macrophages in the draining lymph nodes of mice phagocytose dead tumor cells following subcutaneous vaccination, and these cells are required for vaccine efficacy by cross-presenting tumor antigen and priming antitumor T cells.\textsuperscript{188} Similarly, a study by Pucci et al. showed that CD169+ macrophages within lymph nodes captured extracellular vesicles produced by tumor cells, and this capture prevented activation of tumor-promoting B lymphocytes.\textsuperscript{189}

Several clinical studies have also illustrated the significance of CD169+ lymph node macrophages as predictive biomarkers for cancer progression.\textsuperscript{190,191} In melanoma patients, specifically, higher densities of these macrophages in regional lymph nodes correlated with prolonged survival and greater infiltration of tumor tissue by CD8+ T cells.\textsuperscript{192} Like the mouse models, these studies suggest that CD169+ macrophages in lymphoid tissue are involved in priming antitumor T cells. In the current study, we show for the first time that CD169+ macrophages are not only localized to draining lymph nodes but are also found directly within a solid melanoma tumor. These cells appear in high frequency in the tumor tissue, are phenotypically distinct from skin-resident macrophages, and may phagocytose tumor cell particles more efficiently than CD169- TAMs.
Results:

*Intradermal B16 tumors contain a novel CD169\(^+\) macrophage subset that are distinct from skin resident macrophages*

Given the diversity of myeloid cells seen in different tumors and the potential of CD169\(^+\) macrophages to elicit antitumor immunity in lymph nodes, we asked whether B16F10 melanoma tissue contained CD169\(^+\) TAMs. Surprisingly, B16F10 tumors were inundated with CD169\(^+\) cells (Figure 5-1). These cells were F4/80\(^+\) by immunofluorescence imaging and flow cytometry, confirming their identity as TAMs (Figures 5-1 and 5-2A). CD169\(^+\) TAMs expressed high levels of Fc gamma receptor 1 (CD64) and CSF1R (CD115), further confirming their myeloid identity (Figure 5-2B). Interestingly, CD169\(^+\) TAMs expressed only low levels of CCR2 which is a key chemokine receptor for monocyte recruitment (Figure 5-2B).

Impressively, CD169\(^+\) TAMs uniformly expressed high levels of MHCII, while CD169\(^-\)F4/80\(^+\) TAMs contained a significant MHCII\(^{lo}\) population (Figure 5-2C).

*Intradermal CD169\(^+\) macrophages are phenotypically distinct from skin resident macrophages*

Previous studies have shown that tumor tissue contains both tissue-resident macrophages and monocyte-derived macrophages.\(^{181}\) CD169\(^+\) macrophages have been described in healthy skin, predominately within the dermal layer.\(^{193,194}\) We therefore asked if CD169\(^+\) TAMs were phenotypically distinct from skin resident macrophages. Like previous studies, we detected a subpopulation of macrophages in skin from naïve mice expressing CD169 (Figure 5-3A). Skin resident CD169\(^+\) macrophages contained a distinct CD11c\(^+\)MHCII\(^+\)CCR2\(^+\) subpopulation (Figure 5-3B). In contrast, CD169\(^+\) TAMs uniformly expressed MHCII and low levels of CCR2 (Figure 5-3C), showing that CD169\(^+\) TAMs are phenotypically distinct from skin resident macrophages.
As previously stated, the majority of TAMs in other tumor models are derived from recruited circulating monocytes.\textsuperscript{181} We hypothesize that CD169\textsuperscript{+} TAMs are also derived from circulating monocytes rather than skin resident macrophages. Future studies will examine this more closely.

\textit{CD169\textsuperscript{+} TAMs ingest tumor particles within the tumor microenvironment}

As already stated, CD169\textsuperscript{+} macrophages in lymphoid tissues are thought to efficiently phagocytose tumor derived particles by filtering draining lymph.\textsuperscript{188,189} We therefore hypothesized that CD169\textsuperscript{+} TAMs might also efficiently internalize tumor fragments. To test this, mice were inoculated via intradermal injection with B16F10 expressing Red Fluorescent Protein (B16RFP). Eleven days later, tumor tissue was harvested and processed for flow cytometry. As hypothesized, some CD169\textsuperscript{+} macrophages also expressed low levels of RFP, suggesting internalization of tumor fragments (Figure 5-4A). Impressively, of all F4/80\textsuperscript{+} cells co-expressing RFP, the majority were CD169\textsuperscript{+}, signifying preferential phagocytosis by CD169\textsuperscript{+} TAMs (Figure 5-4B). We also noted high expression of MHCII and the costimulatory ligand, CD80 on CD169\textsuperscript{+} macrophages, suggesting a preferential ability to prime T cells (Figure 5-4C).

While this flow cytometry data suggests internalization of tumor fragments by CD169\textsuperscript{+} macrophages, it is possible that these macrophages take up tumor fragments during processing for flow cytometry. We therefore stained fixed sections of B16RFP tumors for CD169. Imaging revealed many CD169\textsuperscript{+} TAMs in close association with RFP\textsuperscript{+} tumor cells (Figure 5-5) and some contained punctate RFP\textsuperscript{+} signals, confirming internalization of tumor particles \textit{in vivo} (Figure 5-6). Thus, CD169\textsuperscript{+} macrophages may be well-equipped to internalize and process tumor antigen not only at lymphoid tissues but also within the tumor microenvironment.
Discussion:

Infiltration of tumor tissue by macrophages has been associated with poor prognosis for patients with several types of malignancy.\textsuperscript{183,184} The mechanisms behind this observation are probably diverse as TAMs can influence disease progression through several pathways including angiogenesis and immunosuppression.\textsuperscript{186} Tumor tissue can contain several subsets of macrophages with different phenotypic characteristics. Some of these macrophages are derived from proliferating tissue-resident macrophages, but the majority of TAMs are thought to originate from blood-derived monocytes that differentiate within the tumor microenvironment.\textsuperscript{181}

Lymph node resident CD169\textsuperscript{+} macrophages have been implicated in the priming of antitumor immunity in preclinical models by capturing and cross-presenting tumor antigen to prime antitumor T cells.\textsuperscript{188} In patients, the density of these cells in regional lymph nodes correlates with increased T cell infiltration of tumor tissue and increased overall survival.\textsuperscript{190-192} Thus, CD169\textsuperscript{+} cells within the lymph node appear to be critical in regulating antitumor immunity.

In this study, we discovered that a significant number of cells express CD169 within the tumor microenvironment. These cells were confirmed to be TAMs by co-staining for the macrophage markers, CD64 and F4/80. While our data shows that CD169\textsuperscript{+} TAMs are phenotypically distinct from skin resident macrophages, it is still unclear whether CD169\textsuperscript{+} TAMs arise from skin resident macrophages that respond to the growing tumor or whether they differentiate from circulating peripheral monocytes. Future studies will dissect the origin of these cells.

CD169\textsuperscript{+} TAMs uniformly expressed high levels of MHCII and CD80, suggesting a potential ability to activate antitumor T cells. In contrast, CD169\textsuperscript{-} TAMs contained populations of MHCII\textsuperscript{lo} and MHCII\textsuperscript{hi} cells and expressed lower levels of CD80. Surprisingly, our preliminary
data also suggests an ability of CD169+ cells to ingest fragments of tumor cells. With these properties, we hypothesize that CD169+ TAMs have the ability to stimulate rare antitumor T cells within the tumor microenvironment. A previous study using clinical samples from patients with hepatocellular carcinoma has also described CD169+ TAMs which express high levels of HLA-DR and CD86. This same study also showed that the density of CD169+ TAMs correlated with intratumoral CD8 T cell density and overall patient survival.195 Our future studies will test the ability of CD169+ TAMs to stimulate antitumor T cells and investigate the role of these cells in melanoma tumor progression.

Figure 5-1: Intradermal B16F10 tumors contain a high density of CD169+ TAMs. Mice were inoculated with B16F10 tumor cells via intradermal injection. Established tumors were fixed in PLP buffer and cut into 20µm sections before staining antibodies against CD169 (red), F4/80 (dark blue), CD8 (light blue). Scale bar=50µm.
Figure 5-2: Phenotypic characterization of CD169+ TAMs. Intradermal B16F10 tumors were processed into single cell suspension and stained for flow cytometry. A) CD169 staining of tumor infiltrating leukocytes, gated on live CD45+ cells. B) CD169+ TAMs express CD115, CD64, and low levels of CCR2. C) CD169+ TAMs express high levels of MHCII, while CD169- TAMs show diverse MHCII expression. n=3 mice. Representative of at least two independent experiments, except CD64 staining which is representative of one experiment.
Figure 5-3: Skin resident CD169+ macrophages are phenotypically distinct from TAMs.  

A) CD169+ macrophages are found in skin from healthy mice.  B) CD169+ macrophages from healthy skin consist of two populations: MHCII+CCR2+CD11c+ and MHCII+CCR2−CD11c− populations.  C) CD169+ TAMs from B16F10 tumors are uniformly MHCII+ and express low levels of CCR2 and CD11c.  n=3 mice/group.
Figure 5-4: CD169+ TAMs engulf tumor particles in untreated solid tumors. Mice were inoculated with B16RFP. Tumors and spleen were harvested 11 days later for flow cytometry. A) CD169+ F4/80+ TAMs show increased fluorescence in the RFP channel compared to CD169- F4/80+ TAMs from the same tumors. B) The majority of RFP+ TAMs are CD169+. RFP gating was based on staining in spleen which should not contain RFP+ cells. C) CD80 expression on CD169+ TAMs and CD169- TAMs. n=4 mice.
Figure 5-5: CD169+ TAMs interact with B16RFP tumor cells in close proximity. Mice were inoculated with B16RFP tumors and 11 days later, tumors were PLP fixed, sectioned into 20µm sections, and stained for CD169. Top row shows B16RFP tumors without CD169 staining. Bottom two rows show RFP (red) and CD169 (blue) in close proximity within the tumor. Arrows indicate CD169+ TAMs in close proximity to B16RFP tumor cells. n=2 mice; Scale bar=80µm.
Figure 5-6: CD169+ TAMs ingest RFP+ tumor particles within the tumor microenvironment. Mice were inoculated with B16RFP tumor cells and 11 days later tumors were harvested and fixed by PLP fixation. Tumors were cut into 20µm sections and stained for CD169. Arrows indicate CD169+ TAMs (blue) with internalized RFP+ particles (red). n=2 mice; Scale bar=80µm.
Chapter 6: Summary and Future Directions

Cytomegalovirus-based Tumor Vaccines

Cytomegalovirus (CMV) is a ubiquitous herpes virus that generates a unique T cell response termed “memory inflation”. This phenomenon is characterized by the maintenance of a high frequency of virus-specific T cells over the lifetime of the host. In contrast to other chronic infections, CMV-specific T cells maintain effector function over time. Impressively, this unique T cell response is still observed after vaccination with a single-cycle virus, limiting the danger of viral reactivation. These properties make CMV an attractive platform for vaccines targeting infectious diseases and cancer.

Our lab has previously generated a Murine Cytomegalovirus (MCMV) vaccine vector expressing an alter melanoma antigen (MCMVgp100KGP) that delays murine melanoma growth. In this study, we sought to generate a novel MCMV vaccine expressing both gp100KGP and a second altered melanoma antigen. In the process, we show that a MCMV based vaccine expressing both gp100KGP and the native melanoma antigen, Trp2 is unable to delay tumor growth compared to MCMVgp100KGP, highlighting the difficulty of vaccinating against self-antigens. We anticipated this result and therefore simultaneously constructed a MCMV vector expressing gp100KGP and an altered Trp2. However, by altering Trp2 at one amino acid, we drastically reduced the expression of the full-length protein. Not surprisingly, this vector also did not improve antitumor activity compared to MCMVgp100KGP. While this study did not generate a more effective melanoma vaccine, it does highlight a potential pitfall in altering T cell epitopes within full-length proteins in an expression vector. A previous study by Klyushnenkova et al. showed that a MCMV-based vaccine expressing a T cell epitope in prostate-specific antigen was
more effective than one expressing the full-length protein.\textsuperscript{126} Thus, future CMV-based vaccines may be more effective if designed to express shorter peptides corresponding to T cell epitopes rather than full-length proteins. Another potential pitfall for CMV-based vaccines may be the incorporation of several epitopes within the same vaccine vector. Research has shown that inserting a foreign antigen into a MCMV vector reduces the endogenous virus-specific inflationary T cell response. However, when these epitopes are expressed in two different viruses, inflationary T cell responses were not restrained, suggesting that inflationary epitopes compete within infected cells for antigen presentation.\textsuperscript{196} Thus, targeting different tumor antigens using several CMV-based vectors may be more effective than multiple-epitope vectors. Future work will test several MCMV-based vaccines each expressing altered T cell epitopes within established melanoma antigens. In addition, targeting novel epitopes created as a result of tumor mutation (termed neo-epitopes) may yield more dramatic results. As methods for predicting immunogenic neo-epitopes improves, CMV-based vaccines may be an effective vaccine formulation to induce lifelong T cell responses specifically recognizing tumor antigen.

**Combining CMV-based Vaccines with Other Immunotherapies**

This study extends previous work by our lab investigating the impact of MCMV-based vaccines for the treatment of melanoma. Herein, we show that MCMV expressing tumor antigens can delay tumor growth in an aggressive solid tumor model of B16 melanoma. We also show that vaccine efficacy is limited by the precursor frequency of tumor-reactive T cells. As hypothesized, combining MCMV vaccination with adoptive cell therapy significantly delays tumor growth compared to either therapy alone.

In this study, we also show that combination adoptive cell transfer and MCMV vaccination significantly upregulates the expression of several immunosuppressive pathways directly within
the tumor microenvironment. B16 melanoma is generally considered to be a non-immunogenic
tumor model as checkpoint inhibitor monotherapy has little effect on established tumor
growth.\textsuperscript{163,197} Clinical responses to immune checkpoint therapy has been associated with
increased T cell infiltration, mutational load, and PD-L1 expression.\textsuperscript{39,41,76} This has led scientists
to distinguish tumors as “inflamed” or “non-inflamed” based on the presence of T cell infiltrate
and inhibitory pathway expression.\textsuperscript{143} Our data suggests that CMV-based vaccination can at
least partly induce an “inflamed” phenotype in an aggressive tumor model. However, in contrast
to clinical studies suggesting that expression of inhibitory ligands in the tumor
microenvironment, we did not discover enhanced responsiveness to PD-1, NKG2A, or IDO
inhibition following vaccination, despite high expression of several of these pathways. As
previously discussed, several other pathways may be limiting vaccine efficacy, including
expression of other inhibitory receptors like LAG-3 and 2B4 or recruitment of regulatory cells
like MDSCs. Molecules targeting these pathways may be useful in this tumor model. For
instance, CSF1R antagonists have been shown to limit the influence of MDSCs within the tumor
microenvironment.\textsuperscript{178} More broadly, several studies have noted that tumor necrosis and tissue
hypoxia can limit T cell activity within the tumor microenvironment, providing other potential
mechanisms for immune resistance.\textsuperscript{179,180,198} Future studies will determine if inhibitors of these
pathways may enhance antitumor activity of MCMV-based vaccines.

The most promising results from our studies include the eradication of tumors in mice treated
with adoptive cell therapy and MCMVova. This data suggests that CMV-based vaccines
targeting neo-epitopes may be highly effective immunotherapies, even in these highly aggressive
tumors. One study has already tested traditional peptide-based vaccines targeting neo-epitopes
in B16 melanoma, showing a significant delay in tumor growth.\textsuperscript{35} These results may be even
more dramatic in the context of an inflationary T cell response against these epitopes. Thus, future work will seek to generate several MCMV-based vaccines targeting tumor-specific antigens.

**Elucidating the Role of CD169+ Macrophages within the Tumor Microenvironment**

Tumor associated macrophages (TAMs) make up a large frequency of tumor infiltrating leukocytes and have generally been associated with poor prognosis in several cancers.\(^{183,184}\) In contrast, CD169+ macrophages within the regional lymph nodes seem to contribute to antitumor immunity, as higher densities of these macrophages correlate with CD8\(^+\) T cell infiltration of tumor tissue and overall survival in melanoma.\(^{192}\) In preclinical models, these cells are essential for capturing tumor antigen within the draining lymph node and activating CD8\(^+\) T cells while preventing activation of tumor-promoting B cells.\(^{188,189}\)

Here, we have shown that CD169+ macrophages are also found at relatively high frequency directly within B16 melanoma tumors. Few studies have described this subpopulation of macrophages directly within the tumor microenvironment. In one such study, CD169+ TAMs in hepatocellular carcinomas displayed high levels of MHCII and CD86, and CD169+ TAM density correlated with CD8\(^+\) T cell infiltration and overall patient survival, suggesting a critical role for these macrophages in antitumor immunity.\(^{195}\) In our own current study, we show that CD169+ TAMs from B16F10 tumors also express high levels of MHCII and the costimulatory molecule CD80. Impressively, we also show preliminary evidence that CD169+ TAMs can engulf tumor particles directly within the tumor microenvironment. All of these results lead us to hypothesize that CD169+ TAMs may represent a subpopulation of TAMs capable of capturing and presenting tumor antigen to T cells directly within the tumor microenvironment. We have already begun trying to record CD169+ TAM capture of tumor particles using intravital two photon imaging and
CD169 reporter mice to further confirm this ability in these cells. Future work will also test the ability of these cells to stimulate T cells. By FACS sorting CD169+ and CD169- TAMs directly from B16 tumors, we will compare the ability of these two populations to stimulate T cells \textit{ex vivo}. If our hypothesis is correct and CD169+ TAMs do stimulate antitumor T cells, therapies aimed at increasing this TAM subpopulation may greatly impact cancer progression or responsiveness to other cancer immunotherapies.
References


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