Merkel Cell Carcinoma: Immunogenicity and the characterization of CD4 T cell responses to the Merkel cell polyomavirus

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ABSTRACT

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Merkel cell carcinoma (MCC) is a deadly, virus-associated skin cancer with a 5-year relative mortality rate of 46%. The Merkel cell polyomavirus (MCPyV) is clonally integrated into 80% of MCCs and persistent expression of MCPyV T-antigen oncoproteins is required for tumor survival and growth, potentially providing ideal targets for immune based therapies. In the remaining 20% of MCCs that are virus-negative, remarkably high numbers of UV-induced neoantigens are detected, suggesting that both MCC subsets harbor immunogenic epitopes. Over the last few years, this hypothesis has been strongly supported by extraordinarily high response rates to agents blocking the PD-1 pathway in patients with both virus-positive and virus-negative MCC. However, still roughly half of patients do not benefit from these modalities, indicating an urgent need to identify biomarkers predictive of response and immune evasion mechanisms that underlie PD-1 blockade resistance. While much of the work presented here was initiated and/or completed prior to the use of these novel therapies, these data provide the basis for ongoing efforts to delineate predictors and mechanisms of resistance to PD-1 blockade therapy.

Within the opening chapters, we explore pathogen-driven cancers more broadly (**Chapter 1**) before delving specifically into MCPyV-induced MCC, its rising incidence rate (**Chapter 2**) and known mechanisms of immune evasion (**Chapter 3**). Previous studies have indicated that a robust CD8 T cell response is associated with dramatically improved MCC outcomes, therefore, we sought to characterize several mechanisms of CD8 T cell dysfunction. The first is described in **Chapter 4** in which we show that the downregulation of the adhesion molecule E-selectin within MCC tumor vasculature is associated with intratumoral T cell exclusion and reduced survival. However, even if CD8 T cells can infiltrate tumors, there is abundant literature to indicate that effective CD8 T cell responses require CD4 help and that this ‘help' is

often impaired in the setting of cancer. Consequently, **Chapters 5-8** focus upon elucidation of the CD4 helper T cell response against MCC. Specifically, in **Chapter 5** we discuss the multitude of CD4 subtypes that have been described and their relevance in the setting of cancer and cancer therapies. In order to elucidate the phenotype and function of MCPyV-specific CD4 T cells in the context of MCC, we needed to first identify CD4 T cell epitopes within MCPyV and develop reagents enabling their isolation. This work is the focus of **Chapter 6**. In **Chapter 7** we examine an especially fascinating, newly identified CD4 epitope 'WEDLFCDESLSSPEPPSSSE' locating within the MCPyV Large T-antigen. This epitope is highly immunogenic and has several key features which make it an ideal target for immune-based therapies such as a therapeutic cancer vaccine. Discovery of this epitope also resulted in the generation of HLA class-II tetramers allowing for the first time isolation of MCPyV-specific CD4 T cells directly *ex vivo* without antigenic stimulation. However, in many patients the frequency of these cells was found to be below the limit of the detection by standard methods. As a result, in **Chapter 8** we describe the development of a novel method using a digital scanning microscope to specifically and sensitively identify rare antigen-specific T cells. Finally, in **Chapter 9,** we shift away from the CD4 T cell describe a unique subset of MCC patients who present without a detectable primary skin lesion and who have a remarkable 50% higher rate of survival as compared to stage-matched patients with primary skin lesions. These patients have several elevated markers of immunity suggesting that clearance of the primary skin lesion is immune-mediate.

This past year (2017) historically marked the first FDA approval of an agent for the treatment of advanced MCC. Therefore, as we continue to treat more MCC patients with this agent (avelumab; anti-PD-L1) and other immune checkpoint inhibitors, the findings described in this dissertation will allow us to evaluate potential biomarkers of response and resistance including E-selectin downregulation and evaluation of CD4 T cell phenotype and function. For patients who do not respond to PD-1 blockade, these studies will help inform the use of existing therapies in potentially novel combinations and support the development of new approaches, such as a therapeutic cancer vaccine. Ultimately, we believe that these efforts will translate to improving patient outcomes.

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CHAPTER 1: PATHOGEN-DRIVEN CANCERS AND EMERGING IMMUNE THERAPEUTIC STRATEGIES

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Chapter Summary

Infectious agents play an etiologic role in approximately 20% of cancer cases worldwide. Eleven pathogens (7 viruses, 3 parasites, 1 bacterium) are known to contribute to oncogenesis either directly via the expression of their protein products or indirectly via chronic inflammation. While prevention of infection and anti-microbial treatments have helped reduce infection rates and the incidence of associated malignancies, therapies for these cancers remain limited. The importance of immune control over malignant progression is highlighted by the fact that many cancers, particularly those induced by pathogens, occur more frequently among immunosuppressed as compared to healthy individuals. Therefore, therapeutic strategies that can elicit a robust immune response and restore tumor detection may be a beneficial approach for treating these cancers. In addition, the study of immune escape mechanisms utilized by pathogens and their associated cancers may provide insight into the mechanisms of malignant transformation and how to generate therapies against cancer more generally.

Pathogen Mediated Oncogenesis

It is estimated that approximately 1 in 5 cancers worldwide is linked to an infectious agent[5]. To date there are seven oncogenic viruses (Hepatitis virus B and C (HBV and HCV), human papilloma virus (HPV), Epstein-Barr virus (EBV), human T cell lymphoma virus 1 (HTLV-1), Merkel cell polyomavirus (MCPyV) and Kaposi's sarcoma virus also known as human herpes virus 8 (KSVH or HHV8)), one oncogenic bacterium (*Helicobacter pylori)*, and three oncogenic parasites (Schistosoma haematobium, *Opithorchis viverrini* and Clonorchis sinensis) have been identified[5-8]. Four of these agents (HBV, HCV, HPV and *H.* pylori) each account for a remarkable 5% of all cancer cases by leading to hepatocellular carcinoma (HCC), cervical cancer and stomach cancer respectively[5]. While highly varied in their oncogenic mechanisms, these pathogens can generally be divided into direct and indirect carcinogens[8,9]. Currently five viruses (HPV, HTLV-1, EBV, MCPyV, KSVH) are classified as direct carcinogenic

pathogens and share several similarities[8]. At least a critical portion of the viral genome can generally be detected in each cancer cell resulting in expression of viral oncogenes that disrupt cell-cycle checkpoints, inhibit apoptosis and contribute to cell immortalization[7,9]. In contrast the indirect carcinogenic pathogens (HBV, HCV, *H. pylori*, S. haematobium, *O. viverrini* and C. sinensis) do not induce expression of oncogenes but instead their persistent infection leads to a chronic inflammatory state. Persistent inflammation from these pathogens leads to the release of chemokines, cytokines, prostaglandins, and reactive oxygen species, which can result in deregulation of the immune system and promotion of neovascularization[7-9]. Of note, classification of pathogens as direct or indirect carcinogens is simplistic and does not fully capture the likely oncogenic mechanisms of these pathogens. HBV, for example, is an indirect carcinogen that is clonally integrated into almost all HBV related cancers, however it is unclear whether persistent viral gene expression is required for continued cancer cell proliferation[9].

Prevention and Eradication of Oncogenic Infectious Agents

Important strategies for reducing the incidence of pathogen-driven cancers have been prevention of infection or eradication of the infection prior to development of cancer. Large-scale vaccination programs for both HBV and HPV have dramatically reduced infection rates. Specifically, within the US, an 82% decline in HBV infection has been reported since the implementation of the vaccine in 1991[10]. In Taiwan, introduction of HBV vaccines has also shown remarkable efficacy in reducing infection rates and longitudinal studies have also shown a corresponding reduction in the age-specific incidence of hepatocellular carcinoma (HCC)[11]. Since the introduction of HPV vaccines to the US in 2006, the prevalence of the targeted high-risk HPV types has fallen from 11.5% to 5.1%, a 56% reduction among teenage girls[12]. Of note, only 32% of 13-17 year old girls received all 3 doses. Improved administration and access could therefore lead to even greater efficacy. Despite these successes, administration of these vaccines to the developing world remains a challenge due to environmental, cultural and socioeconomic barriers[11]. Vaccinations against other oncogenic pathogens such as HTLV-1, EBV, HCV and H. pylori are in development but will face diverse technological and implementation challenges[11,13-16]. Infection with these microbes will therefore remain a global problem prompting the need for other treatment modalities.

Since persistent infection is a hallmark of oncogenic pathogens, there is a window of opportunity for cancer prevention by treating the pathogen prior to malignant progression[11,14,17]. Anti-viral therapies including interferons, nucleoside/nucleotide analogs and therapeutic vaccines can be used to treat oncogenic viruses prior to malignant progression. Such anti-viral strategies have been successful in reducing HBV and HCV associated cirrhosis and HCC[18]. The combination of zidovudine (a nucleoside analog) and interferon-alpha may reduce the incidence of EBV-induced lymphoma and a worldwide meta-analysis demonstrated a 35% complete response rate and 31% partial response rate in HTLV-1-driven adult T cell leukemia/lymphoma (ATLL)[19,20]. Another anti-viral strategy, currently being tested in clinical trials for HPV treatment, is the use of therapeutic vaccines, which can range from peptide, protein, DNA, RNA, and dendritic cell based vectors[21]. For the non-viral pathogens, several anti-microbial therapies have been successfully used such as the quadruple therapy approach for H. pylori (a proton pump inhibitor, dual antibiotics and bismuth) and praziquantel for the oncogenic parasites[22-24]. Rising antibiotic resistance, re-infection, and lack of access to available treatments have diminished the potential benefit of these approaches[23,24]. Therefore, while effective strategies are being taken to reduce the incidence of oncogenic agents, these infections will continue to occur, as will their corresponding malignancies.

Pathogen-driven cancers are uniquely poised for immunotherapies

While infectious agents contribute significantly to the overall global cancer burden, it is important to realize that oncogenesis is actually an uncommon outcome of infection and is a deviation from the normal life cycle of these pathogens. Pathogen-induced oncogenesis, when it does occur, usually arises many years after the initial infection. This delay indicates that additional steps are required beyond infection by the pathogen[9]. As one would expect, there are increased rates of pathogen-driven cancers where infection rates are higher such as in developing countries, underserved communities and among immunosuppressed populations. A meta-analysis of two immunosuppressed populations (HIV-AIDS patients and transplant patients) demonstrated a significantly increased incidence of several types of cancer, most of which were pathogen-driven[25]. Higher rates were reported of EBV-lymphoma/leukemia, HBV and HCC-hepatocellular carcinoma, HPV- cervical cancer and H. pylori associated gastric

carcinoma whereas rates of most common epithelial cancers were equivalent or reduced as compared to the general population[25]. This pattern of increased cancer risk in two different immunosuppressed populations suggests that immunodeficiency, rather than other risk factors, is responsible for the increased cancer incidence[25]. An additional example of immune regulation of pathogen-driven cancers is seen in the setting of Merkel cell carcinoma (MCC). Approximately 10% of MCC patients have chronic immunosuppression, which is a significant overrepresentation of the general public[26]. In addition, immunosuppressed MCC patients have a significantly reduced MCC-specific survival (40% at 3 years) as compared to non-immunosuppressed MCC patients (74% at 3 years)[26]. This indicates that immunosuppressed patients are both more likely to develop MCC and are more likely to succumb to the disease, underscoring the importance of immune function in regulating this pathogen-driven cancer[26].

The idea that the immune system has the capacity to control malignancy is not a new concept. In the 1890's a New York neurosurgeon, William Coley, documented complete regression of a sarcoma lesion in a patient who had developed a concurrent infection. He went on to treat many more patients with bacteria or bacterial products (which became known as Coley toxins) to induce an immune response and saw some responses[27]. However, this technique was highly criticized and immunotherapeutic approaches remained in the background until other studies documented improved cancer outcomes via manipulation of the immune system. One example included the discovery that IL-2 administration had efficacy against melanoma and renal cell carcinoma[28]. Specifically, 15-20% of patients exhibited objective regression following treatment with high dose IL-2, with half of responding patients experiencing complete regression despite bulky metastatic disease[28]. The mechanism for this observed effect is likely due to expansion of anti-tumor lymphocytes. Indeed it has been shown in several cancer types that T cell intratumoral infiltration can positively influence survival outcomes indicating that a cellular rather than humoral response mediates cancer progression[29]. As a result, enhancing cell-mediated immunity using antigen-specific T lymphocytes has received significant attention and has emerged as an increasingly effective treatment for advanced cancer patients[28,30].

Adoptive T cell transfer therapy involves the collection and expansion of antigen-specific T cells and subsequent infusion of these cells back into the patient where they can traffic to the tumor and promote targeted tumor cell death. Tumor-specific antigens presented on MHC-I molecules provide an excellent target for discriminating malignant from normal cells. T cells targeting MART-1 were first proven effective in the treatment of metastatic melanoma[31]. This method has since been applied to lymphomas associated with EBV. Specifically, post-transplant lymphoproliferative diseases (PTLD) arise following administration of immunosuppressive agents, which can lead to a reactivation of latent EBV. PTLDs encompass a range of disorders from reactive, polyclonal hyperplasia to aggressive non-Hodgkin's lymphomas [32]. The highly immunosuppressed state seen in these patients allows for immune escape despite expression of highly immunogenic viral latency proteins (EBNA3 family proteins) on the surface of tumor cells[32,33]. Targeting of these EBV-specific proteins using T cell therapy resulted in complete responses in 10 of 24 PTLD patients[32]. The expression of EBV-specific antigens on malignant cells provides an example of how tumor-specific antigens can make such cancers particularly suited for targeted cellular therapies.

This immunotherapeutic approach has also has been used prophylactically in transplant patients as well as in the treatment of other EBV-related malignancies such as nasopharyngeal carcinoma (NPC) and Hodgkin's lymphoma (HL). Treatment of NPC and HL using T cell therapies was not as successful as responses seen in PTLD, perhaps due to the reduced expression of EBNA3 family proteins, expression of cytokines promoting Th2 responses and a higher expression of T regulatory cells[32]. Targeting another EBV protein (LMP2), expressed on several EBV associated tumors, has been shown to mediated successful resolution of some Hodgkin's, non-Hodgkin's lymphomas and severe chronic active EBV infection patients[32] while the use of polyclonal CTL lines resulted in several complete and partial remissions in nasopharyngeal carcinomas. Unfortunately, these results were often short lived most likely due to lack of persistence and proliferation of infused cells *in vivo*. Adoptive T cell strategies are being investigated for the treatment of Merkel cell carcinoma (MCC) patients. 80% of MCC tumors require persistent expression of immunogenic polyomavirus tumor-antigen oncoproteins. MCC thus has highly desirable tumor-specific antigens for T cell therapy.

The shortcomings of treating EBV and MCPyV associated malignancies with virus-specific T cells highlights some of the challenges currently facing this approach including the insufficient persistence of infused T cells, down-regulation of antigen presentation and T cell exhaustion. One method to enhance the persistence of transferred T cells is to administer low dose IL-2 following T cell infusion although this approach can induce T-regulatory cells[34,35]. Another approach called lymphodepletion has been used in combination with IL-2[36]. Lymphodepletion involves destruction of host lymphocytes prior to T cell infusion using cyclophosphamide or anti-CD45. This approach eliminates host T regulatory cells, improves access to cytokines such as IL-7 and IL-15, and thus promotes the ability of infused T cells to persist *in vivo*[32,36]. The necessity of this approach, however, remains unclear as some studies have shown that with sufficient numbers of infused T cells, complete regression of a tumor can occur in either lymphodepleted or lymphoreplete hosts[36]. Another challenge for adoptive strategies is the down-regulation of HLA-I molecules on the surface of tumor cells, thereby obscuring the intended target of the infused tumor-specific T cells. HLA down-regulation has been shown to be reversible in patients by treatment with either interferon or single fraction radiation[37-39]. These strategies are currently being tested in conjunction with T cell therapy for MCC patients. In addition, epigenetic modulators such as histone deacetylase inhibitors and a methyltrasferase inhibitor (5-aza-2′-deoxycytidine) have been shown to up-regulate HLA and cancer testis antigen expression on tumor cells[40,41]. These agents are under active investigation and could significantly increase tumor immunogenicity and clinical responses to concurrent immunotherapies[41].

Aside from increasing immunogenicity, ensuring that tumor-specific T cells retain their effector function is another essential component of T cell therapy. Studies of chronic infection have shown that upon persistent exposure to a specific antigen, T cells can progressively lose their ability to kill target cells, in part through a process known as T cell exhaustion. T cell exhaustion has been best described in LCMV (lymphochoriomeningitis virus)-infected mice. Over the course of chronic LCMV infection, virus-specific T cells lost effector function most significantly when viral burden was high and CD4+ helper T cells were lacking[42]. Markers of T cell exhaustion have been extensively investigated and co-inhibitory molecules such as PD-1 and CTLA-4 have been shown to be up-regulated and contribute to this phenotype,

although through different mechanisms[42,43]. CTLA-4 attenuates early activation of naïve and memory T cells whereas PD-1 interaction with PD-L1 serves to modulate T cell activity in peripheral tissues including the tumor microenvironment[44]. Importantly, antibodies targeting CTLA-4 and PD-1/PD-L1 reverse exhaustion and mediate clinical activity against melanoma, renal cell carcinoma and non-small cell lung cancer[44,45]. In 2011, ipilimumab (anti-CTLA-4) was approved by the FDA for treatment of unresectable malignant melanoma. Because these two molecules act in a non-redundant fashion, combined blockade may achieve enhanced anti-tumor activity[44]. It is plausible that the combination of antigen-specific T cell infusion with agents that activate T cells and prevent their exhaustion, may be a particularly effective approach to treating pathogen-associated cancers.

While therapies targeted to specific tumor antigens have shown success in the treatment of some cancers, immunotherapies that aim to stimulate a more general cellular response against malignancies may prove beneficial. A promising therapeutic cytokine is interleukin-12 (IL-12), which is considered to be a highly potent trigger of anti-tumor immune responses[46]. IL-12 is required for optimal differentiation of naïve CD4 T cells into type 1 T helper cells and promotes cell-mediated immunity making it an ideal candidate for immunotherapies. Subcutaneously injected IL-12 as a monotherapy has shown a 71% response rate in Kaposi's sarcoma patients and a 43% response rate in patients with various non-Hodgkin's lymphomas, however, minimal responses were observed in several other cancer types[46]. Localized low-level production of IL-12 following plasmid DNA electroporation intratumorally has shown benefit in the treatment of malignant melanoma[47].

Future Directions

The development of treatments for pathogen-driven cancers is an important goal due to their high prevalence. Immunotherapies may offer particularly appealing therapeutic options for many such cancers due to their expression of microbial products. In addition, development of immunotherapies targeting pathogen-driven cancers may provide insight into targeted immune therapies for other cancers. However, it is important to note that while antigen-specific T cell therapy shows promise in treating pathogen-driven cancers, several challenges limit the efficacy of this approach, including the inability to treat patients who

Table 1: Prevalence and characteristics of pathogens known to promote cancer development
*Data for infection prevalence derived primarily from the WHO[8]. Abbreviations: HPV (human papilloma virus), HTLV-1 (human T cell lymphotropic virus 1), EBV (Epstein-Barr virus), MCPyV (Merkel cell polyomavirus), KSVH (Kaposi's sarcoma herpes virus), HBV (hepatitis B virus), HCV (hepatitis C virus), BL (Burkitt's lymphoma), NPC (nasopharyngeal carcinoma), HL (Hodgkin's lymphoma), NHL (non-Hodgkin's lymphoma).

Pathogen	Prevalence of infection*	# of new pathogen attributable cancers worldwide[6]	Notable Cancers	% Attributable to infection[6]	Oncogenic mechanism/oncogenes expressed[11,48]
Direct Carcinogens					
HPV	~10%[49] (in women by cytology)	610,000	Cervical carcinoma Penile Anal Vulva Vaginal Oropharynx	100 50 88 43 70 13-56	Viral integration/E6 and E7 expression
HTLV-1	~5-10 million infected [50] (by serology)	2,100	Adult T -CLL	100	Viral integration/Tax
EBV	>90% (by serology)	110,000	BL (Sub-Saharan Africa) BL (Other regions) NPC HL (developing-children/adults) HL (developed)	100 20-30 80-100 90 60 40	Viral integration/EBNA1, EBNA2, EBNA3C, LMP1, LMP2 + EBER (oncogenic RNA)
MCPyV	60-80%[51] (by serology)	2,500 (US)	Merkel cell carcinoma	80	Viral integration, deletion of C-terminus of LT/ LT
KSHV	<10% (Northern Europe, USA, Asia) 10-30% Mediterranean >50% Sub-Saharan Africa	43,000	Kaposi's sarcoma	100	No integration/LANA, vFLIP
Indirect Carcinogens					

HBV	240 million infected worldwide with highest incidence in Sub-Saharan Africa[52] (by serology)	380,000	Hepatocellular carcinoma	23-59	Viral integration, inflammation/ HBX
HCV	2.2% (by serology)	220,000	Hepatocellular carcinoma	20-33	Uncertain/NS3, NS5A
H. Pylori	~50% (by serology)	660,000	Non-cardia gastric cancer NHL of gastric location	90 86	Oncoprotein inject./Cag A Oncogene insertion/mutated core protein
S. Haemoatobium	200 million infected in Africa Less common elsewhere	6,000	Bladder cancer	40	Irritation, inflammation, immunomodulation
O. viverrini, C. sinensis	~10 million infected ~45 million infected	2,000	Cholangiocarcinoma	NA	Irritation, inflammation, immunomodulation

do not have particular HLA types compatible with therapy. One approach that does not limit which patients can be treated based on their HLA type is the use of cytokine-induced killer (CIK) cells. These cells are CD3+CD56+ T cells that express both NK and T cell markers and target stress-inducible molecules including MIC A/B that are expressed on many tumor types yet are usually not present on normal tissues[53]. This method has shown promise in the treatment of several cancers[53]. Interestingly, CIK therapy for the treatment of hepatitis B-associated HCC has been shown to significantly reduce viral DNA levels in addition to the eradication of residual cancer cells, prevention of recurrence, and improved progression-free survival rates[53,54]. Sub-optimal persistence of infused cells does, however, remain a challenge and will require further investigation[53]. Another therapy that is not limited to patients with particular HLA types, is the use of chimeric antigen receptors (CARs), which combine the specificity of an antibody with the effector function of CD8 T cells. B cell malignancies expressing CD19 were the first malignancies treated with CARs and demonstrated several complete responses, however their effect on solid tumors has to date been less encouraging[55]. While challenges facing the development of treatments for pathogen-driven cancers are significant and diverse, there is ample reason for optimism. Moreover, it

is likely that mechanisms of immune escape used by pathogen-driven cancers will continue to provide valuable clues in the treatment of cancer more generally.

CHAPTER 2: MERKEL CELL CARCINOMA; IT'S RISING INCIDENCE

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Chapter Summary:

Background: Merkel cell carcinoma (MCC) incidence rates are rising and strongly age-associated, relevant for an aging population.

Objective: Determine MCC incidence in the United States and project incident cases through 2025.

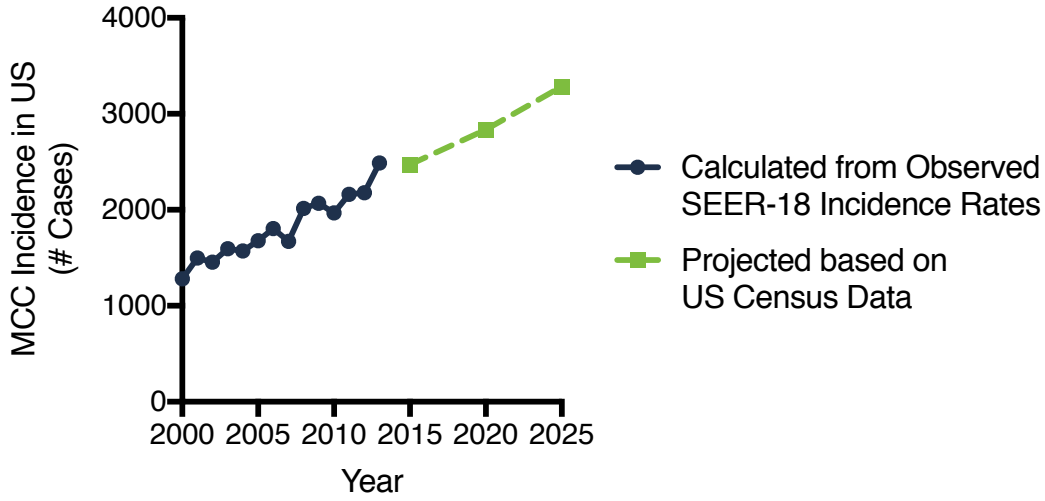
Methods: Registry data were obtained from the SEER-18 database, containing 6,600 MCC cases. Age and sex-adjusted projections were generated utilizing US census data.

Results: Between 2000-2013, there was a 95% increase in the number of reported MCC cases, as compared to 57% for melanoma and 15% increase for cases of all 'solid' cancers. Indeed, by 2013 the MCC incidence rate was 0.7 per 100,000 person-years in the US, corresponding to 2,488 cases. MCC incidence increased exponentially with age, from 0.1 to 1.0 to 9.8 (per 100K person-years) between age groups 40-44, 60-64, 85+ years respectively. Due to aging of the "baby-boom" generation**,** projected US MCC incidence is predicted to climb to 2,835 cases in 2020 and 3,284 cases in 2025.

Limitations: Projections assume the age-adjusted incidence rate stabilizes and thus may be underestimates.

Conclusions: Given upcoming demographic shifts, an increasing number of individuals are likely to be diagnosed with MCC in the US. Given this trend, high recurrence risk, and availability of new immunotherapies, more MCC awareness is justified.

GRAPHICAL ABSTRACT



CAPSULE SUMMARY

- Updated Merkel cell carcinoma (MCC) incidence statistics are needed.
- US incidence was 2488 cases in 2013. Given the disproportionate age-associated risk and the aging of baby-boomers, ongoing increases are likely.

>3000 US MCC cases/year are forecast by 2025. Given this and newly available therapies, more MCC-specific provider education is needed.

INTRODUCTION:

Merkel cell carcinoma (MCC) is a neuroendocrine skin cancer with high metastatic potential, with one-third to one-half of patients developing recurrence or metastasis. In 2007, annual incidence of MCC in the US was estimated at 1500 cases per year[56]. 80% of MCCs are caused by a common virus (Merkel cell polyomavirus),[57,58] and the remaining 20% by extensive UV-mediated damage[59-63]. MCCs that are diagnosed at early stage have better outcome, and high dermatologist density has been associated with improved MCC-specific survival suggesting provider familiarity with MCC may positively impact patient outcomes[64]. A population-based cohort study on the association of dermatologist density and Merkel cell carcinoma survival. For patients with metastatic disease, immunotherapies have been recently demonstrated to be effective in MCC[65-67] and there is emerging evidence that these are most effective if given in first-line, highlighting the importance of proper up front systemic therapy[1]. Therefore, updated incidence numbers can allow for better appreciation of the true impact of MCC and if increasing, proportionally increase its prominence in education for providers including those in primary care, dermatology, surgery and medical oncology, with hopes of improving patient outcomes.

From its first description by Toker in 1972[68], the observed incidence of MCC grew rapidly and this trend was sustained into the new millennium[69,70]. Increases were felt to initially represent an underappreciation/misdiagnosis of MCC cases that was improved in the 1990s with the widespread adoption of CK20 antibody immunohistochemistry. Over the past 10 years, the MCC incidence rates have been reported to continue to rise worldwide: in France[71], Sweden[72], Germany[73], Australia[74], China[75], and the United States[76]. However, to our knowledge no estimates of total annual US incidence (number of cases) have been published within the last five years. Furthermore, a large population shift is anticipated, with most “baby boomers” passing the 65 year threshold, at which the risk of MCC markedly increases. Indeed, the percentage of Americans >65 years of age is expected to dramatically increase from 13% of the population in 2015 to 20% in 2025[77]. Therefore, we used the SEER-18 registry, which captures approximately 28% of the US population[78]. In order to estimate current MCC incidence, and cross reference these data with US census projections to forecast incidence in 10 years.

MATERIALS AND METHODS:

SEER Database

De-identified national registry data from the Surveillance, Epidemiology, and End Results (SEER-18) database[79,80] was accessed using SEER*Stat 8.3.2 software in February 2017. Incidence data were collected from a SEER-18 "rate session". The SEER-18 registry contains information from registries that are geographically represented across the US (Atlanta, Connecticut, Detroit, Hawaii, Iowa, New Mexico, San Francisco-Oakland, Seattle-Puget Sound, Los Angeles, San Jose-Monterey, Rural Georgia, Alaska Native Tumor Registry, Greater California, Greater Georgia, Kentucky, Louisiana, and New Jersey). At the time of database access, data were available from 2000-2013. Rates were age and sex adjusted to the 2000 US Standard population (19 age groups – Census P25-1130). Data were *selected* for cases in the research database with known sex and age and tumors with SEER defined "malignant behavior". Data were *extracted* for MCC (ICD-O-3 Hist/behavior code 8247/3), malignant melanoma (codes 8720/3-8761/3) and for the SEER defined site recode B ICD-O-3/WHO 2008 grouping "All Solid Tumors" (http://seer.cancer.gov/siterecode).

US Census data

For the years 2000-2013, US Census Population Data were accessed through a frequency session utilizing SEER*Stat 8.3.2 software (Populations- Total US 1969-2015 Katrina/Rita Adjustment). For the years 2015, 2020, and 2025 US population estimates were downloaded from the 2014 national population projections publicly available at census.gov.[77]

Statistical Analyses

Statistical analyses were performed in SEER*Stat software and standard errors/confidence intervals generated with the Tiwari et al 2006 modification for confidence intervals[81]. Projected incidences were calculated using 2011-2013 incidence rates for each age and sex bracket (with multiple years allowing for reduced error in incidence rate) and total projected incidence was summed (**Supplemental Table 1**). Graphs were created in GraphPad Prism software.

RESULTS:

Trends in MCC incidence rate and reported cases

A total of 6,600 cases of Merkel cell carcinoma (MCC) were reported to SEER between 2000 and 2013 (the most recent year for which data are were available at the time of extraction in February 2017). Age and sex adjusted incidence rates were calculated and normalized to the 2000 US standard population.

For all solid cancers, there was a significant decrease in the standardized incidence rate between 2000 (429 cases per 100,000, 95% CI 427.5-430.5) and 2013 (379.8 cases per 100,000, 95% CI 378.6-381.1). In contrast, for the most aggressive skin cancers (melanoma and Merkel cell carcinoma), incidence rates significantly increased. For MCC, the incidence rate rose from 0.5 cases per 100,000 in 2000 (95%CI 0.4-0.5) to 0.7 per 100,000 in 2013 (95% CI 0.7-0.8)(**Figure 1A**).

Next, we determined changes in the total number of cases reported annually to the SEER-18 database (28% of US population captured). The number of cases reflects the incidence rate, the population at risk, and the database capture efficiency. For all solid tumors, there was a modest 15.5% increase in total number of cases reported to SEER-18 (from 313,683 in 2000 to 362,397 in 2013). In contrast, for MCC a 95.2% increase was observed (from 334 cases captured by SEER in 2000 to 652 in 2013) (**Figure 1B**); this impressive increase exceeded even the 56.5% increase seen with melanoma (from 13,945 to 21,824 reported cases).

Association of Demographic Factors with MCC

The incidence rate of MCC increases dramatically with age (**Figure 2A; n = 6,600 MCC cases**) and this effect is more pronounced than for melanoma (**Figure 2A**; n = 251,437 melanoma cases) or for solid tumors in general (**Supplemental Figure 1**). Specifically, the MCC incidence rate increases 10-fold between ages 40-44 (rate 0.1 cases/100,000/year, 95% CI 0-0.1) and 60-64 (rate 0.9/100,000/year, 95%CI 0.8-1) and 10-fold again between ages 60-64 and 85+ (rate 8.3 cases/100,000/year, 95% CI 7.9-8.7). This trend has been sustained, and data from 2011-2013 (the most recent years with data available, n=1778) are consistent: 0.1 cases/100,000 for ages 40-44, 1.0/100,000 for ages 60-64, and 9.8/100,000

for ages 85+. Unlike the rate of most cancers that decrease among the oldest (85+) individuals, the rate of MCC continues its significant rise. Consistent with this, in 2013 the median age at diagnosis for MCC was between 75-79 years for both men and women, as compared to 65-69 years for men with melanoma and 60-64 years for women with melanoma. 84% of persons with MCC were 65 years or older at diagnosis.

Across all age groups in the US, the incidence rate of Merkel cell carcinoma is higher in men than in women, and this effect is most pronounced at the oldest age groups (**Figure 2B**). For melanoma, incidence rates are higher in men than women over the age of 50, and higher in women than men under age 50[82], suspected to be due in part to changing patterns of UV exposure including indoor tanning[83]. MCC incidence below the age of 50 is too low to evaluate whether this trend towards increased risk in younger cohorts of women ("Gen-X" and "millennial" generations) will also hold true for MCC. Approximately 2/3 of cases of MCC are currently diagnosed in men and this was stable between 2000-2013.

Ultraviolet light is a well-established MCC risk factor[84]. Consistent with this, observed MCC incidence rates were highest in non-Hispanic white individuals. In the most recent years for which data is available (2011-2013, n=1778) the age- and sex-adjusted incidence rate of MCC in non-Hispanic whites was 0.8 per 100,000 (95% CI 0.8-0.9) as compared to 0.3 per 100,000 (95% CI 0.3-0.4) in Hispanics and 0.1 per 100,000 (95% CI 0.1-0.2) in non-white, non-Hispanic individuals. The proportion of individuals presenting with MCC that were minority (defined as either Hispanic or non-white) increased significantly between 2000-2002 and 2011-2013 (from 7.5% to 9.7%, $p = 0.045$) and increases in MCC incidence rate were seen across all racial and ethnic groups.

Estimates and Forecasts of Number of Merkel Cell Carcinoma Incident Cases in the US

Data from the SEER-derived incidence rates were combined with US census population data to estimate the total US MCC incidence (cases per year) from 2000-2013 and project incidence for 2015, 2020 and 2025. For these analyses, for the years 2000-2013 we utilized the incidence rate for each

individual age and sex bracket observed for that particular year. For the years 2015 and later, we used the incidence rate observed for each individual age and sex bracket in 2011-2013 (the most recent years for which data was available; **Supplemental Table 1**). In order to be conservative (erring towards underestimate), the adjusted incidence rate was not increased but instead held rate stable; thus, projections reflect only anticipated changes in population demographics.

Based on US census reports, due to the aging of the "baby boom" generation there is anticipated to be a large and disproportionate increase in the population aged 65 and older between 2015 and 2025 (**Figure 3A**[85]). These individuals will increase from 13% of the US population to 20% of the total population. This means that there will be a large increase in the individuals who are at higher risk for MCC.

In 2013, the total US incidence of MCC (comparing age and sex bracketed observed incidence rates to US census report of population at risk) was calculated as 2488 cases (**Figure 3B**). Given the rise in the aging population, and assuming incidence rates for any given age group remain stable, the total incidence of MCC in 2020 is projected to be 2,835 cases. Given the further increases in populations at higher risk of MCC, the projected annual incidence of MCC in the US increases to 3,284 cases in 2025 (**Figure 3B**).

To determine the approximate accuracy of our approach, we retrospectively performed similar forecasts (projecting 2008 using 2003 data, and 2013 using 2008 data). When we performed such calculations, the observed numbers of incident cases were 9-13% greater than our projections, indicating that our methods were underestimating true incidence. This was due to increases in the age and sex adjusted incidence rate (assumed to be stable for the projections). If one were to instead allow for a 10% increase in incidence rate, the projected annual incidence of MCC would increase to approximately 3,500 cases per year in 2025.

The methods of Bashir and Esteve were next utilized to determine the proportion of increase in incident cases due to increased population size versus the proportion due to the aging of the population[86]. From 2015 to 2025, we forecast a total increase in incident MCC cases of 812 cases per year (from 2,472 cases per year in the US in 2015 to 3,284 incident cases per year in the US in 2025). Of this increase, only 200 cases are explained by growth in population. The remaining 612 cases are instead due to the aging of the population, largely the aging of the baby boomers.

Ideally, incidence forecasts would effectively control for race and ethnicity. However, due to the relative rarity of MCC in non-white populations, forecasts accounting for each racial and ethnic group could not be performed with adequate precision. We did perform forecasts in the largest subset of patients with MCC (non-Hispanic whites) using race- and ethnicity- specific (as well as age- and sex-specific) incidence rates and population forecasts. By these methods, the number of incident cases in non-Hispanic white individuals in the US is predicted to be 3,077 cases in 2025. Assuming this represents approximately 90% of total cases of MCC (based on current data from 2011-2013, as above), this brings the total estimate of MCC incident cases in the US to 3,419 cases in 2025, which is roughly concordant with our projected annual incidence in 2025 of 3,284 cases as derived above.

DISCUSSION:

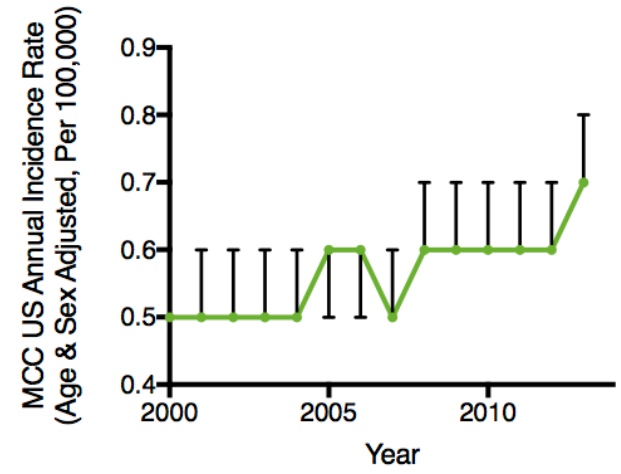
Merkel cell carcinoma is an aggressive skin cancer that is associated with Merkel cell polyomavirus and sun exposure. The incidence of MCC has risen over the past several decades. Here we report ongoing increases in incidence, with the number of incident cases rising by >95% since the year 2000, which is well above the increase in incident cases of all solid tumors (15%) and even above that of the rapidly increasing melanoma (56%). We further project incident cases over the next 5 and 10 years, utilizing population projections from the US census. We estimate current annual incidence at 2,500 cases per year in the US, rising to approximately 3,250 cases in the year 2025 based on the established relationship of age and MCC risk.

Merkel cell carcinoma particularly affects the elderly; this relationship to age is much more pronounced than for melanoma or solid tumors in general. This relationship is observed despite the fact that infection with Merkel cell polyomavirus often occurs before adulthood[87-90]. Given the critical role that the immune system plays in MCC surveillance as evidenced both by the observation of worse outcomes in immunosuppressed populations[26] and better outcomes in patients with brisk immune responses[91], as well as the excellent responses to immunotherapy amongst patients with MCC[1,65,92], it is plausible that the predilection of MCC for older individuals may represent diminished immunity in these populations. Indeed, immunosenescence is a well characterized phenomenon with diminished B and T cell function as well as response to vaccination in older individuals[93].

Our study had several limitations. Although large, including more than 6,000 patients from a database encompassing more than one-quarter the US population, there may be some geographic differences in incidence not reflected in the available data. Projections are limited to the US. Future studies could consider doing similar projections in other US (eg. National Cancer Data Base or National Program for Cancer Registries) or European/worldwide databases. For the projections of MCC incidence, we held the rate of MCC incidence for any given age steady despite the observed increases in adjusted-rates over the past decade, and thus the projected incidence of 3,250 cases may be an underestimate of true incidence. Our data was standardized to the 2000 census as the 2010 census data was not yet integrated into SEER*STAT. Our projections cannot take into account skin tone or changes in sun exposure pattern that may occur across the next ten years, although changes in these factors are unlikely to have substantial effect in the short term. In addition, we lack immunosuppression data which can affect risk, although patients with immunosuppression currently represent <10% of those diagnosed with MCC[84].} Finally, our data report on incidence only, not prevalence or mortality.

In conclusion, the incidence of Merkel cell carcinoma is increasing and will very likely continue to rise as the baby boom population enters the higher-risk age groups for MCC. We estimate this will exceed 2,800 MCC cases per year in 2020 and 3,250 cases per year in 2025 in the US. Because of its high propensity for spread, the need for adjuvant radiation in many cases[94], and the clear role for early

immunotherapy in the metastatic setting, both early detection and optimal management will be critical for improved outcomes. These ongoing increases in MCC incidence strongly advocate for increased specialty-appropriate MCC-specific education to the broad set of providers that care for MCC patients.



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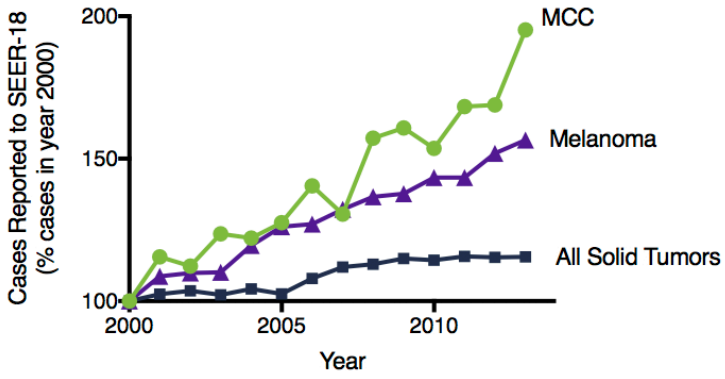
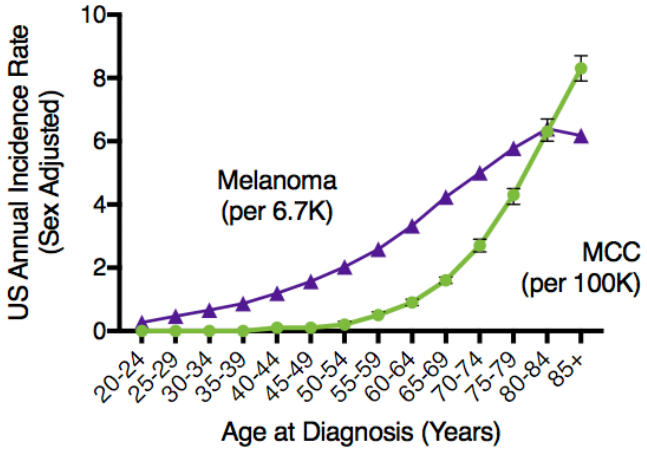


B.

Figure 1. Changes in incidence of Merkel cell carcinoma (MCC) as compared to all solid tumors and melanoma, 2000-2013. Data were extracted from the SEER-18 database, which captures 28% of the US population. A) US annual incidence rate of Merkel cell carcinoma The US annual incidence rate, age and sex adjusted to the 2000 US standard population (cases per 100,000 persons per year). Bars represent 95% confidence intervals. B) Cases reported to SEER with year 2000 as reference. The change in number of cases reported to SEER-18 (which reflects incidence rate and number of persons at risk in SEER catchment area) are shown, normalized to year 2000. The total number of solid tumors reported (blue squares) increased by 15% between 2000 and 2013, as compared 57 percent for melanoma (purple triangles), and 95% for MCC (green circles).



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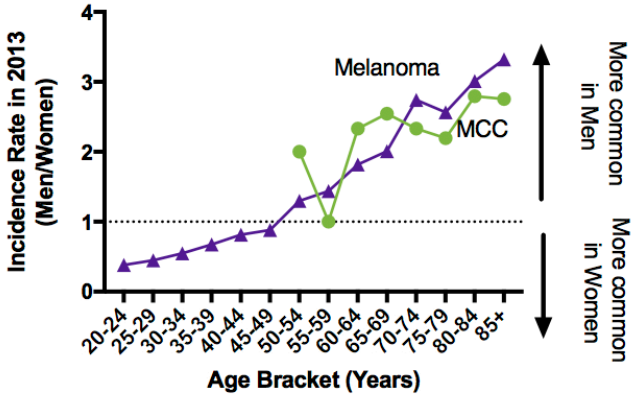

B.

Figure 2. Merkel cell carcinoma disproportionately impacts individuals >=65 years of age. A) Incidence rate by age. Incidence rate by age is shown for Merkel cell carcinoma (green circles, per 100,000 persons) and melanoma (purple triangles, per 6,667 persons). Unlike for melanoma, the incidence rate of MCC increases in individuals >= 85 years of age. N=6,600 cases of Merkel cell carcinoma and 251,437 cases of melanoma (all cases reported to SEER between 2000-2013 with associated age and sex information). 95% confidence intervals are shown. B) Relative incidence in men and women by age. Both MCC and melanoma have a strong male predominance in the oldest individuals. There are insufficient cases of MCC below age 50 to determine whether women in the 'Gen-X' and 'Millenial' generations will be at higher MCC risk relative to men, as they are for melanoma. Year 2013 only is shown due to rapid changes in melanoma risk for young women.

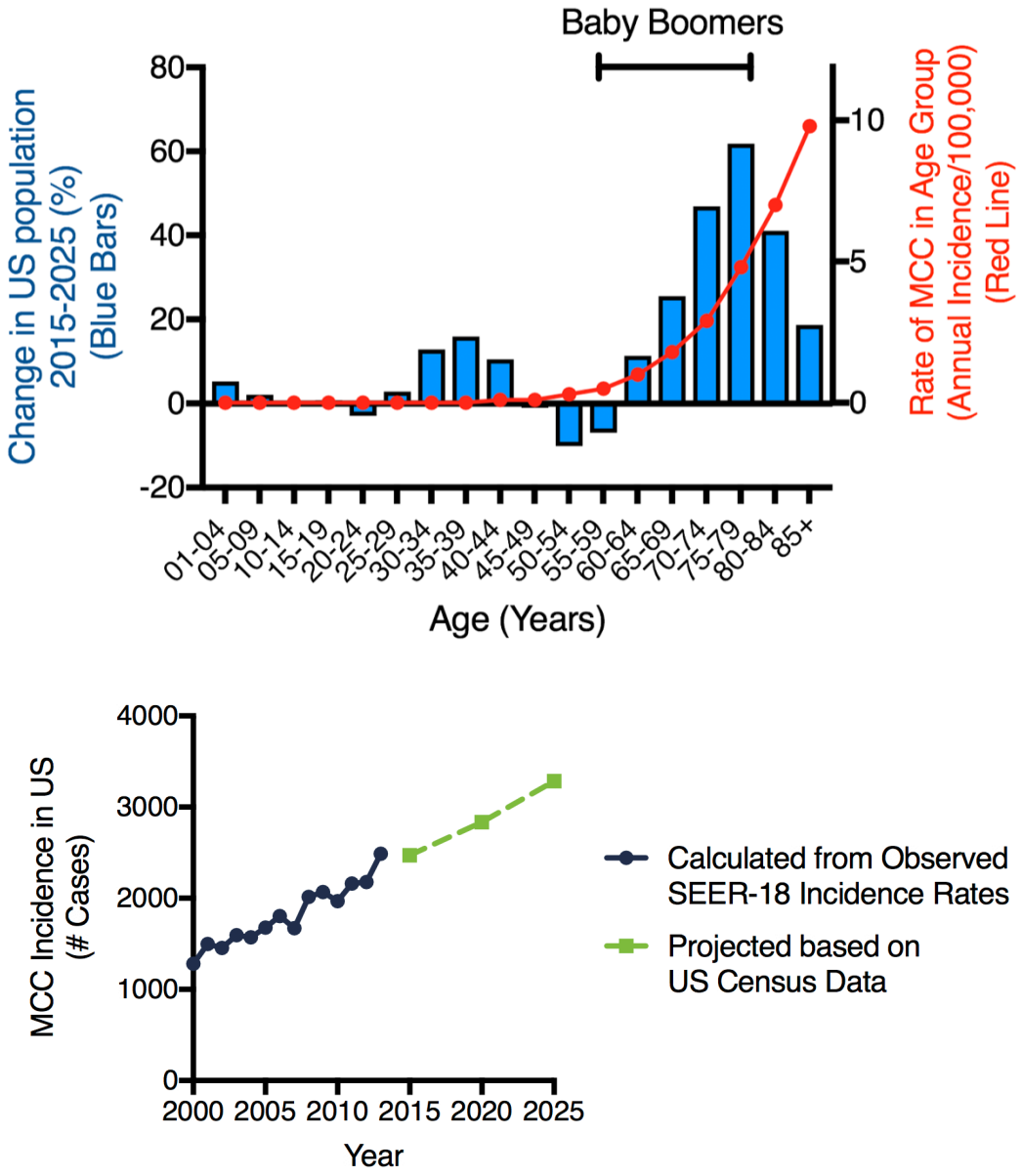

Figure 3. Observed and projected MCC incidence. A) Explanation for ongoing brisk rise in MCC incidence. Projected change in US population based on US census projections (bars) with MCC incidence rate per 100,000 from 2011-2013 (red line) (most recent years of available data) overlaid. The baby boom generation in 2025 is indicated by the bracket and account for much of the anticipated rise in MCC incidence. B) Observed incidence and projected annual incidence for MCC from 2000-2025, based on SEER-18 data and US census projections. Estimated number of cases in 2015 in the US is 2,472 cases and in 2025 3,284 new cases per year.

CHAPTER 3: MERKEL CELL CARCINOMA ETIOLOGY, IMMUNOGENICITY AND CURRENT TREATMENT STRATEGIES

Adapted from: Vandeven N, Nghiem, P: Rationale for immune-based therapies in Merkel polyomavirus-positive and -negative Merkel cell carcinomas. Immunotherapy. 2016 Jul;8(8):907-21.

Chapter Summary:

Merkel cell carcinoma (MCC) is a rare but often deadly skin cancer that is typically caused by the Merkel cell polyomavirus (MCPyV). Polyomavirus T-antigen oncoproteins are persistently expressed in virus-positive MCCs (~80% of cases), while remarkably high numbers of tumor-associated neoantigens are detected in virus-negative MCCs, suggesting that both MCC subsets may be immunogenic. Here we review mechanisms by which these immunogenic tumors evade multiple levels of host immunity. Additionally, we summarize the exciting potential of diverse immune-based approaches to treat MCC. In particular, agents blocking the PD-1 axis have yielded strikingly high response rates in MCC as compared to other solid tumors, highlighting the potential for immune-mediated treatment of this disease.

Modifications to published text:

A few modifications have been made to the published text listed above (Vandeven & Nghiem[95]). Modifications include the addition of several figures so as to provide a more detailed introduction to this dissertation. All added figures reference their publication of origin within the figure legend. Additionally, in the published article, two anti-phagocytic molecules (CD47 & CD200) were listed as mechanisms of immune evasion under the heading “other candidate mechanisms of immune evasion”. Within the context of this disseratation, these molecules are now discussed in more detail as we have recently reported the finding that MCC express elevated CD200 expression[96]) and preliminary data indicates that CD47 expression is associated with poor outcome in MCC. These findings were unknown at the time of publication. Finally, we have added in extended data pertaining to response rates from three MCC clinical trials treating patients with pembrolizumab (anti-PD-1), avelumab (anti-PD-L1) or autologous T cell therapy. These data expand upon the preliminary work that was reported last year (2016) and now include significantly longer follow-up times.

Introduction

Merkel cell carcinoma (MCC) is a rare and often lethal skin cancer with an incidence of ~2,000 new cases per year in the US[97]. While infrequent, the reported incidence of MCC has tripled in the last 30 years[69,70]. This increased incidence is partially attributable to the identification of cytokeratin-20 **(Figure 1C)** as an immunohistochemical marker of MCC in 1992, which has greatly enhanced the detection of MCC[98]. Additionally, a rising prevalence of known risk factors for MCC including immune suppression, age over 50 and extensive prior sun exposure likely contribute to the increased number of reported MCC cases[84]. Clinically, MCCs present as painless, red or purple nodules **(Figure 1A)** and are commonly misdiagnosed as benign cysts or as another malignant neoplasm[69,84]. The vast majority of cases arise in Caucasians, predominantly in males and in sun-exposed areas, suggesting that UV-induced skin damage is a major contributing factor in the development MCC[84]. While the single most common site of presentation is on the head and neck, accounting for nearly half of cases, MCC can arise on non-sun exposed regions including on the skin of the buttocks as well as rarely on the oral and genital mucosae[69]. Our understanding of the etiology of MCC has expanded dramatically over the past several decades, most notably with the discovery of the Merkel cell polyomavirus (MCPyV) which is causative in ~80% of MCC cases[57].

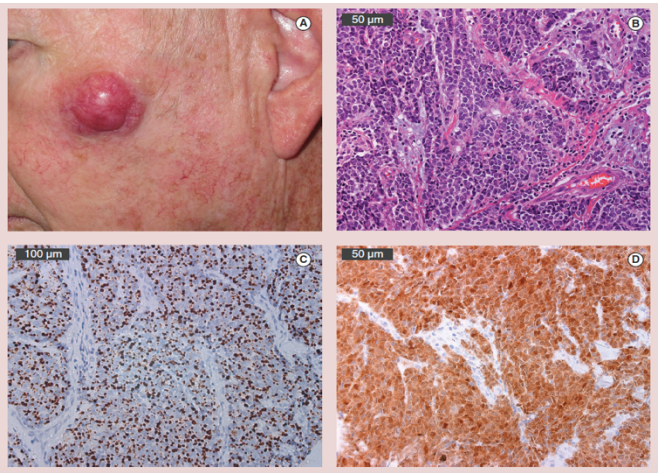

Figure 1. Clinical and pathologic presentation of Merkel cell carcinoma. (A) A 2.5 cm primary MCC on sun exposed skin of the left cheek. **(B)** Hematoxylin & eosin magnification of MCPyV-positive MCC tumor. Bar indicates 50 µm. **(C)** Cytokeratin-20 immunohistochemical staining of an MCPyV-positive MCC demonstrates characteristic perinuclear dot-like expression. Bar indicates 100 µm. **(D)** Viral oncoprotein expression limited to tumor (not adjacent stroma). MCPyV LT-antigen expression detected using CM2B4 antibody. Bar indicates 50 µm. Photos courtesy of Chris Lewis.

The majority of MCCs are associated with the Merkel cell polyomavirus (MCPyV)

MCC occurs more frequently in patients with immunodeficiency, including AIDS, suggesting that MCC may have an infectious etiology similar to Kaposi's sarcoma and EBV-induced Burkitt's lymphoma [26,99-101]. This was confirmed in 2008 when MCPyV was discovered in 8 of 10 tested MCC tumors using Digital

Transcriptome Subtraction, a high-throughput cDNA sequencing platform that aligned MCC tumor transcripts against reference human sequences[57]. MCPyV was found to be clonally integrated in these tumors, suggesting that viral integration is a critical and early event in MCC development (**Figure 2**)[57]. Viral integration occurs throughout the genome without apparent specificity[102] and therefore likely does not require perturbation of specific host cell genes to mediate oncogenesis. Furthermore, integration is probably a rare biological event as it prevents viral transmission and renders the MCC tumor cell a dead-end host for MCPyV[103].

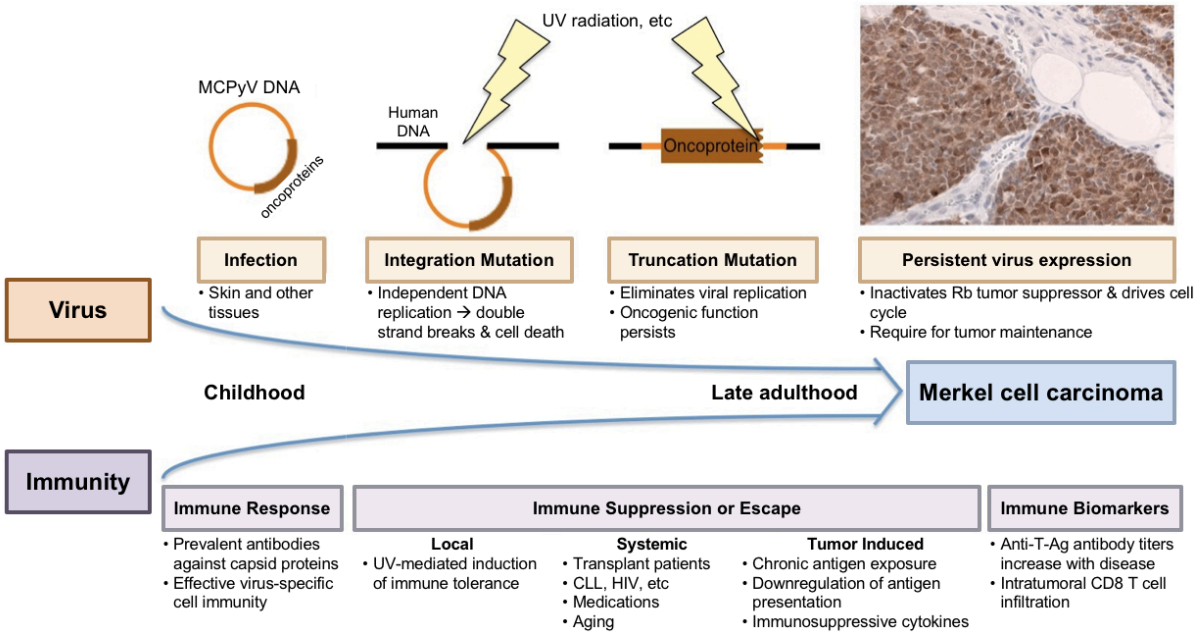

Figure 2: Although MCPyV infection is common, several rare mutagenic events are required to develop MCC. Infection with MCPyV occurs early in childhood, is clinic all asymptomatic and likely induces humoral and cellular immune responses. UV radiation and other mutagens may mediate viral integration and LT truncation leading to MCC pathogenesis. Local, systemic, or tumor induced loss of immune surveillance may allow for unsupervised increase in viral burden and T-Ag drive MCC. Adapted from Bhatia et al., Curr Oncol Rep, 2011.

MCPyV infection is widely prevalent and appears to be asymptomatic, with the exception of rare occurrences of MCC[104,105]. Seropositivity against the viral capsid protein VP1 as well as viral DNA from cutaneous swabs indicate that 40-88% of healthy adults have been infected, with primary exposure often occurring during childhood[90,104,106-108]. Viral DNA has also been detected in the respiratory tract, saliva, urine and the gastrointestinal system, suggesting possible fecal-oral transmission[105]. Fascinatingly MCPyV is currently the only human polyomavirus known to be oncogenic, despite numerous studies investigating the carcinogenic potential of the 12 other human polyomaviruses[109].

MCPyV biology

MCPyV is a small (~5kb), double stranded DNA virus that consists of both early and late gene regions (**Figure 3**)[110]. Current evidence suggests that LT and sT are the major oncoproteins mediating MCPyV-driven tumorigenesis as knockdown of these T-antigens results in cell cycle arrest and death in MCPyV-positive MCC cell lines[3,58,111].

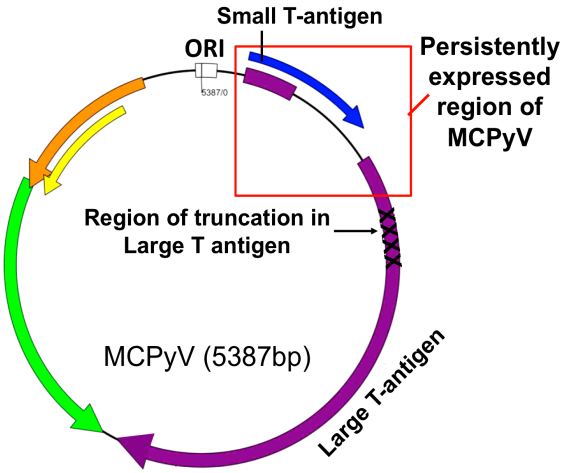

Figure 3: Schematic of the Merkel cell polyomavirus. The MCPyV genome encoding characteristic features of a polyomavirus, including the large T-antigen (purple) and small T-antigen (blue). The persistently expressed region is denoted by the red box, and the region of truncation observed in MCCs is indicated by the XX's. Adapted from Feng et al., Science, 2008.

MCPyV LT promotes oncogenesis partially through the highly conserved LXCXE motif, which binds to retinoblastoma protein (Rb)[112]. Rb normally sequesters the transcription factor E2F, however, LT binding to Rb releases E2F resulting in increased expression of cyclin E and CDK2. This promotes entry into the S-phase of the cell cycle and subsequent cellular proliferation[110]. While it appears that the LXCXE motif is critical for MCPyV-driven oncogenesis, mutation of LT resulting in C-terminal truncation is another crucial event in MCC tumor development. This hallmark truncation event within MCCs eliminates uncontrolled viral replication, as is seen in other virally-driven cancers, thereby preventing initiation of DNA damage response and cell death[103,110].

While MCPyV sT shares the first 78 N-terminal residues with MCPyV LT, expression of sT alone mediates *in vitro* transformation of rodent fibroblasts independent of LT expression and can induce hyperplasia and transformation in transgenic mice[113-116]. MCPyV sT alters cap-dependent translation through inhibition of 4E-BP1 and can prevent degradation of MCPyV LT as well as other key oncoproteins including cyclin E, c-Myc, c-Jun, Notch, mTOR, MCL-2 and NF-κB2 through suppression of the E3 ubiquitin ligase, SCF^{Fbw7}[117]. Detailed summaries of the currently known functions of LT and sT are presented in several recent reviews[110,118]. Importantly, these viral oncoproteins are persistently expressed

in MCC tumors **(Figure 1D)** and are absent in normal tissues, thereby providing ideal targets for immune therapy.

IMMUNE RESPONSE AGAINST MCC

Immune suppression leads to a dramatically increased risk of developing MCC[26,84,99,119]. While 90% of MCC patients do not have clinically apparent immune dysfunction, patients on immunosuppressive regimens following organ transplantation or with compromised cell-mediated immunity (such as those with chronic lymphocytic leukemia and HIV/AIDs) are 10-30 fold more likely to develop MCC and suffer a higher MCC-specific mortality rate than the general population[84,119-122]. This suggests that impaired cellular immunity predisposes individuals to not only developing MCC, but also to poorly controlling their disease.

Additionally, MCCs can regress following withdrawal of immune suppressive treatment [123,124] and spontaneous regression of MCCs is associated with T cell and foamy macrophage infiltration suggesting that regression may be immune-cell mediated [125,126]. While rare, spontaneous regression in MCC is much more common (1.3 per 1,000 cases) than in other malignancies (1 in 60,000-100,000 cases) [126]. Furthermore, a subset of advanced stage MCC patients present with unknown primary tumors (no primary skin lesions are detectable) likely as the result of immune-mediated clearance of the primary lesion and these patients have markedly improved overall and disease-specific survival[127].

Humoral response

The immune response against MCC encompasses both the humoral and cellular arms of adaptive immunity. While MCPyV infection is almost ubiquitous, MCC patients have significantly higher capsid protein antibody titers and higher MCPyV DNA levels on their skin than healthy controls, suggesting that these individuals have reduced viral control [87,106,108]. Humoral recognition of MCPyV T-antigen oncoproteins on the other hand is restricted to MCC patients. Among MCPyV-positive MCC patients, ~40% are seropositive for the oncoproteins at the time of diagnosis while these antibodies are detected in <1% of healthy controls [107]. MCPyV oncoproteins are not expressed within MCPyV virions, however, viral

integration in the setting of MCC results in persistent intracellular expression of LT and sT, potentially explaining why the presence of oncoprotein antibodies is restricted to MCC patients[128]. Oncoprotein antibody titers have been found to fluctuate with tumor burden and a clinical test monitoring oncoprotein antibody titers is now being used as a tool to monitor disease progression **(Figure 4)**[129]. It is unclear whether MCPyV oncoprotein antibodies affect (positively or negatively) tumor clearance, however, oncoprotein seropositivity at the time of diagnosis is associated with reduced risk of recurrence and may reflect a broader immune response against MCC[129].

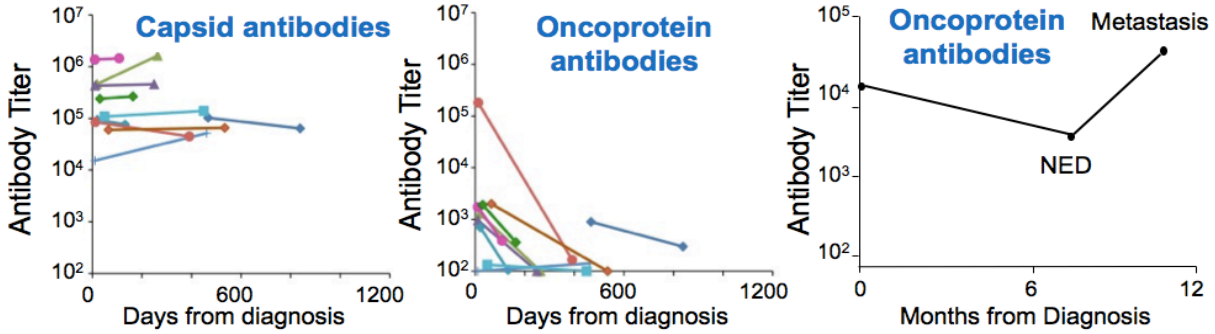

Figure 4: Titers of IgG to MCPyV T-Ag, but not VP1, dynamically reflect MCC disease burden. A: Patients without MCC recurrence (these 10 cases represented in both the VP1 and T-Ag graphs). These patients did not progress in the time between draws or subsequently develop MCC recurrence after the second blood draw (available follow-up range, 0–6 mo). **B:** Patients with progression often have a rise in titer preceded the clinical detection of metastasis by 1 to 6 mo. Disease status at time of draw is indicated on the graph. NED, no evidence of disease; MET, metastasis/ disease progression. Adapted from Paulson Can Res 2010.

T cell response

The production of oncoprotein-specific antibodies implies the presence of a MCPyV-specific CD4 response. In an effort to identify MCPyV-specific T cells, Iyer *et al.* described an initial set of 24 epitopes within the persistently expressed region of the T-antigen oncoproteins[130]. Five of the 24 were recognized specifically by CD4 T cells and subsequently an additional CD4 epitope was reported[130,131]. Therefore, 6 MCPyV-specific CD4 epitopes have been described, however, limited information regarding the HLA restriction of these epitopes as well as phenotypic and functional analysis of CD4 T cells specific to these epitopes is required in order to understand the role of these cells in the context of MCC. Investigation into the CD4 T cell response will be address in detail in **Chapters 5-8.**

The importance of the CD8 T cell response against MCC is highlighted by the finding that robust intratumoral (not peritumoral) infiltration of CD8+ TILs is associated with a striking 100% survival in a

study of 146 patients[91]. Additional studies have also indicated that MCC TILs, including CD3+, CD8+ T cells, are associated with improved overall and disease-specific survival[132,133]. Furthermore, expression of genes encoding granzyme A, B, H, and K, CCL19, lymphocyte activation genes (SLAMF1 and NKG2D) and CD8α are associated with favorable prognoses, independent of stage[91]. To date, 17 MCPyV-specific CD8 epitopes have been identified, for which 14 HLA-I tetramers have been generated and 7 have been validated in our lab (**Table 1**), enabling functional and phenotypic analysis[130,134-136]. Importantly, while robust CD8 responses have been associated with improved outcome in MCC, only 4-18% of MCC patients present with significant CD8 infiltration, suggesting that most MCCs block intratumoral CD8 infiltration as a means of evading immune detection[91,92].

Table 1. Current validated CD8 MCPyV-specific tetramers. Additional epitopes are known[130] and unpublished.

Name	Type	HLA locus/allele	Protein, AA	TIL (+)?[2]	Notes, references
A2KLL[3]	CD8	A*0201	CT15-23[3]	yes	~20% of MCPyV(+) A*0201 (+) MCC pts are PBMC (+)[134,137]
A2KTL	CD8	A*0201	ST171-181	yes	found in TIL[135] but not yet PBMC. 172-181 also active
A24EWW	CD8	A*2402/A*23[1]	LT92-101	yes	~50% of MCPyV(+) A*2402 (+) MCC pts are PBMC (+)[130,134]
B7APN	CD8	B*0702	CT20-29	yes	Unpublished
B35FPW	CD8	B*3502	ST83-91	yes	Used to isolate cells for CD8 therapy by Project 2 team (NCT01758458)[136]
B37KEW	CD8	B*3701	LT91-101	yes	Unpublished
C2FSF	CD8	C*0202	LT99-107	yes	[136]

MCC TUMOR EVASION MECHANISMS. Over the past few years, studies have reported several immunological barriers that often occur within the MCC tumor microenvironment (**Figure 5**).

MHC-I down-regulation

In order for a tumor to be immunologically detected by CD8 T cells, tumor-associated antigens must be presented in the context of MHC-I molecules. However, immunohistochemical evaluation of 114 MCC tumors indicated that 84% downregulated expression of MHC-I, with 51% being markedly downregulated (**Figure 6)** [92]. Furthermore, mRNA expression levels of MHC-I closely correlated with expression levels of antigen processing machinery, including proteins involved in the antigen processing complex TAP. This suggests that multiple components involved in antigen processing and presentation are downregulated in MCC and may impair T cell recognition of MCC tumors [92]. Importantly, treatment of MCC cell lines with

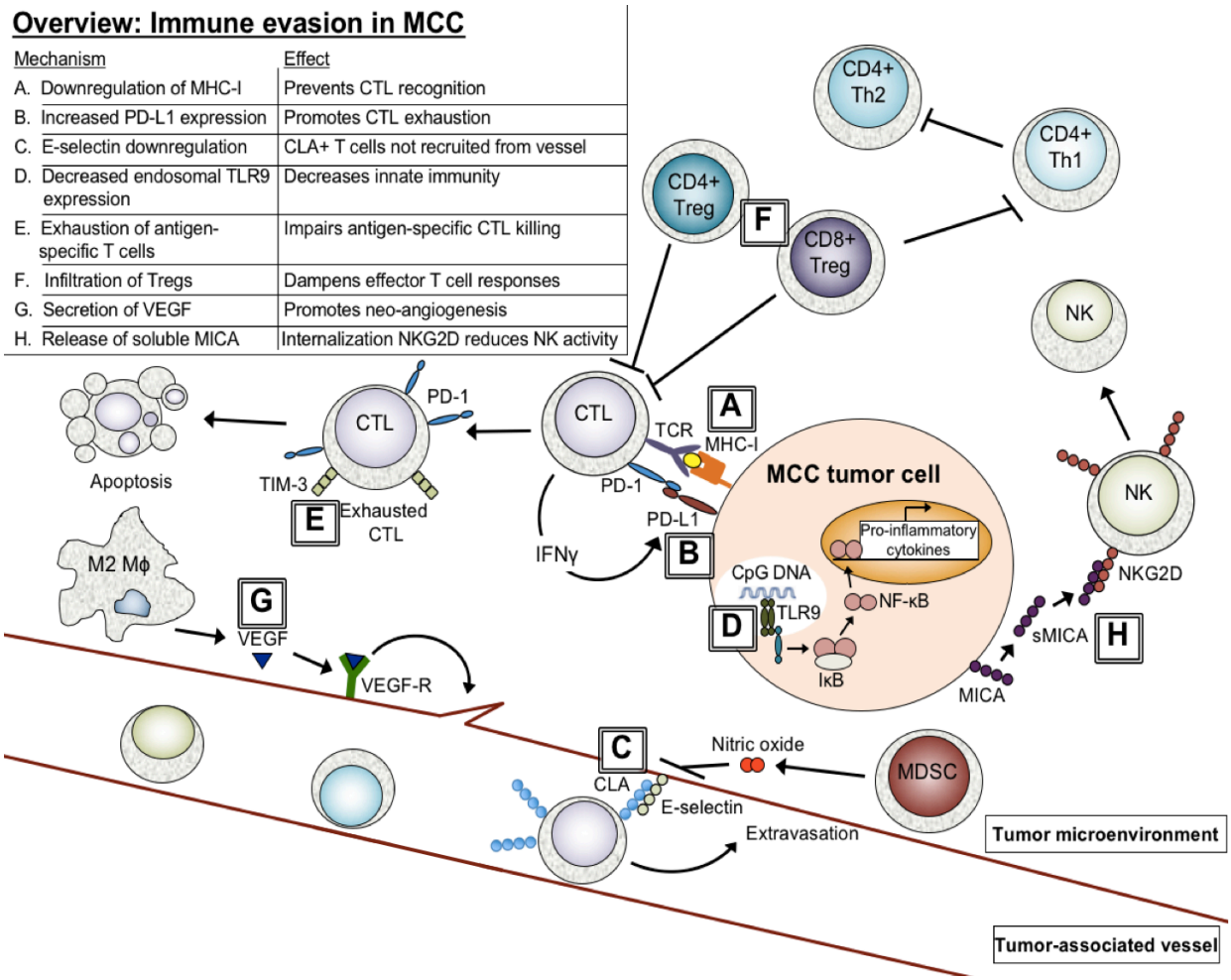

Figure 5: Schematic of documented and putative mechanisms of immune evasion in MCC. The letters in the key above (A-H) indicate critical mechanisms implicated in immune evasion for MCC, which are detailed in the text.

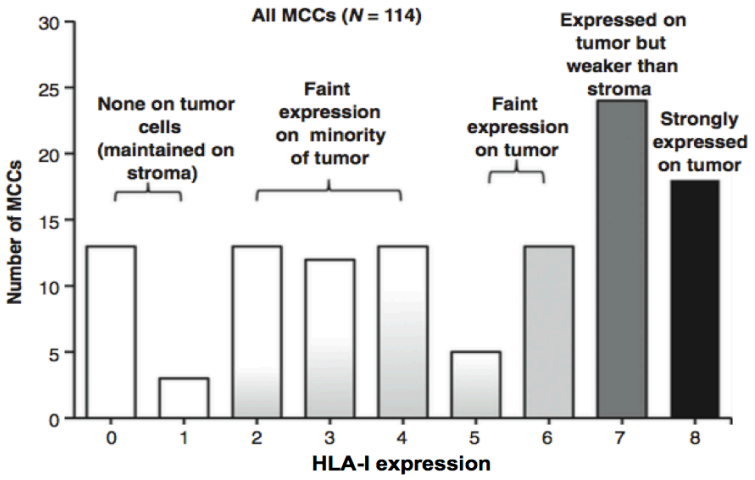

Figure 6: HLA-I downregulation is frequent in MCC tumors. HLA-I expression among 114 human MCC tumors as determined by immunohistochemistry and Allred scoring. HLA-I was downregulated (Allred score ≤7) on 84% of MCCs. Adaptive from Paulson *et al*., Cancer Immunol Res, 2014

type-I interferons, etoposide (a standard MCC chemotherapeutic) and radiation, can all induce MHC-I upregulation *in vitro*[92]. Notably, *in vitro* treatment of MCC cells lines with type-I interferons also reduced expression of MCPyV LT, which may further promote tumor destruction[138]. Downregulation of MHC-I can also be reversed *in vivo* and will be discussed subsequently in the context of intralesional IFN treatment.

Programmed cell death ligand-1 (PD-L1)

PD-L1 is a member of the B7 immunoglobulin superfamily[139] and is a ligand for the programmed death-1 (PD-1) receptor expressed primarily on T lymphocytes[140]. PD-L1 binding to PD-1 limits T cell expansion, promotes functional exhaustion of T cells by inhibiting IL-2 and IFNγ production and decreases survival[141,142]. This mechanism is thought to play an important physiological role in facilitating tolerance and suppressing autoimmunity, however, evidence suggests that cancers and viruses (including HBV, HPV, EBV, HTLV-1) can induce PD-L1/PD-1 expression to promote local immune suppression[141,143]. Expression of PD-L1 within the tumor microenvironment in gastric carcinoma, RCC, and esophageal cancer is associated with poor prognosis[144-146]. Conversely, in melanoma and MCC, PD-L1 expression is associated with improved overall survival[143]. An evaluation of 67 MCC specimens from 49 MCC patients found that 49% of tumor cells and 55% of TILs expressed membranous PD-L1 **(Figure 5: process "B")**[143]. All of these PD-L1 expressing tumors had tumor infiltrating lymphocytes (TILs) while TILs were detected in only 47% of PD-L1 negative tumors[143]. Similarly, in another study PD-L1 protein and mRNA expression correlated with the presence of intratumoral CD8 T cells[134]. Therefore, while increased PD-L1 expression may be preventing a complete anti-tumor response, detection of intratumoral PD-L1 indicates some degree of immune activity against MCC and suggests that PD-1 blockade may be a promising therapeutic approach for this disease[143].

Downregulation of E-selectin

While MHC-I downregulation and PD-L1 expression may reduce activation of tumor-specific T cells, another mechanism of immune evasion is to prevent recruitment of T cells into the tumor microenvironment. Cutaneous lymphocyte antigen (CLA) is expressed on skin-homing T cells and is critical for T cell extravasation from the vasculature into the tissue[147]. CLA binds to E-selectin and/or P-

selectin expressed by endothelial cells. In squamous cell carcinomas (SCCs), E-selectin downregulation is mediated through nitric oxide (NO) signaling that is released by tumor-associated myeloid derived suppressor cells (MDSCs)[148]. Nitration of proteins is a marker of NO production and evaluation of nitrotyrosine expression in MCCs indicated that increased levels of nitrotyrosine was associated with decreased E-selectin expression and CD8 T cell infiltration, suggesting that a similar mechanism is being employed within MCC tumors **(Figure 5: process "C")**[134]. Notably, elevated expression of E-selectin was correlated with improved survival in MCC patients, implying that T cell extravasation into the tumor microenvironment is critical for optimal immune function against MCC[149]. These findings are described in detail in **Chapter 4**.

Decreased expression of TLR9

While most of the described mechanisms have related to adaptive immune responses to MCC, innate immune signaling can also elicit antitumor effects. Toll-like receptor 9 (TLR9) is expressed within the endosomal compartment and activates the NF-κB pathway in response to viral and bacterial CpG-DNA motifs thereby promoting a pro-inflammatory response[150]. MCPyV-LT and –sT have been shown to inhibit TLR9 expression in an epithelial and MCC cell line *in vitro* which may reduce inflammatory responses **(Figure 5: process "D")**[151]. Several other oncogenic viruses (including HPV, EBV and HBV) have also been shown to alter TLR9 expression, suggesting that this is a common strategy to limit immune activation[152].

CD8 T cell exhaustion

T cells can become dysfunctional or exhausted within a few weeks after infection if the infectious agent persists and is not cleared by the host[153]. This has been extensively described in the setting of persistent viral infections or more recently in cancer[153]. The obligate expression of viral T-antigens in MCC, therefore may similarly induce a state of exhaustion in virus-specific T cells. Exhausted T cells have distinct transcriptional programs, impaired proliferative capacity, decreased cytokine production and reduced cytotoxicity[153]. A hallmark of exhausted T cells is the increased expression of various inhibitory receptors including PD-1, TIM-3, Lag-3, and 2B4[153]. MCPyV-specific T cells isolated from MCC tumors

and peripheral blood have been shown to express elevated levels of the inhibitory markers PD-1 and TIM-3 relative to control CMV- or EBV-specific cells **(Figure 5: process "E")**[134]. Additionally, MCPyV-specific CD8 T cells often have a limited ability to secrete the effector cytokine IFNγ following antigenic stimulation and tumor infiltrating lymphocytes (TILs) within MCC tumors have markedly lower expression of the early activation marker CD69 relative to T cells isolated from normal skin, supporting the notion that these cells are dysfunctional and exhausted[130,154,155].

CD4 T cell polarization

In several cancer types, intratumoral infiltration of Th1 CD4 T cells is strongly associated with good clinical outcomes while infiltration of other CD4 subtypes (Th2 and Th17) is associated with mixed outcomes[156](discussed in more detail in **Chapter 6**). Th1 cells produce large amounts of IFNγ, which facilitate priming and expansion of CD8 T cells[157]. Th1 CD4s also serve to recruit NK and type-I macrophages (pro-inflammatory) to the tumor site, thereby orchestrating robust antitumor immunity[157]. In the setting of MCC, secretion of Th1 and Th2 type cytokines by bulk intratumoral CD4 T cells was observed from one MCC patient[130]. Whether a significant bias towards a particular subtype occurs in MCPyV-specific CD4s or in additional MCC tumors has not been investigated. Importantly, several therapeutic approaches (discussed below) that promote a Th1 type response have shown clinical promise in treating MCC suggesting that a Th1 response may be beneficial in MCC.

T regulatory cells

T regulatory cells (Tregs), typically identified through expression of CD25 and FOXP3, play a crucial role in mediating peripheral tolerance to self-antigens under normal conditions. However, in the setting of cancer they are generally thought to be tumor promoting[158,159]. It has been shown that high percentages of CD25+FOXP3+ T cells infiltrate MCC tumors relative to normal skin **(Figure 5: process "F")**[155]. Notably, among FOXP3+ T cells, a discrete population of CD8+FOXP3+ T cells was observed in MCC tumors[155]. These CD8 Tregs are associated with disease progression in several other cancers including malignant melanoma, prostate, ovarian and colorectal[155]. These cells preferentially target Th1 CD4 cells while sparing Th2 cells which may contribute to a tumor-promoting polarization within the tumor

microenvironment[160]. While Dowlatshahi *et al*. reported that intratumoral FOXP3 expression was not correlated with survival in MCC, a study by Sihto *et al*. indicated that increased FOXP3 expression was associated with improved survival[79]. Therefore, it is unclear whether Treg function is a decisive factor in immune evasion in MCC.

Infiltration of M2 macrophages

M2 macrophages are typically induced by type II cytokines (IL-4, IL-10 and IL-13) and have reduced antigen presentation capacity, promote angiogenesis through secretion of vascular endothelial growth factor (VEGF), facilitate tissue remodeling and ultimately tumor progression[161]. Evaluation of immune cell infiltrates in 21 MCC tumors, found that nearly all of the macrophages present within MCC stained positive for CD163, a marker often used to identify M2 macrophages. Immunohistochemical analysis of MCC tumor samples from 29 patients indicated that VEGF-A, VEGF-C as well as the VEGF-receptor-2 (VEGF-R2) are highly expressed (>75%) within MCC tumors **(Figure 5: process "G")**, suggesting that angiogenesis via VEGF-VEGF-R ligation may be occurring in this disease[162]. Importantly, CD163 expression alone is likely insufficient to fully identify M2 macrophages[163], therefore a more detailed analysis including additional markers could more definitively characterize macrophage phenotypes in this disease.

Inhibition of NK cell killing

NK cells can induce cytotoxicity against certain tumor types without prior stimulation and high levels of infiltrating NK cells have been correlated with favorable outcomes in patients with several types of solid tumors[164]. NK cells are regulated by a complex balance of inhibitory and stimulatory signals[165]. Inhibitory killer Ig-like receptors (KIRs) expressed on NK cells bind MHC-I molecules and prevent NK-mediated killing of normal tissues[165]. Stimulation occurs primarily through MHC class I-related chain –A and –B (MICA/MICB) binding of NKG2A and NKG2D expressed on NK cells[165]. Cancer cells have been shown to evade NK cell activation by cleaving surface MICA/B into a soluble form, which transiently activates NK cells non-specifically, but ultimately causes inhibition by inducing downregulation of NKG2D **(Figure 5: process "H")**[165]. The presence of soluble MICA in patient sera has been associated with poor outcome in

some cancer types[165] but has not been reported in MCC. Infiltration of NK cells intratumorally has been reported in MCC[79] and the development of tumors despite significant downregulation of MHC-I implies that mechanisms of NK cell evasion are being employed within MCC tumors.

Impaired phagocytosis

One key anti-tumor function of macrophages is the ability to phagocytose tumor cells. Regulation of phagocytic function is tightly controlled via activating signals and inhibitory signals[166]. One such inhibitory signal is delivered via ligation of signal regulatory protein alpha (SIRPa, expressed on the surface of phagocytes) to CD47, a transmembrane protein expressed on the target cell surface[167]. Overexpression of anti-phagocytic signals such as CD47 have been reported on numerous tumor types including hematologic and solid tumors and is associated with poor prognosis[166,167]. Our preliminary evaluation of CD47 expression in 23 MCC tumors recapitulates these findings **(Figure 7)**. These findings may be due to the fact that Myc can drive the expression of CD47 and MCC tumors often upregulate Myc isoforms[168]**.** These data await validation in a larger cohort.

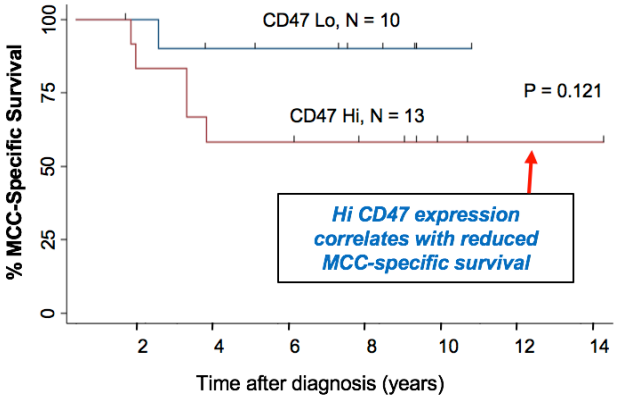

Figure 7: Elevated CD47 expression is associate with poor MCC-specific survival. MCC patients were divide into $CD47^{hi}$ and $CD47^{lo}$ subgroups based upon whether their CD47 tumoral expression was above or below the median expression score respectively. (Vandeven SITC 2016).

A second molecule that is known to play an anti-phagocytic role is CD200. This molecules is a membrane bound glycoprotein expressed by many cell types and we recently showed that 95% of MCCs express CD200[96]. It is currently unknown whether CD47 or CD200 play a role in pathogenesis of MCC, however, the elevated expression of both of these molecules in MCC support therapeutic targeting of these anti-phagocytic axes in order to facilitate increased antigen uptake and presentation to T cells[96,166].

Mechanisms of immune evasion in other cancers

Numerous additional mechanisms of evasion have been reported in other cancers that have yet to be investigated in MCC. Secretion of immunosuppressive cytokines such as TGFβ, IL-10, and Fas-L within the tumor microenvironment can promote the expansion of Tregs and decrease the activation of cytotoxic T cells and NK cells[159]. Tumor cell secretion of indoleamine 2,3-dioxygenase (IDO) and galectins can impair antitumor T cell responses[159]. Additionally, other cellular infiltrates, including myeloid derived suppressor cells (MDSCs), can promote tumor growth through numerous mechanisms including increased angiogenesis and disruption of antigen presentation[169]. Notably, several of these mechanisms are targetable for therapeutic purposes and therefore may merit further investigation in MCC[159,167,169].

Virus-negative MCCs and UV-induced neoantigens

While the study of MCC has primarily focused upon the immunobiology of tumors caused by MCPyV, several studies have recently investigated the ~20% of MCCs that do not contain the virus. The prognosis and overall survival of these two subsets of MCC patients has been debated. Two studies have indicated that patients with virus-negative MCC experience decreased survival as compared to patients with virus-positive MCC[170,171]. Conversely, several others have reported no significant survival difference between the two groups[172-174]. Importantly, genetic analysis indicates that these two subsets are etiologically distinct[59,60,62]. Specifically, several recent studies have shown that MCPyV-negative MCCs have a very high mutation burden (median 1,121 somatic single nucleotide variants per exome). These are dominated by C > T transitions, characteristic of UV-induced DNA damage[59,60,62]. This UV-induced signature was not observed in MCPyV-positive tumors and the mutation burden was 19-fold lower (median 12.5 somatic single nucleotide variants per exome) indicating that these tumor types arise through distinct mechanisms[59,60,62]. High mutational burdens seen in melanoma, colorectal and several types of lung cancer have been associated with a higher prevalence of tumor-associated neoantigens, greater immunogenicity and improved response to immune-based therapies[175]. Strikingly, on average, MCPyV-negative tumors were found to contain more tumor neoantigens than either melanomas or non-small cell lung cancers (NSCLC) suggesting that these virus-negative MCCs have the potential to be highly immunogenic[60]. Furthermore, among virus-negative tumors, a subset expressed PD-L1 and these PD-L1-

positive tumors harbor a higher mutational burden as compared to PD-L1-negative tumors, which may reflect immune recognition within these tumors[176]. Notably there were also varying grades of tumor infiltrating lymphocytes (TIL) within virus-negative tumors and an increased TIL infiltration correlated with improved survival, as has been described for MCC more generally[62,79,91]. These findings indicate that among virus-negative MCCs, tumors with higher mutational burdens have increased immune recognition which may indicate that this subset may be particularly responsive to checkpoint inhibitors as has been similarly described in other cancers[177,178].

Treatment of MCC

The standard initial management of MCC typically involves surgical excision and radiation therapy for local and regional disease while patients presenting with distant disease are primarily managed with systemic therapy[179]. Although virtually all patients are rendered free of detectable disease, roughly half of these patients will recur[180,181]. Once distant metastatic disease arises, cytotoxic chemotherapy leads to a response in >50% of cases, but the median time to progression is only 3 months and durable responses are exceedingly rare[182]. There is thus an urgent need for improved therapies.

Local immune therapies

The delivery of targeted therapies specifically into a tumor has proven efficacious for several immune-based agents and can significantly reduce toxicity that is observed with systemic treatment [183].

Single fraction radiation

Radiation therapy has been shown to increase antigen presentation and to diversify the T cell receptor repertoire of intratumoral CD8 T cells[184,185]. Preclinical models using targeted single-fraction radiation therapy (SFRT) indicate that SFRT enhances antitumor immunity more effectively than fractionated radiation[186]. SFRT has been reported for treatment of bone metastases in other cancers and because of the known immunogenicity of MCC, SFRT has been used for palliative treatment of MCC patients who either developed chemotherapy-resistant disease or who were unable to receive fractionated radiation for logistical reasons[187]. This approach has yielded a remarkable 94% objective response rate of irradiated

lesions in 26 patients who received SFRT to 93 tumors[187]. Complete responses were reported in 45% of tumors with no progression of 77% of tumors with a median follow-up time of 277 days[187]. Importantly, this approach is limited to “in field” lesion control and therefore may not be appropriate for all tumors. However, SFRT may be combined with other immune-stimulating agents as a means of lowering tumor burden and increasing antigenicity to enhance systemic immune responses[184,187].

Intralesional IL-12 DNA electroporation

IL-12 is a Th1 promoting cytokine that can facilitate antitumor immune responses by inducing IFNγ secretion and increasing proliferation and effector function of NK cells and T cells[188]. Furthermore, IL-12 can induce increased expression of MHC-I, MHC-II and ICAM-1 on human melanoma cells thereby enhancing antigen presentation and T cell recruitment[189]. However, systemic administration is extremely toxic and can lead to temporary immune suppression and even death[188]. Localized delivery of plasmid-IL-12 using electroporation has significantly reduced toxicity and has shown promising results in melanoma[188]. Subsequently, a phase II clinical trial using electroporation of intratumoral IL-12 DNA for MCC has fully enrolled and has had promising results in some patients (NCT01440816)[190].

Intralesional TLR4 agonist (GLA) injection

Activation of toll-like receptor signaling can lead to secretion of pro-inflammatory cytokines and type-I IFNs promoting adaptive and innate immune responses[191]. Glucopyranosyl Lipid-A is a recently generated synthetic TLR-4 agonist that is administered within a stable emulsion (GLA-SE) and specifically induces Th1 responses while minimizing Th2 responses[191]. A phase I/II clinical trial for treating MCC patients has completed enrollment and has been efficacious in some patients (NCT02035657)[192].

Intralesional IFNβ

The majority of MCCs downregulate MHC-I expression thereby evading cellular immune responses, however, several case reports have described MHC-I upregulation on MCC tumors following either intralesional IFNβ injections or local radiation therapy[154,193]. The use of IFNβ injections clinically has also

induced lesion shrinkage, which may be due to enhanced T cell recognition of these tumors[154,193,194]. Notably, a woman in Japan with stage II MCC on the right arm was treated with IFNβ injections and experienced a complete response that continued for more than 8 years, indicating the potential efficacy of this approach[194].

Topical dinitrochlorobenzene

Dinitrochlorobenzene (DNCB) forms stable protein conjugates that can stimulate T cells to secrete Th1 type cytokines and induce contact sensitization[195]. One patient with multiple local and regional MCC metastases experienced a complete response following 4 weeks of topical application of DNCB[196]. Adjuvant radiation of the whole scalp was performed following regression and the patient had remained in remission for more than a year at the time of the report[196].

Systemic immune therapies

While several local immune therapies have shown clinical promise, the treatment options for patients with advanced distant disease remain severely limited, therefore systemic immune approaches are being intensively investigated.

Anti-4-1BB (CD137)

The TNF-family receptor, 4-1BB, is expressed on activated T cells and antibodies binding this receptor increase NF- κB activity resulting in cytokine production, leukocyte proliferation and antitumor efficacy in preclinical models[197]. MCPyV-specific T cells express elevated 4-1BB on their surface relative to other virus-specific cells suggesting that these cells may be particularly responsive to 4-1BB agonism[134]. A phase-I trial in solid tumors (including MCC) and B-cell Non-Hodgkin's lymphoma using a 4-1BB agonist (PF-05082566) has completed enrollment. The drug was well tolerated and had promising antitumor activity in an MCC patient[198].

Allogeneic NK cell therapy

NK cells can be dysfunctional or suppressed in the setting of cancer, which may be augmented by the infusion of allogeneic (non-self) NK cells[164]. Unlike autologous NK cells, allogeneic NK cells are not inhibited by host MHC-I expression and can therefore overcome NK cell suppression[164]. One such cell line is NK-92, a continuously growing, IL-2 dependent line that is highly cytotoxic against several tumor cell types *in vitro* and *in vivo*[199]. Not only are NK-92 cells allogeneic, they also lack expression of most inhibitory receptors, thereby enhancing their cytolytic function[199]. A phase II clinical trial is currently recruiting MCC patients and the preliminary data looks promising. An MCC patient with advanced MCC that was refractory to several prior therapies, including pembrolizumab, experienced a complete response following aNK infusions (**Figure 8).** This response was immediate and dramatic tumor regression began about ~14 weeks after treatment, suggesting induction of an adaptive immune response from ongoing innate immune stimulation.

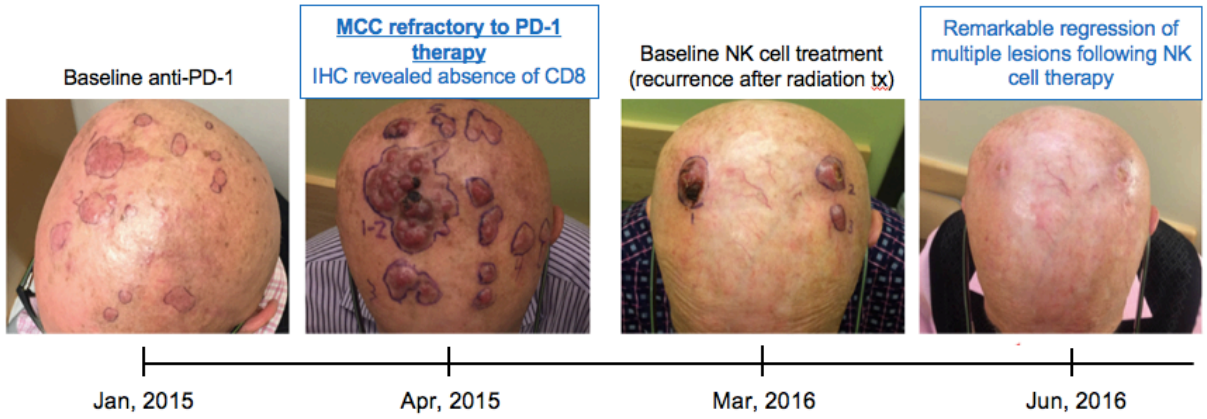

Figure 8. Regression of anti-PD-1 resistant after NK cell therapy in one patient. Patient did not respond to anti-PD-1 therapy and recurred after radiation therapy but exhibited striking regression of after NK cell therapy.

IL-2 fusion protein targeting tumor stroma

Tenascin C is expressed on reactive stromal cells in many solid tumor types predominantly around vascular structures[200]. The use of a monoclonal antibody (F16) targeting tenascin C fused to IL-2, enables targeted delivery of IL-2 to reactive tumor vasculature which may help mediate intratumoral immune activation[201]. Administration of F16-IL2 has been well tolerated and has shown clinical efficacy in trials of certain solid tumors. A phase II trial using F16-IL2 in combination with a chemotherapeutic agent,

paclitaxel, is currently enrolling for metastatic MCC patients in Europe (NCT02054884) under the auspices of IMMOMEC (http://www.immomec.eu).

Targeting CD47 with TTI-621

A clinical trial utilizing intratumoral injections of TTI-621 (anti-CD47) in patients with distant metastatic MCC (NCT02890368) recently opened and is currently enrolling. TTI-621 is a fully humanized recombinant fusion protein composed of SIRPa (the CD47 ligand expressed on macrophages) fused to an IgG1 Fc domain. This fusion protein thereby inhibits CD47 signaling (blocks the 'don't eat me signal') promoting phagocytosis which is further enhanced by the addition of an Fc domain[166]. The hypothesis is that TTI-621 will facilitate phagocytosis and antigen presentation in the TME and augment the adaptive immune response through increased intratumoral T cell infiltration, expansion of T cell reactivity (epitope spreading), and diversification of the T cell repertoire. Preliminary in vitro studies indicated that incubation of MCPyV-positive and –negative cells lines with TTI-621, increased phagocytosis of these MCC cell lines[2] suggesting that targeting this pathway may be beneficial in mediating increased antigen-presentation in MCC.

Ipilimumab (anti-CTLA-4)

Over the last decade, the use of monoclonal antibodies targeting checkpoint inhibitors CTLA-4, PD-1 and PD-L1 has revolutionized clinical oncology. These agents have proven remarkably efficacious in treating a range of liquid and solid tumors[202-207]. Ipilimumab, an IgG1 monoclonal antibody targeting cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), was the first checkpoint inhibitor to be approved by the FDA for treating advanced melanoma[202]. Ipilimumab therapy augments the T cell response through inhibition of T-regulatory cells and enhanced T cell priming thereby expanding the T cell repertoire[142,184,208]. Treatment of several cancer types with ipilimumab has shown promising results and a randomized clinical trial utilizing ipilimumab in the adjuvant setting for the treatment of MCC is currently enrolling in Europe (NCT02196961).

Nivolumab (anti-PD-1)

In melanoma, agents targeting the PD-1/PD-L1 axis have tended to show higher response rates than those targeting CTLA-4[44]. PD-1 axis blockade, like ipilimumab, enhances T cell function, though through a distinct mechanism. Instead of priming new responses, PD-1 blockade facilitates the expansion of pre-existing quiescent T [142,184]. A trial using nivolumab, an anti-PD-1 human IgG4 monoclonal antibody, was better tolerated than ipilimumab and reported a 28% response rate in melanoma patients. Responses were durable, with patients continuing to benefit even after drug discontinuation[203,209]. A phase I/II clinical trial utilizing nivolumab is open for patients with virus-associated tumors including MCC is showing promising results to date (NCT02488759).

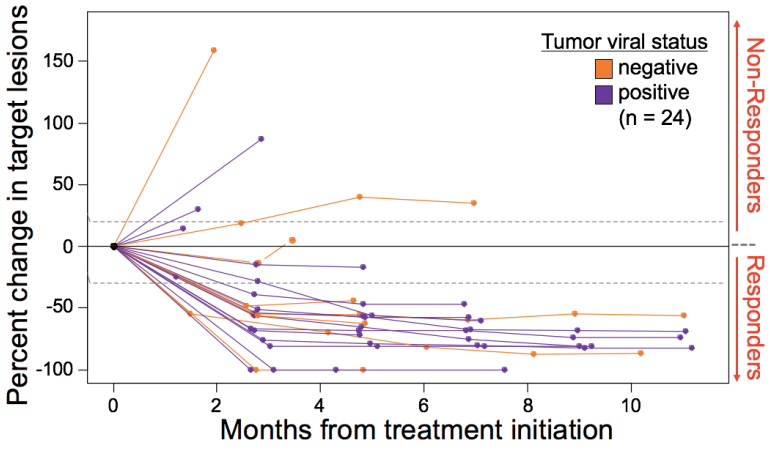

Figure 9: Response to pembrolizumab (anti-PD-1) in MCC. Overall response rate (ORR) of MCC patients to anti-PD-1 was 56%. Patients with virus-positive and virus-negative tumors experienced responses (62% and 44% respectively; *Nghiem et al., N Engl J Med 2016*)[1].

Pembrolizumab (anti-PD-1)Pembrolizumab (MK3475), a humanized IgG4 antibody, has been most well studied in the context of melanoma but has shown promising clinical results in several tumor types[202]. In a recent phase II study of pembrolizumab for advanced solid tumors (NCT01295827), the most dramatic response was observed in the single MCC patient who experienced a complete response that was ongoing at the time of last follow-up, reflecting 100+ weeks of durable response[210]. In a clinical trial using pembrolizumab for first line treatment for advanced virus-positive and virus-negative MCC (NCT02267603) striking response rates were observed (at 44% and 62% respectively; **Figure 9**) and indicate some of the highest observed in solid tumors to date[65]. However, ~50% of patients remain unresponsive to PD-1 blockade, and currently there are no clinically useful indicators of who will and will not respond. Therefore, ongoing goals in the Nghiem lab are to 1) identify biomarkers predictive of response, 2) identify immune evasion mechanisms

that underlie PD-1 blockade resistance, and 3) explore novel immune therapies that can rescue non-responders.

Avelumab (anti-PD-L1)

The PD-1 axis can also be inhibited through antagonism of PD-L1, the ligand for PD-1 expressed primarily on cells of the monocyte lineage[139]. Avelumab (MSB0010718C) is a human monoclonal IgG1 antibody that binds to PD-L1 [176]. Binding of PD-L1 instead of PD-1 may reduce toxicity as anti-PD-1 blockade prevents PD-1 interaction with both PD-L1 and PD-L2[211]. PD-L2 is expressed on normal parenchymal cells in the lung and kidneys and prevents autoimmunity against these tissues [211]. Indeed, anti-PD-1 agents such as nivolumab have induced adverse reactions in these tissues including severe pneumonitis[211]. Avelumab, however, retains PD-1/PD-L2 signaling, thereby preserving these potentially important mechanisms for avoiding autoimmunity. A phase II clinical trial of avelumab has recently completed enrollment of 88 MCC patients who were refractory to chemotherapy (NCT02155647).

Autologous T cell therapy

Autologous T cell therapy involves isolating tumor-specific or tumor-infiltrating T cells from a patient, expanding them in culture and infusing them back into the patient. This approach has shown efficacy in treating several cancers including other virally-induced malignancies and melanoma[212-214]. In 2013, an MCC patient received three infusions of MCPyV-specific CD8s in combination with subcutaneous administration of IL-2 and HLA-I upregulating agents (single-dose radiation and IFNβ injections)[154]. This patient experienced mixed tumor responses but did not develop a recurrence for 535 days, significantly longer than median time (200 days) to next metastasis experienced by historical controls[154]. It appears that this immune therapy may have conferred benefit, in part because functional infused T cells persisted for > 200 days and preferentially accumulated within tumor tissue[154]. A phase I/II trial utilizing avelumab (anti-PD-L1) and HLA-I upregulation with or without the infusion of autologous T cells is currently enrolling (NCT02584829) and initial response rates are highly promising. Four patients with metastatic MCC have been treated with the triple therapy of Avelumab, autologous T cells and HLA upregulation and all are showing clinical response with three patients experiencing durable complete responses for over 12

months **(Table 2;**Paulson ASCO 2017). Importantly, toxicities using triple therapy were similar in grade to those observed with double therapy with the most common adverse event reported being lymphopenia which was expected and not sustained.

Table 2: Addition of Avelumab (Triple Tx) had Acceptable Toxicity Profile and High Response Rate

Patient	Prior Treatments	HLA-upregulation method	Targeted T cell epitope of T cell Tx	Reported Grade 3/4 AE's	Best Response per RECIST
Patient 1: 57 y/o F	Surgery, XRT	XRT	HLA-A24-'EWW' HLA-B07-'APN'	Lymphopenia	CR ongoing at 18 months
Patient 2: 56 y/o M	Surgery, XRT and GLA	IFN	HLA-A02-'KLL'	Lymphopenia	CR ongoing at 19 months
Patient 3: 60 y/o M	Surgery, XRT	IFN & XRT	HLA-A02-'KLL' HLA-B35-'FPW'	Lymphopenia, cytokine release syndrome	CR, ongoing at 16 months
Patient 4: 64 y/o M	XRT	XRT	HLA-A02-'KLL'	Lymphopenia	PR, progressed at 3 months

Conclusions and future perspectives

Remarkable early responses have been observed among MCC patients treated with PD-1 axis blockade as well as other immune-based therapies. However, approximately 50% of patients with advanced MCC are either not candidates for immune checkpoint blockade or will require the addition of other therapies to achieve meaningful clinical benefit. Studies of potential predictive biomarkers will be important to identify patient subsets that are either particularly likely or unlikely to respond to a given therapy. One potential biomarker for response to PD-1 axis blockade is the expression of PD-L1 within tumors. In melanoma, PD-L1 expression within the tumor closely correlates with clinical response in several studies, however, some patients with PD-L1-negative tumors can also respond[215]. Notably, Tumeh *et al.* also reported that the presence of pre-existing CD8 T cells at both the invasive tumor margin as well as within the tumor, was associated with PD-L1 expression and response to PD-1 axis blockade[216]. However, in our initial cohort of 26 patients treated with pembrolizumab, intratumoral CD8 infiltration and PD-L1 expression did not correlate significantly with response[1,217], which may partly be due to the limited sample size. Interestingly, we have recently found that peritumoral infiltration (not intratumoral) of CD8 T cells may be a better predictor of response to PD-1 blockade (**Figure 10**).

Excitingly, elevated numbers of PD-1+ cells (peritumoral). This promising finding now requires rigorous validation and hence, we plan to explore PD-1 expression in an expanded cohort. moral or intratumoral) is even more strongly correlated with PD-1 blockade response **(Figure 10).** Additionally, a recent study indicated that among malignant melanoma patients, those with higher neoantigen load, and expression of cytolytic markers responded better to CTLA-4 blockade[218]. Consequently, virus-negative MCCs with higher mutational burdens may respond better to checkpoint inhibitors than those with lower mutational burdens.

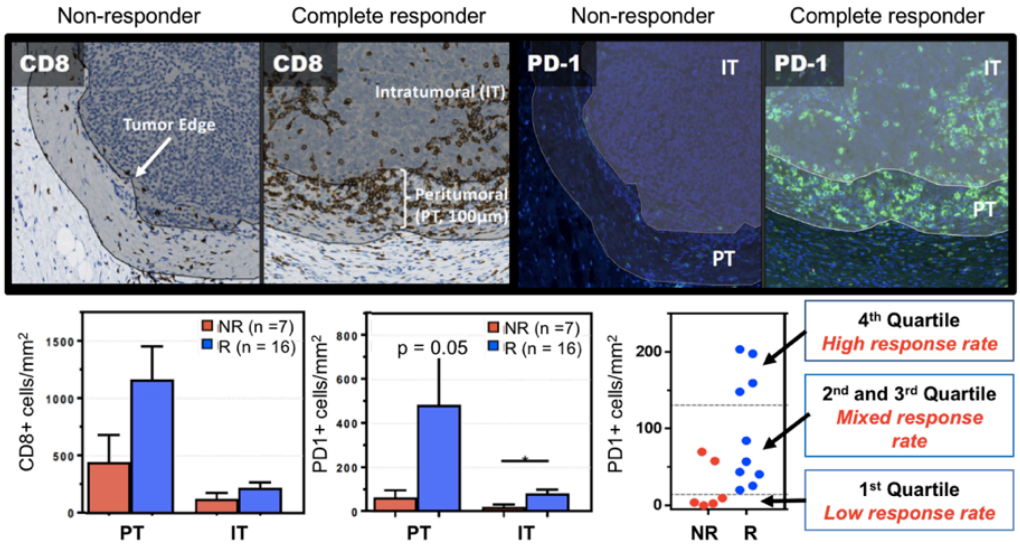

Figure 10: Patients who responded to PD-1 blockade therapy have elevated peritumoral and intratumoral infiltration of PD-1+ cells[2]. Representative images for CD8 (brown) and PD-1 (green) staining from a non-responder (NR) and complete responder (CR). The peritumoral edge (PT, 100 μm) and intratumoral (IT) regions are represented. Responders had higher mean densities (±SEM) of CD8+ and PD-1+ cells, the latter of which was statistically significant. This relationship is also evident when subdividing the cohort by PD-1+ cell density quartiles. *p <0.05.

While identifying predictive biomarkers is of great significance, for patients that are refractory to monotherapeutic approaches, numerous immune-combination therapies have been reported for other cancers that may also be beneficial in MCC[219]. In melanoma, the combination of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) has shown markedly increased response rates and longer progression-free survival than monotherapy[44,220]. This is likely because these agents act through non-redundant mechanisms[142]. A preclinical model described by Twyman-Saint Victor *et al*[184]*.* also reported that the triple therapy of radiation, anti-CTLA-4 and anti-PD-L1 yielded superior response rates as compared to dual

checkpoint blockade without radiation, suggesting that triple therapy such as this may also be beneficial in human subjects.

CHAPTER 4: VASCULAR E-SELECTIN EXPRESSION CORRELATES WITH CD8 LYMPHOCYTE INFILTRATION AND IMPROVED OUTCOME IN MERKEL CELL CARCINOMA

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Chapter Summary:

Merkel cell carcinoma (MCC) is an aggressive, polyomavirus-linked skin cancer. Although CD8 lymphocyte infiltration into the tumor is strongly correlated with improved survival, these cells are absent or sparse in most MCCs. We investigated whether specific mechanisms of T-cell migration may be commonly disrupted in MCC tumors with poor CD8 lymphocyte infiltration. Intratumoral vascular E-selectin, critical for T-cell entry into skin, was downregulated in the majority (52%) of MCCs (n = 56), and its loss was associated with poor intratumoral CD8 lymphocyte infiltration ($P < 0.05$; n = 45). Importantly, survival was improved in MCC patients whose tumors had higher vascular E-selectin expression ($P < 0.05$). Local nitric oxide (NO) production is one mechanism of E-selectin downregulation and it can be tracked by quantifying nitrotyrosine, a stable biomarker of NO-induced reactive nitrogen species (RNS). Indeed, increasing levels of nitrotyrosine within MCC tumors were associated with low E-selectin expression ($P < 0.05$; n = 45) and decreased CD8 lymphocyte infiltration ($P < 0.05$, n = 45). These data suggest that one mechanism of immune evasion in MCC may be restriction of T-cell entry into the tumor. Existing therapeutic agents that modulate E-selectin expression and/or RNS generation may restore T-cell entry and could potentially synergize with other immune-stimulating therapies.

INTRODUCTION

Merkel cell carcinoma (MCC) is an increasingly common neuroendocrine skin cancer that is at least twice as likely to be lethal as melanoma[221]. Although surgery and/or radiation therapy may be curative for patients with localized MCC in the absence of distant metastases, relapses are common and often incurable, with no disease-specific therapies available. Investigation of mechanisms involved in MCC pathogenesis and progression could offer rational targets for future therapies. The cellular immune response against MCC is particularly relevant in light of the recently discovered causal link between this cancer and the Merkel cell polyomavirus (MCPyV)[57], as well as the increased MCC incidence among

immune suppressed individuals with HIV, chronic lymphocytic leukemia, or solid organ transplantation[84,99,120]. Indeed, MCPyV oncoproteins that are persistently expressed in MCC tumors have recently been shown to be targets for CD8 and CD4 T cells[130] Furthermore, several studies suggest that CD8 and CD3 lymphocyte infiltration into MCC tumors is strongly linked to survival[79,91]. However, this advantageous robust lymphocytic infiltration into MCC tumors is only present in 20% of patients[91]. Thus, we hypothesized that the inability of the immune response to control MCC may in part be because of blockade of lymphocyte entry into MCC tumors. One mechanism of T-cell exclusion from tumors is downregulation of adhesion molecules on tumor vasculature or on lymphocytes, thereby blocking recruitment of T cells from blood vessels. In skin, expression of endothelial E-selectin adhesion molecule is the earliest step of tethering, rolling, and emigration of cutaneous lymphocyte antigen (CLA)-positive T cells from blood vessels to sites of inflammation[147] and cancer[222]. Indeed, human squamous cell carcinomas have been shown to evade the immune response by downregulating E-selectin on tumor vasculature [222]. A recent report suggests that E-selectin expression in squamous cell carcinomas is downregulated by nitric oxide (NO) produced by tumor associated myeloid-derived suppressor cells[148]. Protein nitration is a stable biochemical marker of NO production and inducible NO synthase/arginase pathway activation and thus can be tracked in archival tissues using an antibody against nitrotyrosine. Indeed, several human cancers, including prostate, colon, and hepatocellular carcinomas[223], show markedly elevated levels of nitrotyrosine, which are associated with a lack of functional tumor-infiltrating lymphocytes[224,225]. We therefore investigated the role of nitrotyrosine and its association with E-selectin downregulation in, and CD8 lymphocyte exclusion from, MCC tumors.

In this study, we found that increased numbers of E-selectin positive vessels in the tumor are associated with greater intratumoral CD8 lymphocyte infiltration and improved MCC-specific survival. The downregulation of E-selectin may be a consequence of the high levels of nitrotyrosine expression in MCC tumors. These findings have mechanistic and potential therapeutic implications for MCC.

MATERIALS AND METHODS

Tissue and blood samples

This study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board and conducted according to the Declaration of Helsinki Principles. Written informed consent was received from participants before inclusion in the study. A total of 248 formalin-fixed, paraffin-embedded tumors from 192 patients were analyzed (Table 1). Blood samples were collected from MCC patients (n = 11) and healthy volunteers (n = 10) who were used as control subjects.

Immunohistochemistry and immunofluorescence

Serial tumor sections were stained with hematoxylin and eosin, and with antibodies against E-selectin (clone 16G4, 1:50 dilution; Novocastra, Buffalo Grove, IL), CD31 (clone JC70A, 1:100 dilution; Dako, Carpinteria, CA), CD8 (clone 4B11, 1:200 dilution; Novocastra), CLA (clone HECA-452, 1:100 dilution; BioLegend, San Diego, CA), and nitrotyrosine (rabbit polyclonal, 1:250 dilution; Millipore, Billerica, MA). The specificity of the nitrotyrosine antibody was validated using colon tissue treated with peroxynitrite as a positive control and degraded peroxynitrite as a negative control (Supplementary Figure S1 online)[226,227]. Scoring for all studies was performed by observers who were blinded to all subject characteristics. Intratumoral and peritumoral E-selectin-positive vessels were scored among 56 MCC tumor specimens from 55 patients using a three-tiered system: absent/low (<0.1%), moderate (0.1–5%), and high (>45%), expressed as a percent of CD31-positive vessels in serial sections. The score represented the average fraction of E-selectin-positive vessels in the entire intratumoral or peritumoral areas with at least 8–10 tumor fields scored when possible. To compare peritumoral and intratumoral areas, the fold difference (ratio of the percentages of E-selectin-positive vessels) was calculated for each tumor, and then averaged over all tumors. Intraobserver variability was evaluated in a random sample of 13 tumors. Observed agreement was 80% and weighted k-statistic was 0.55, consistent with fair to good agreement between observers[228].

Fifty-six MCC tumors from 55 patients were assessed for CD8 lymphocytes using a previously described scoring system[91]. Briefly, intratumoral and peritumoral CD8 infiltrates were scored separately on a 0–5 scale with 0 representing no CD8 cells and 5 representing a strong CD8 infiltrate. Approximate

numbers of CD8. cells per mm^2 were quantified for each 0–5 bin (with an average of 0, 90, 306, 508, 675, 732. CD8 cells per mm^2, respectively). Intratumoral CD8 lymphocytes were those that were surrounded by tumor cells and did not have direct contact with stroma. Tumor CD8 lymphocyte infiltration patterns were categorized as sparse (intratumoral CD8 score p2), brisk (intratumoral CD8 score X3), or stalled (intratumoral CD8 score p1 and peritumoral CD8 score X3).

For dual staining of CLA and CD8 immunofluorescence studies, sections were incubated with anti-CLA as above followed by biotinylated goat-anti-rat (1:50; Jackson ImmunoResearch) and streptavidin AlexaFluor-568 (1:200; Invitrogen, Grand Island, NY). The same sections were stained with anti-CD8 (1:50, clone C8/144B; Dako) followed by goat-anti-mouse AlexaFluor-647 (1:50; Invitrogen). 4,6-diamidino-2-phenylindole was used for nuclear staining. CLA/CD8 co-expression was quantified as the number of cells with CLA and CD8 colocalization as a percent of total CD8- positive cells. The fraction of CD8 lymphocytes co-expressing CLA was assessed in the whole tissue specimen and was categorized as none/low (o5%), moderate (5–50%), or high (X50%). Sections were captured using ScanScope model FL (Aperio, Vista, CA), acquired and analyzed with Spectrum version 11.1.1.764 (Aperio), and confirmed with Definiens Architect XD Tissue Studio IF software version 2 (Definiens).

Three observers assessed nitrotyrosine staining. Tissue microarrays of tumor cores were scored using a semi quantitative integrated assessment of intensity and proportion staining and categorized as follows: none, low, moderate, or high staining. The median of the observers' scores was calculated. Tissue microarray cores contain mostly tumor cells, but both tumor and stroma areas were included in the score.

Flow cytometry analysis

Peripheral blood mononuclear cells were thawed' from cryopreserved heparinized blood separated with Ficoll/Hypaque. Lymphocytes were incubated with allophycocyanin-conjugated HLA/peptide tetramers specific for MCPyV (A24/MCPyV.LT-92-101), cytomegalovirus (A2/ CMV.pp65.495-503), or Epstein–Barr virus (A2/EBV.BMLF1.280-288) for 30 minutes at 37°C. Fc receptor blocking reagent (Miltenyi Biotec,

Auburn, CA) was then added for 10 minutes at 4°C. Next, cells were stained with CD3-Qdot605 (clone 7D6/S4.1; Invitrogen), CD8-V500 (clone RPA-T8; BD Biosciences, San Jose, CA), and CLA-FITC (clone HECA-452; BioLegend) for 30 minutes at 4°C. Cells were washed and fixed. Events were collected on a FACSAriaII machine (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Analysis and gating were carried out on CD3+CD8+ or CD3+CD8+Tetramer+ T cells from the blood of MCC patients or control subjects.

Statistical analysis

Wilcoxon rank-sum test was used to assess significance among categorically ordered groups. Cuzick's nonparametric test for trend[229] was used to assess trend across ordered groups. Student's t-test was performed when comparing means among two groups. Fisher's exact test was used to determine associations between two categorical variables. Kaplan–Meier survival curves of cause-specific survival were generated using preselected E-selectin category cutoffs (low, moderate, high) and statistical significance was determined using log-rank test for trend. The P-value of o0.05 was considered significant. All analyses were performed with Stata software (StataCorp, College Station, TX).

RESULTS

The fraction of E-selectin-positive blood vessels is decreased in the majority of MCC tumors. Vascular E-selectin is critical for the entry of CLA-positive T cells into the skin under both normal and inflamed conditions[147]. To determine the presence of E-selectin in MCC tumor vasculature, we stained serial sections of MCC tumors with antibodies to E-selectin and CD31. A total of 56 MCC tumors from 55 patients were analyzed. Vascular structures, as identified by staining for CD31, were apparent both within the tumor and in the adjacent peritumoral areas (**Figure 1**). Among the 56 tumors, the mean number of vessels was similar in intratumoral (35 ± 19 CD31-positive vessels per 200 magnification field) and peritumoral areas (44 ± 24). Strikingly however, when tumors were compared for the fraction of E-selectin-positive vessels within versus outside the tumor, there was a 4-fold decrease in the proportion of E-selectin-positive vessels within the tumor as compared with that in the tumor periphery ($P < 0.05$; representative tumor seen in **Figure 1B**). Further analyses were carried out after stratifying intratumoral

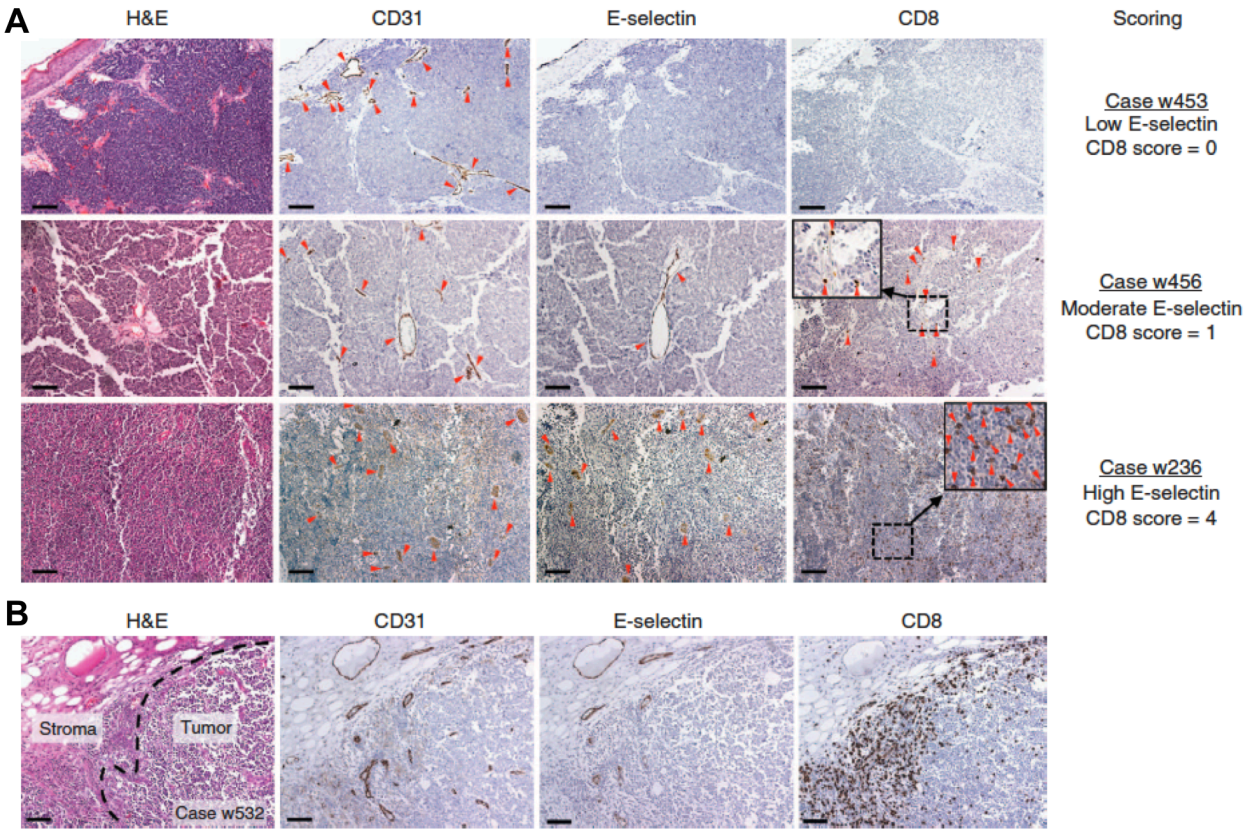


Figure 1. Vascular adhesion molecules and CD8 infiltration on representative serial tumor sections. (A; left to right) Serial sections stained as indicated from three patients (cases w453, w456, and w236) with the specified intratumoral CD8 and E-selectin scores. Red arrowheads indicate areas of positive staining on immunohistochemistry for the indicated antibody. Bar.100 mm. (B; left to right) Serial sections from a Merkel cell carcinoma (MCC) tumor (case w532) with both stromal and tumor components stained with specified antibodies. Black dashed line indicates the junction between tumor and stroma. The sections shown are representative of staining patterns in the stroma and tumor. H&E, hematoxylin and eosin. Bar.100 mm.

or peritumoral areas into low, moderate, or high bins (<1%, 1–5%, and 45% of vessels being E-selectin-positive, respectively; **Figure 2A**). Among 56 MCC tumors, the fraction of E-selectin-positive vessels inside the tumor was often 'low' (52% of MCCs) as compared with the fraction of E-selectin positive vessels in peritumoral areas (29% of MCCs; $P < 0.05$). In contrast, intratumoral areas of MCCs were less likely to have a high fraction of E-selectin-positive vessels as compared with peritumoral areas (14% vs. 32%, respectively; $P < 0.05$; **Figure 2A**). Next, to investigate if there was a correlation between E-selectin expression and MCC-specific survival, we compared the fraction of intratumoral E-selectin-positive vessels among MCC patients. We observed a significant trend toward improved survival among patients with tumors expressing a higher fraction of E-selectin-positive vessels within the tumor vasculature ($P < 0.05$ by log-rank test for trend; **Figure 2B**). There were no significant associations between E-selectin and

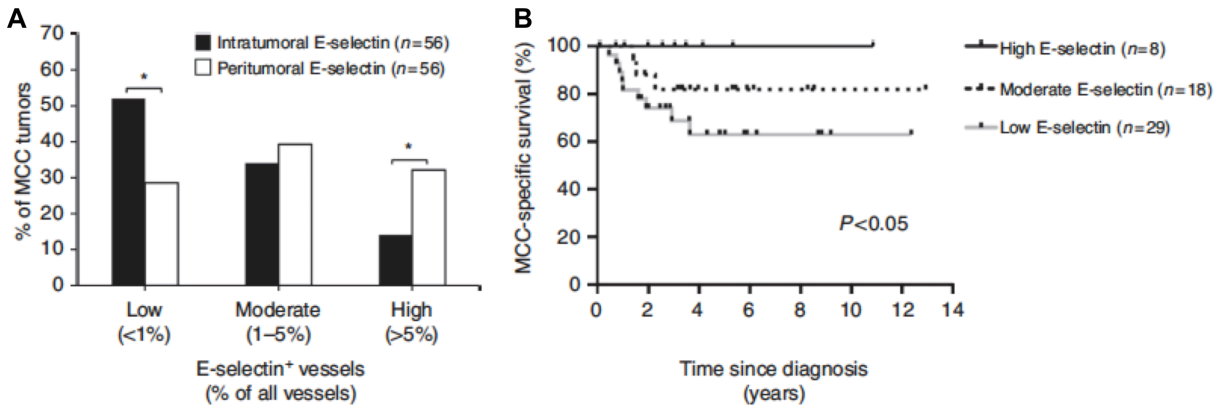


Figure 2. Merkel cell carcinoma (MCC) tumors often have decreased E-selectin-positive vessels, which correlate with worse survival. (A) Percent of MCC tumors with low (<1%), moderate (1–5%), or high (>5%) fraction of intratumoral (black bars) or peritumoral (white bars) E-selectin-positive vessels.*P < 0.05, Fisher's exact test. (B) Kaplan–Meier curves showing MCC-specific survival of patients with low (n = 29), moderate (n = 18), or high (n = 8) fractions of intratumoral vessels that were E-selectin-positive. P-value determined by log-rank test for trend.

stage, gender, age, or lesion type. Intratumoral CD8 infiltration is correlated with E-selectin-positive vessels

To analyze the relationship of T-cell infiltration with vascular E-selectin patterns described above, we stained serial sections of 56 MCC tumor specimens from 55 patients for CD8 and the indicated vascular markers (**Figure 1**). Tumor CD8 lymphocyte infiltration patterns were categorized as previously described into six bins of density in intratumoral and peritumoral sites and subsequently into three infiltration patterns (brisk, sparse, or stalled)[91]. Of the 56 tumors, 25% had a brisk CD8 infiltrate (intratumoral CD8 score of 3–5), whereas 75% had a sparse CD8 infiltrate (intratumoral score of 0–2). Among 34 tumors with no or very low CD8 intratumoral infiltrate (intratumoral score of 0 or 1), 41% exhibited a prominent stalled phenotype with high numbers of peritumoral CD8 cells (peritumoral score 3–5) accumulating within the tissue immediately adjacent to the tumors (representative example seen in **Figure 1B**). Among all analyzed tumors, an increasing fraction of intratumoral E-selectin-positive vessels was associated with an increasing intratumoral CD8 lymphocyte score ($P < 0.05$; **Figure 3A**). Tumors with a high fraction of E-selectin-positive vessels had a median CD8 lymphocyte score of 3.5, with CD8 scores ≥ 3 previously reported to be associated with excellent MCC-specific survival in a large cohort study[91]. In contrast, tumors with a low fraction of E-selectin-positive vessels had a median CD8 lymphocyte score of zero.

In order to explain the distinct CD8 infiltration patterns in MCC tumors (brisk, sparse, or stalled as described above), we compared the relative E-selectin-positive fractions between intratumoral and

peritumoral vessels. Among tumors with sparse or stalled CD8 infiltrates, the intratumoral E-selectin positive fraction was selectively decreased as compared with the peritumoral E-selectin fraction ($P < 0.01$; **Figure 3B**). In contrast, among tumors with robust numbers of CD8 lymphocytes in the tumor and surrounding stroma (brisk infiltrate), E-selectin positivity was preserved among both intratumoral and peritumoral vessels. Altogether, these studies suggest that the restriction for CD8 lymphocyte entry into some tumors may be mechanistically linked to the low E-selectin-positive proportion of tumor vessels.

Expression of the skin-homing receptor CLA is retained on MCC-targeting lymphocytes Because of its key role in facilitating lymphocyte adhesion to E-selectin and entry into the skin, we determined whether CLA was expressed on lymphocytes in and around MCC tumors. MCC tumor sections were costained with CLA and CD8 and colocalization of these proteins was quantified as a percent of total CD8 lymphocytes by immunofluorescence analysis (**Figure 4A**).

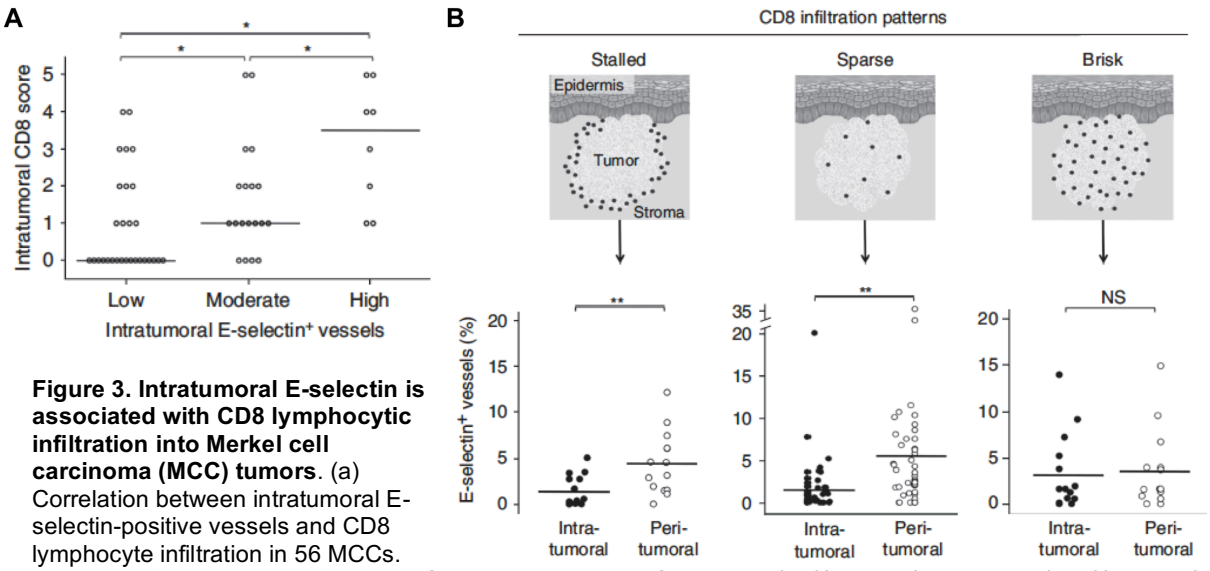


Figure 3. Intratumoral E-selectin is associated with CD8 lymphocytic infiltration into Merkel cell carcinoma (MCC) tumors. (a) Correlation between intratumoral E-selectin-positive vessels and CD8 lymphocyte infiltration in 56 MCCs. E-selectin was scored as a percent of all vessels and stratified as low (<1%, n = 29), moderate (1–5%, n = 19), or high (>5%, n = 8). CD8 cells were scored a 0–5 scale (Paulson et al., 2011). Black bar indicates median. *P < 0.05, Wilcoxon's rank-sum test. (B) Comparison of intratumoral (filled circles) with peritumoral (empty circles) E-selectin-positive vessels among tumors with CD8 infiltrates characterized as stalled (intratumoral CD8 score ≤1 and peritumoral CD8 score ≥3, n = 14), sparse (intratumoral CD8 score ≤2, n = 42), or brisk (intratumoral CD8 score ≥3, n = 14). Black dots in schematic indicate CD8 lymphocytes. Black bar indicates mean. **P < 0.01, Student's t-test. Adapted from Afanasiev et al 2013.

Of the 20 tumors, 80% had CLA/CD8 co-expression that was moderate (n = 9, defined as 5–50% CLA-positive CD8 cells) or high (n = 7; >50% CLA-positive CD8 cells; **Figure 4A**). CD8 T cells from blood had similar levels of CLA expression in MCC patients and control subjects, suggesting no global dysregulation of CLA expression (**Figure 4B**). In a small cohort of MCC patients in which it was possible

to examine MCPyV oncoprotein-specific CD8 T cells from blood, on average, CLA expression was observed in 39% of these virus-specific T cells as compared with 7% and 4% on T cells specific for cytomegalovirus and Epstein–Barr virus, respectively (P<0.05; **Figure 4B**). The CLA-negative MCPyV-specific T cells may represent populations that have been primed in other non-skin compartments or cells with central memory rather than effector memory function. Overall, these data suggest that MCC-targeting CD8 lymphocytes, especially those in the MCC tumor microenvironment, often express CLA and would

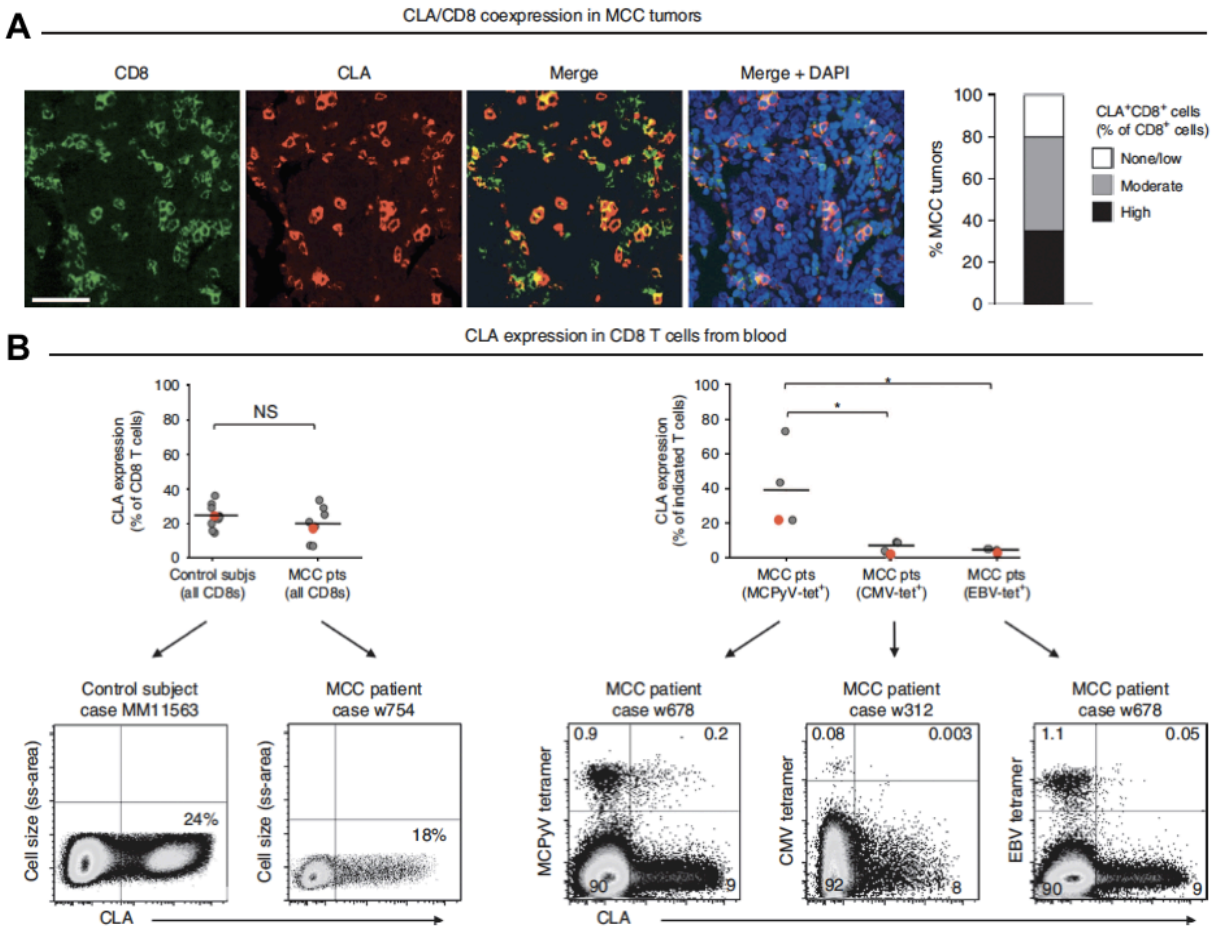


Figure 4: Cutaneous lymphocyte antigen (CLA) expression on Merkel cell carcinoma (MCC)-specific lymphocytes. A: CLA/CD8 coexpression as evaluated by immunofluorescence with the indicated stains in a tumor (high CLA/CD8 coexpression; case w588). (A, right) Fraction of MCCs with CLA/CD8 coexpression categorized as none/low (n = 4, <5% CLA-positive CD8 cells), moderate (n = 9, 5-50% CLA-positive CD8 cells), or high (n = 7; 50% CLA-positive CD8 cells). Bar = 50um. B: CLA expression in blood as evaluated by flow cytometry. (B; top right) CLA expression among CD3+CD8+Tetramer+ cells specific for MCPyV (n = 4), cytomegalovirus (CMV; n = 4), and Epstein-Barr virus (EBV; n = 3). The red dot on each graph indicates the representative sample selected for flow plot display below. Black bar indicates mean. DAPI, 4,6-diamidino-2-phenylindole; MCPyV, Merkel cell polyomavirus; NS, nonsignificant; Tet+, tetramer positive. *P, 0.05, Wilcoxon's rank-sum test.

thus be capable of binding its E-selectin ligand when expressed on blood vessels. High nitrotyrosine levels are associated with low E-selectin-positive vessels and poor CD8 lymphocyte infiltration.

Recent studies have reported that local production of NO leads to downregulation of vascular E-selectin and impairment of T-cell trafficking into tumors[148]. Thus, we stained 236 MCC tumors from 181 patients using an anti-nitrotyrosine antibody to evaluate protein nitration[227], which is a consequence of local NO mediated production of reactive nitrogen species (RNS)[226,230-233]. Approximately 43% of MCC tumors (n = 101) had moderate or high expression of nitrotyrosine, with only 6% of tumors completely lacking nitrotyrosine staining within the tumor microenvironment (**Figure 5A**). Furthermore, increasing levels of nitrotyrosine were associated with lower number of E-selectin-positive vessels within MCC tumors ($P < 0.05$; **Figure 5B**). Higher nitrotyrosine levels were also associated with lower intratumoral CD8 lymphocyte scores ($P < 0.05$; $n = 45$; **Figure 5B**). These data suggest that metabolic pathways involving NO and RNS production may be one of the several mechanisms regulating T-cell extravasation into MCC tumors.

DISCUSSION

The cellular immune system is particularly important in controlling MCC given that immune dysfunction is associated with increased incidence[84,99,120] and diminished survival for MCC[26]. Sparse lymphocyte infiltration observed in the majority of MCCs suggests that defective T-cell entry may play a role in the inability to control this highly immunogenic cancer. Indeed, we report that vascular E-selectin, required for the recruitment of CLA-positive T cells into the skin, is downregulated in the majority of MCCs. Tumors with a higher number of E-selectin-positive vessels are associated with increased intratumoral CD8 lymphocyte infiltration and improved MCC-specific survival. Furthermore, we provide evidence that metabolic pathways leading to production of nitrotyrosines are associated with E-selectin downregulation and poor CD8 T-cell infiltration into MCC tumors. Vascular adhesion molecule expression has clinically significant implications in a number of human cancers. Depending on the cancer type, the presence of E-selectin may be associated with an improved or worsened prognosis. In breast, colon, and lung cancers, elevated E-selectin expression on tumor vasculature recruits protumorigenic immune infiltrates and

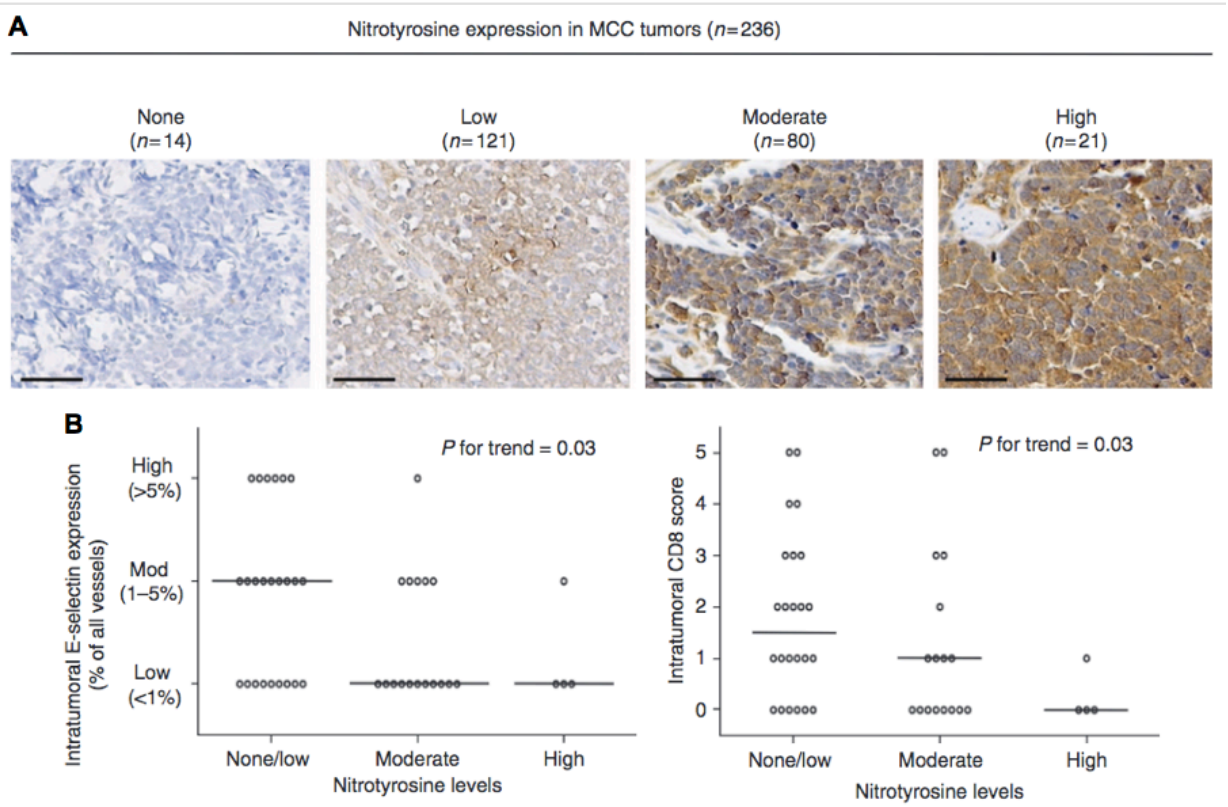


Figure 5: High levels of tissue nitrotyrosine are associated with a reduced fraction of E-selectin-positive vessels and poor CD8 lymphocyte infiltration. A: Representative Merkel cell carcinoma (MCC) tumor cores stained for nitrotyrosine (brown). Nitrotyrosine scores took into account both intensity and proportion and were categorized as none (n = 14), low (n =121), moderate (n = 80), and high (n = 21) expression. Bar = 50um. **B**: Correlation between nitrotyrosine levels and intratumoral E-selectin (left) and CD8 (right) scores among 45 MCC tumors. Black bar represents median. P-value determined by Cuzick's nonparametric test for trend.

facilitates attachment and transmigration of tumor cells through the endothelium, effectively promoting cancer progression, metastasis, and poorer survival[234]. In contrast, in other cancers, including squamous cell carcinoma and melanoma, the proportion of E-selectin-positive vessels is markedly decreased and is associated with a lack of protective T cells within tumor nodules[148,222,235]. The known strong association between intratumoral lymphocyte infiltration and improved survival of MCC patients and the predominant absence of protective lymphocytes in most tumors suggested that vascular endothelium might play an important role in MCC tumor immune escape. Thus, we investigated the association between vascular E-selectin expression, lymphocyte infiltration patterns and survival in MCC. This study expands the limited number of reports on E-selectin relevance and its association with survival in skin cancers. In contrast to other cancers, where E-selectin is often reported as a biomarker of metastatic potential and a predictor of worsened outcome, to our knowledge, the link between intratumoral vascular E-selectin expression and

improved survival has not been previously reported. The presence of E-selectin in the tumor vasculature may be particularly important for immunogenic cancers that are targets of cytotoxic immune cells.

There are several known mechanisms that can contribute to cellular immune escape and diminished lymphocyte infiltration. Loss of E-selectin on the tumor vasculature may prevent adequate leukocyte capture and rolling mediated by E-selectin/CLA interactions on T cells that are capable of reaching the tumor periphery. Recent evidence suggests that there is a strong link between vascular E-selectin downregulation and NO production by myeloid-derived suppressor cells in squamous cell carcinomas[148]. It is plausible that similar mechanisms of E-selectin regulation are at play in MCC. Indeed, we observed that nitrotyrosine, a surrogate marker of NO and RNS production, is associated with E-selectin downregulation and deficient CD8 lymphocyte infiltration. Beyond E-selectin downregulation, additional nitrotyrosine-mediated mechanisms of T-cell immune evasion include: (1) block of signaling and responsiveness to antigen via TCR/CD3z nitration[225], (2) block of TCR/HLA interactions and tumor recognition by TCR/CD8 nitration[225], and (3) prevention of T-cell migration via nitration of chemokines that renders them dysfunctional[227]. Suboptimal clinical outcomes of adoptive T-cell therapy for immunogenic cancers may be in part due to lack of T-cell recruitment into tumors. Downregulation of vascular E-selectin and tumor protein nitration present obstacles for appropriate tumor entry and activity of therapeutic tumor-targeting T cells. Importantly, studies in a variety of cancers suggest that improved T-cell infiltration and function may be achieved by therapeutic modulation of pathways regulating E-selectin[222] and protein nitration[227]. Specifically, E-selectin induction has been observed in vitro with tumor necrosis factor-a and IL-1 cytokines[236], angiostatins[237], and topical imiquimod[222]. Recent studies also showed that inhibitors of NO synthase activity were effective in both E-selectin upregulation[148] and reversal of nitrotyrosine-associated T-cell dysfunction[224,238]. Furthermore, drugs that block the generation of RNS can increase tumor-specific CD8 T-cell recruitment and reduce tumor growth when given in combination with adoptive immunotherapy in mice[227].

This study was limited to the examination of formalin-fixed, paraffin-embedded human MCC tissues. Future investigations on fresh or frozen MCC tumors using multicolor immunofluorescence markers may

reveal the phenotypic identity of cells that induce protein nitration of MCC tumors. All of our studies were on human tissue, and hence reflected human disease, but this posed obstacles in determining causality of our observations. Although an animal model would have advantages, existing MCC xenograft models require profoundly immune deficient mice, and thus would not be able to address most of the relevant aspects of the immune response in MCC. It is plausible that future studies in transgenic mouse models that mimic MCC pathogenesis (such as the spontaneous carcinogenesis model induced by sporadic SV40 polyomavirus oncoprotein expression[239]; may be useful in studying immune responses to molecules that target E-selectin, nitrotyrosine, and elucidating other relevant mechanisms involved in T-cell trafficking such as NF-kB regulation of adhesion molecules and chemokines[240]. Furthermore, trials in MCC patients using E-selectin upregulating agents as discussed above may validate the observed associations between E-selectin upregulation, enhanced CD8 lymphocyte infiltration, and improved survival. Although we have limited our studies of cutaneous immunosurveillance to investigation of E-selectin, other contributory mechanisms include the recruitment of CCR8. T cells by constitutively expressed CCL1 in the skin[241] and platelet (P)-selectin mediated cutaneous T-cell migration[242].

In summary, this study provides insight into immune evasion mechanisms that likely play a role in diminishing lymphocyte entry into MCC tumors. As it is feasible to target these pathways using existing or emerging agents, it may be appropriate to combine such treatment with adoptive T-cell therapy to improve migration of T cells into tumors and thereby augment the efficacy of future immune therapy.

CHAPTER 5: WHY ARE CD4 T CELLS IMPORTANT IN CANCER IMMUNOTHERAPY

By: N. Vandeven review of CD4 T cell subtypes and their role in cancer

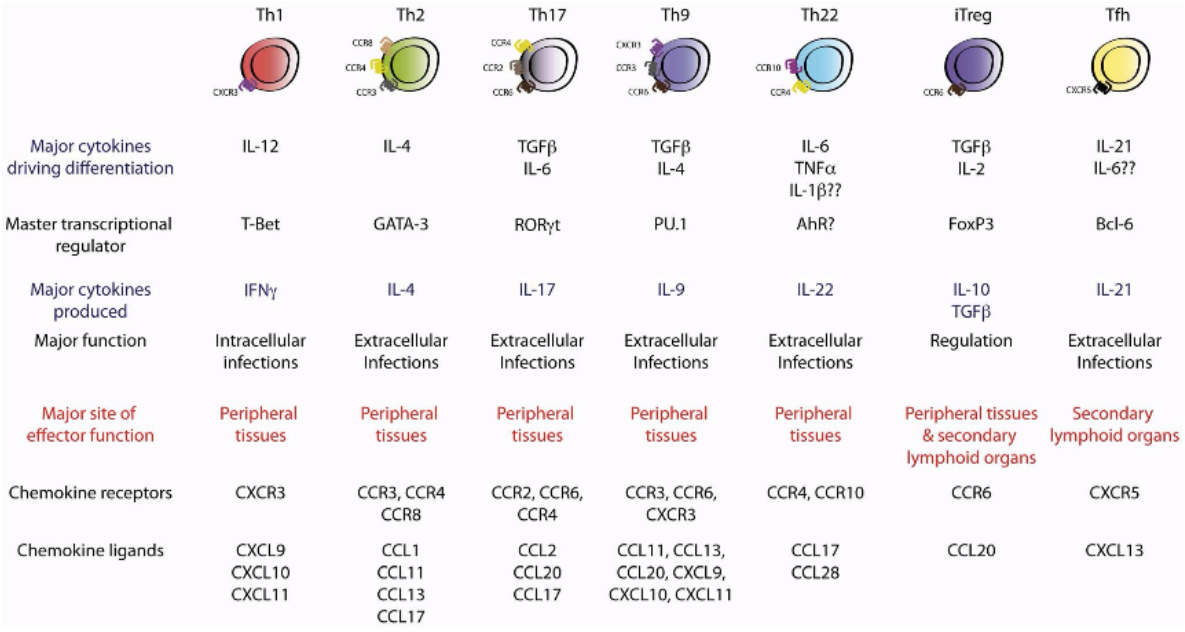
CD4 T CELLS AND CANCER IMMUNITY

Historically, the majority of tumor immunology research has focused upon improving CD8 T cells responses, largely because CD8 T cells can directly lyse and kill target cancer cells expressing HLA class-I. However, genetic instability within tumor cells often leads to reduced expression of HLA class-I and the ability to process and present endogenous antigens rendering cancer cells unreliable targets for CD8 T cells[243,244]. CD4 T cells on the other hand, recognize antigen in the context of HLA class-II molecules that are primarily expressed by professional antigen presenting cells (APCs). In contrast to tumor cells, APCs are capable of providing appropriate costimulatory and activation signals within lymph nodes outside of the immunosuppressive tumor microenvironment[245]. CD4s can then in-turn license APCs to provide the appropriate costimulation for CD8s as well as initiate a broad immune response via recruitment and activation of numerous additional type of immune cells. Consequently, while CD8 based therapies targeting cancer-associated antigens have yielded durable responses in some cases[154,246,247], numerous lines of evidence indicate that harnessing tumor-specific CD4 T cells may improve immune-based therapies[245]. Specifically, treatment with cancer-specific CD4 T cells has induced tumor regression[214,248] and recent and successful therapeutic cancer vaccination strategies for melanoma have indicated that CD4 T cell responses were more strongly induced than CD8 T cells[249,250]. Therefore, further investigation into the role of anti-cancer CD4 T cells is necessary. Here we discuss the various CD4 subtypes described to date and discuss the immunotherapeutic approaches that have been developed that either directly or indirectly target the CD4 T cell response.

CD4 SUBTYPES

CD4 T cells can differentiate into numerous helper subtypes enabling induction of broad and effective immune responses via both the innate and adaptive arms[4,251]. Because of this, CD4 T cells can modulate almost every aspect of an immune response[4]. While initially CD4 T cells were thought to be comprised of only two subtypes T-helper 1 (Th1) and T-helper 2 (Th2[252], numerous additional CD4 subtypes have been described including T-helper 9 (Th9), T-helper 17 (Th17), T-helper 22 (Th22), T follicular helper (Tfh), T-

regulatory cells (Treg). Induction of these discrete subtypes is largely determined during CD4 priming (**Figure 1**)[4,253]. Dendritic cells (DCs) are the most potent APC responsible for CD4 T cell priming. Like most immune cells, DCs are also composed of discrete subsets that can produce varied cytokine profiles depending upon which pathogen associated molecular patterns are recognized via surface or intracellular receptors such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) like receptors[254]. For example, DCs expressing CD8a secrete IL-12 and IFNγ and induce a Th1 response while CD8a-negative DCs secrete IL-6 and induce Th2 differentiation[254]. Co-stimulatory signals are also critical for productive T cell effector function, the most critical of which is the ligation of CD80/86 and CD28 expressed by DCs and T cells respectively. Other co-stimulatory molecules include inducible co-stimulator (ICOS), CD27, 4-1BB and OX40[254]. In combination, the cytokine milieu and co-stimulatory profile present during the process of CD4 T cell priming results in the induction of varied transcription factors (TF) which govern CD4 lineage differentiation. For Th1 induction, T-bet is the predominant TF, while GATA-3, RORγt, Bcl-6 and FOXP3 promote Th2, Th17 and Treg differentiation respectively[254]. The role of these CD4 subsets in cancer immunity will herein be described.



	Th1	Th2	Th17	Th9	Th22	iTreg	Tfh
Major cytokines driving differentiation	IL-12	IL-4	TGFβ IL-6	TGFβ IL-4	IL-6 TNFα IL-1β??	TGFβ IL-2	IL-21 IL-6??
Master transcriptional regulator	T-Bet	GATA-3	RORγt	PU.1	AhR?	FoxP3	Bcl-6
Major cytokines produced	IFNγ	IL-4	IL-17	IL-9	IL-22	IL-10 TGFβ	IL-21
Major function	Intracellular infections	Extracellular Infections	Extracellular Infections	Extracellular Infections	Extracellular Infections	Regulation	Extracellular Infections
Major site of effector function	Peripheral tissues	Peripheral tissues	Peripheral tissues	Peripheral tissues	Peripheral tissues	Peripheral tissues & secondary lymphoid organs	Secondary lymphoid organs
Chemokine receptors	CXCR3	CCR3, CCR4 CCR8	CCR2, CCR6, CCR4	CCR3, CCR6, CXCR3	CCR4, CCR10	CCR6	CXCR5
Chemokine ligands	CXCL9 CXCL10 CXCL11	CCL1 CCL11 CCL13 CCL17	CCL2 CCL20 CCL17	CCL11, CCL13, CCL20, CXCL9, CXCL10, CXCL11	CCL17 CCL28	CCL20	CXCL13

Figure 1. Currently known CD4 T cell subsets. Polarizing cytokines encountered during CD4 cell differentiation drive the expression of subset-specific transcription factors, which imprint subset-specific transcriptomes in the CD4 T cell. These transcription factors define the effector function and migratory capability of CD4 T cells via regulation of subset-specific cytokines and chemokine receptors. Adapted from Kara et al., PLoS 2014[4]

Th1 Cells

Th1 cells are primarily responsible for clearing intracellular pathogens such as viruses through the secretion of the cytokines IL-2, IFNγ , TNFα and lymphotoxin alpha[157,251]. This subtype is also generally thought to be the most effective in mediating anti-cancer function as infiltration of Th1 cells in multiple cancer types has been associated with prognostic benefit while other helper subtypes have varied prognostic impacts[255,256](**Table 1).** This association may be due to the ability of Th1 cells to promote a robust memory CD8 T cell responses[257] which is primarily facilitated through 'licensing' of dendritic cells

Table 1: The association of immune cell infiltrates with cancer prognosis.
Adapted from Fridman et al. Nature Reviews Cancer, 2012

Cells	$CD8^+CD45RO^+$ T cells	Th1 cells	Th2 cells	Th17 cells	Tregs
Melanoma	Good				None Poor
Head and neck cancers	Good			None	Good
Breast cancer	Good	Good None	Good None		None Poor
Bladder cancer	Good				Good
Ovarian cancer	Good	Good	Poor	Good	Good Poor
Esophageal cancer	Good	Good		Good	
Colorectal	Good	Good	None	Poor	Good None
Renal cell carcinoma	Good Poor	Good			Poor
Prostatic adenocarcinoma	Good				
Lung carcinoma	Good Poor	Good		Poor	Poor
Pancreatic cancer	Good		Poor		Poor
Cervical cancer		Good			
Anal squamous cell					None
Brain cancer					None
Hepatocellular carcinoma	Good Poor	Good		Poor	Poor
Gastric cancer		Good	Poor	Good	
Medulloblastoma		Good			
Merkel cell carcinoma	Good				
Urothelial cell carcinoma	Good				
Follicular lymphoma and Hodgkin's lymphoma			Good		Good None Poor

(DCs) and secretion of IFNγ. Licensed DCs secrete chemokines such as CCL3 and CCL4, which serve to recruit naïve CD8 T cells to secondary lymphoid tissue, facilitating interaction between these naïve CD8 T cells and DCs expressing their cognate antigen and high levels of co-stimulatory molecules thereby inducing CD8 T cell priming. Several studies have indicated that in the setting of cancer, CD4 T cells can enhance the recruitment, proliferation and effector function of CD8 T cells and that these effects are

mediated primarily via IFNγ which is predominantly secreted by Th1 cells[258,259]. Additionally, Th1 T cells can also recruit and activate additional inflammatory cells such as monocytes, eosinophils and NK cells thereby augmenting the tumor microenvironment and promote antitumor immunity[260].

Th2 cells

First described in 1986[252] Th2 cells, secrete IL-4, IL-5 and IL-13 and are classically described as mediating humoral immunity and promoting allergic inflammatory responses[157]. IL-4 signaling at the time of CD4 priming is responsible for Th2 differentiation by directly transactivating the transcription factor GATA-3[261]. The antitumor capacity of Th2 cells remains unclear. IL-4 secretion has been shown to enhance intratumoral infiltration of eosinophils and macrophages and in melanoma models, elimination of metastatic disease was observed in pulmonary tissue through eosinophil-dependent mechanisms[157]. However, IL-5 secretion by Th2 cells has been correlated with progressive growth of renal cell carcinoma and melanoma[157]. Furthermore, Th2 cells can promote M2 macrophage polarization resulting in immune tolerance tumor-specific Th2 T cell expansion has been associated with advance stage of disease or very aggressive disease[255,262].

Th17 cells

While Th1 CD4s are generally considered to be the most efficacious in mediating anti-cancer immunity, there have been reports indicating that other CD4 subtype can have important anti-cancer activity. Th17 cells were the third major CD4 subtype to be described when these cells were found not to produce the canonical Th1 and Th2 cytokines[4]. Instead, these cells predominantly produce IL-17A and IL-17F. Th17 lineage commitment is largely driven by IL-6 which induces expression of the transcription factor RORgt[263-265]. IL-17 secretion by these cells is critical in immune defense against extracellular bacteria and fungi[4]. However, recent work has suggested that they may play a role in antitumor immunity as well. One study reported that infusion of melanoma-specific Th17 cells resulted in superior tumor rejection as compared to infusion of Th1 cells in a melanoma mouse model[245]. Th17 cells largely produce IL-17A as well as some IL-17F, IL-21 and IL-22, which mediate responses to certain microbial invaders and fungi, however, IL-17A can also promote tumor growth and angiogenesis. Interestingly, infusion of cancer-

specific Th17 cells in a murine melanoma model resulted in a phenotypic shift of infused Th17 cells to Th1-like cells, leading to reduced expression of IL-17A and increased expression of IFNγ[245]. These melanoma tumors were completed eradicated and this effect was IFNγ-dependent and independent of IL-17A. Notably, Th17 cells are less terminally differentiated, exhibiting superior self-renewal capacity and higher plasticity relative to more terminally differentiated Th1 cells[157,266]. Therefore the ability of these cells to shift to a Th1-like response while maintaining a less differentiated phenotype may result in superior tumor rejection in some cases[245].

Tfh cells

T follicular helper cells (Tfh) secrete predominantly IL-21 and are essential for the generation of neutralizing antibody responses to viral infections[157]. Unlike the other CD4 subtypes described here, these cells predominantly reside in secondary lymphoid tissues and provide essential support to B cell populations to facilitate their differentiation into memory B cells and plasma cells[4]. Consequently, the presence of Tfh cells in the setting of lymphoid tumors is associated with poor prognosis and they facilitate the growth of these cells[267]. Conversely, in solid tumor types including, colorectal, hepatocellular, and breast cancers, increased infiltration of Tfh was associated with improved patient survival[157,268,269,270]. Tfh cells can facilitate and/or maintain B cell rich tertiary lymphoid structures and therefore it is hypothesized that infiltration of these cells may promote the organization or pro-inflammatory microenvironments within tumors to facilitate antitumor immunity[270]. Tfh cells may also promote antitumor immunity through IL-21 mediated expansion of tumor-specific CD8 populations[271].

Th9 cells

Identified as a discrete T-helper subset in 2008[272,273], Th9 cells largely produce the cytokine IL-9 and arise from reprogramming of Th2 cells via stimulation of TGFβ. These cells appear to play a role in autoimmune and allergic disease[274,275], but also can have potent anti-cancer functions[276]. Like Th2 cells, Th1-related cytokines such as IFNγ and IL-27 inhibit Th9 differentiation, while IL-10 and IL-25 promote Th9 lineage commitment[276]. The TFs required for Th9 differentiation are not fully elucidated, however, STAT6, GATA3, Pu.1 and IRF4 appear to be involved and a small fraction of Th9 cells express FOXP3,

though FOXP3 expression does not appear to be essential for Th9 commitment[276]. The anti-cancer effects of Th9 cells reported to date are IL-9 dependent. In B16 melanoma mouse models, RORγt deficient mice produced elevated IL-9 which was associated with reduced tumor growth and antibody neutralization of IL-9 ablated this effect[277]. In addition, adoptive transfer of tumor-specific Th9 T cells into B16/F10 mice (an ovalbumin-expressing melanoma tumor model) resulted in a 75% reduction in the number of lung metastases as compared to PBS control. Infusion of Th1 tumor-specific cells on the other hand, resulted in only a 50% reduction in lung foci[278]. The authors postulated that this effect was mediated by CCL20 produced by these Th9 cells. CCL20 attracts CCR6+ dendritic cells and promotes antigen uptake and presentation within the tumor. In support of this notion, the authors did find an increased number of CD8a+DCs (the most potent antigen-presenting DC subtype) within the tumor tissue following Th9 adoptive transfer relative to PBS control or the infusion of Th1 cells[278]. CD8 T cell activity was also required for the induction of antitumor immunity in this model as Th9 T cell infusion with CD8 depletion radically increased foci development relative to Th9 infusion alone[278]. These findings suggest that in some settings Th9 cells may have anti-cancer efficacy that is CD8-dependent, however, some tumor cells such as certain lymphomas can express the IL-9 receptor which results in increased survival and proliferation of lymphoma cells and therefore this cytokine is associated with poor prognosis in this tumor type[279].

Th22 helper cells

Like Th9 cells, Th22 cells have only recently been recognized as a discrete T-helper subtype[280,281]. These cells predominately secrete IL-22, a proinflammatory cytokine related to the IL-10 family[4] which is also produced by Th17 and NK cells[282]. However, Th22 cells appear to produce IL-22 in the absence of IL-17 and express the chemokine receptor CCR10, thereby differentiating them from Th17 T cells[282]. Th22 lineage commitment is governed by IL-6 and TNFα-dependent mechanisms and is transcriptionally regulated by the TF aryl-hydrocarbon receptor (AhR)[280,281]. IL-22 secretion by Th22 cells within tissues (particularly the skin, intestine, liver, kidney, pancreas and lung), serves to upregulate several inflammatory chemokines to promote neutrophilia at the site of infection, facilitate tissue regeneration, wound healing and induce expression of anti-apoptotic proteins[283,284]. While these cells are generally

thought to mediate responses against extracellular pathogens within tissues, a few studies have implicated Th22 cells as protective against viral infections including influenza and dengue[285-287]. Within the setting of cancer, initial reports suggest that Th22 cells may be associated with the development of colorectal cancer in a mouse model[288]. In human subjects, increased presence of Th22 cells has been reported in hepatocellular carcinoma and chronic myelogenous leukemia patients relative to healthy controls[289,290]. In cervical cancer patients, elevated levels of Th22 cells and an increased concentration of IL-22 were detected relative to patient with cervical invasive neoplasia or healthy controls[291]. Furthermore, lymph node metastases were correlated with increased prevalence of Th22 cells while there was a negative correlation between Th1 cells and Th22. Collectively, there findings suggest that the Th22 subtype may be detrimental in the setting of cancer[291]. However, these results do not identify a mechanism of action, indicating that further elucidation of the role of this CD4 T cell subtype is required in the setting of cancers.

T regulatory cells

It is well known that T-regulatory cells (Tregs) express the transcription factor FOXP3 and are critical in maintaining immunological homeostasis and preventing autoimmunity by reducing excessive immune responses[157]. Tregs can reduce effector CD8 T cell priming via consumption of IL-2 thereby limiting its availability to CD8 T cells and preventing the generation of a functional memory populations[257]. Tregs can also impair CD8 T cell stimulation by DCs through their regulation of expression of CD80 and CD86 on DCs through ligation with CTLA-4[257]. Additionally, secretion of inhibitory cytokines such as IL-10, TGFβ and IL-35 promote conversion of conventional T cells to T regulatory 1 cells (Tr1) and further impair DC function[292]. Notably, Tregs are critical in regulating the size of the proliferative burst of CD8 T cells in response to cognate antigen, to prevent bystander inflammation and to regulate the number of cells surviving into the memory phase thereby serving a crucial role in the development of memory T cell responses and prevent chronic T cell stimulation[257].

In the context of many solid tumors, infiltration of Tregs has been associated with poor prognosis in many cancer types including melanoma, non-small cell lung, gastric, hepatocellular, pancreatic, renal cell,

breast and cervical cancers[156]. However, in colorectal, head and neck, and bladder cancers, increased FOXP3+ T cell infiltration is associated with improve prognosis[156]. Treg depletion has shown therapeutic benefit in some preclinical models [293] and in breast cancer patients. Furthermore, targeted depletion of Tregs using an anti-CD25 monoclonal antibody (daclizumab) in combination with tumor-associated peptide vaccination resulted in favorable clinical responses in metastatic breast cancer patients[294]. Another trial utilizing tumor-associated peptide vaccination and GM-CSF, compared the efficacy of this approach with and without cyclophosphamide, a known Treg-depleting agent. In this study, the addition of cyclophosphamide reduced the Treg frequency and extended patient survival from a median of 14.8 months to 23.5 months in advanced renal cell carcinoma patients[295]. These results suggest that the combination of Treg depletion and effector T cell stimulation can be efficacious in some settings.

EXHAUSTION IN CD4 T CELLS

While CD4 T cells can be phenotypically described by their subtype, these cells can also characterized by their 'exhaustion' status, a dysfunctional state that can occur in the setting of chronic viral infection or cancer (**Figure 2)**[296]. Characteristics of exhausted T cells are elevated expression of inhibitory molecules such as PD-1, CTLA-4, 2B4, altered metabolism and impaired effector function[296]. Long term survival of exhausted T cells is also differentially regulated as compared to memory T cells[296]. The majority of the literature describing T cell exhaustion has detailed the phenotype of dysfunctional CD8 T cells, however, CD4 T cells actually become dysfunctional prior to CD8 T cells and their impairment is directly linked to the promotion of CD8 T cells exhaustion[297-299]. In the context of chronic viral infections, depletion of CD4 T cells during chronic lymphocytic choriomeningitis virus (LCMV) infection results in persistent, uncontrollable viremia and a loss of Th1 transcriptional signatures[298,300]. Early reports into the investigation into the effects of chronic antigen stimulation on CD4 T cell subtype differentiation suggests that in addition to a loss of Th1-type cells, exhausted CD4s appear to skew towards a T follicular helper phenotype with increased expression of the transcription factor Bcl-6[296] (**Figure 2**).

As an example of the importance of CD4 T cells in the setting of chronic antigen exposure, an elegant study of chronic HCV infection showed a strong correlation between the presence and function of HCV-

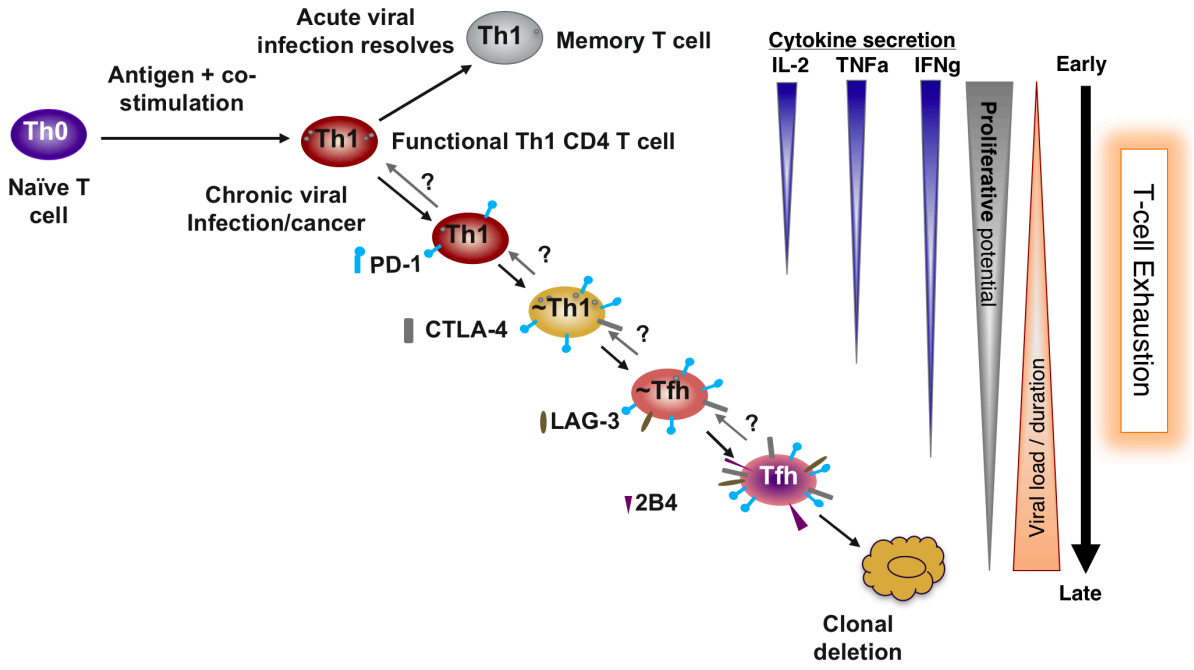


Figure 2: Hierarchical CD4 T cell exhaustion during chronic infection. Naive T cells are primed by antigen during initial infection and differentiate into effector T cells. Resolution of infection and antigen allows T cell contraction and differentiation into highly polyfunctional memory T cells. In the setting of chronic infection, however, as antigen and/or viral load increases, T cells progress through stages of dysfunction, losing effector functions and other properties in a hierarchical manner. CD4 T cells have been shown to progress from effective anti-tumor Th1 responses to more Tfh-like. T cell exhaustion is also accompanied by a progressive increase in the amount and diversity of inhibitory receptors expressed. If the severity and/or duration of the infection is significantly protracted, antigen-specific T cells can be clonally deleted, leading to loss of antigen-specific T cell responses. Adapted from Wherry Nat Immunol 2011 & Crawford Immunity 2014.

specific CD4 T and viral control[301]. PBMC from patients chronically infected with HCV and patients who had resolved HCV infection (resolvers) were screened for HCV-specific CD4s T cells using HLA-DRB1*0101 and HLA-DRB1*1501 tetramers. Chronically infected patients were found to have significantly reduced numbers of HCV-specific CD4 T cells as compared to resolvers[301]. Furthermore, HCV-specific CD4 T cells from chronically infected patients expressed significantly higher levels of PD-1 and CTLA-4 than resolvers. Importantly, *in vitro* treatment with PD-L1/2 blocking antibodies cells from chronically infected patients resulted in dramatically increased levels of the Th1 cytokines IFNγ, IL-2 and TNFα from HCV-specific CD4 T cells[301]. These findings indicate that the function of these cells can be rescued through PD-1 axis blockade. These studies strongly suggest that further investigation into the exhaustion phenotype and function of CD4 T cells is necessary to more accurately determine the role of these cells in chronic viral infection and cancer and to improve immune based therapies targeting these cells.

CANCER THERAPY WITH CD4 T CELLS

Autologous CD4 T cell therapy

Living cellular therapy is capable of intelligently sensing and adapting to a target environment. To date, autologous T cell therapies (ACT) have largely focused on the use of CD8 T cells alone, however, the use of autologous tumor infiltrating lymphocytes (TIL) which contain both CD4s and CD8 T cells, have shown markedly higher response rates (50-70%) in treating malignant melanoma[246,247]. These data suggest that CD4 T cells may be critical to mediate successful T cell antitumor function.

Indeed, though less broadly described, the use of CD4 T cells for ACT has been of interest since the 1980s when several preclinical models showed promise including CD4 T cell-mediated eradication of large sarcomas[302] and disseminated murine leukemia[303]. A study in 2007 showed that cancer antigen-specific CD4 T cells can actually mediate enhanced tumor regression and improve survival as compared to CD8 T cells[304]. In this study[304], the authors found that tumor-specific CD4s were able to prevent the establishment of a wide variety of tumors (bladder, pancreatic, prostate, salivary gland and melanoma) that were resistant to CD8 T cells rejection. Importantly, CD4 T cells were effective at preventing tumor outgrowth independent of tumoral HLA class-II expression[304]. This implies that these CD4s were acting indirectly on the tumor. This indirect effect at least in part was NK cell-dependent as NK cell depletion abrogated this effect[304]. Other preclinical studies have also shown CD4 T cell efficacy against murine melanoma tumors[305,306] and an interesting study by Zhu *et al*., reported that CD4 T cells are particularly important for promoting induction of high avidity, antitumor CD8 T cells but preventing their tolerization[307]. This effect could be critical as it has been shown in several models that high avidity CD8 T cells are the most efficacious at clearing tumors[308].

Aside from promising preclinical data describing the antitumor capacity of CD4 T cells, studies in humans have also yielded encouraging results. Infusion of NY-ESO-1-specific CD4 T cells in a patient with metastatic melanoma resulted in complete clinical remission[214]. Additionally, the use of mutation-specific CD4 T cells for treating a patient with epithelial cancer also mediated regression of distant metastatic disease[248]. These results indicate that cellular therapy utilizing CD4 T cells has powerful

therapeutic potential, while their clinical use has remained limited indicating that further studies are needed.

Transgenic T cell therapy

The use of transgenic T cells whereby a selected TCR specific for a particular tumor-associated antigen (TAA) is genetically transferred into autologous primary T cells, has been a promising strategy for cancer treatment as it provides an opportunity to rapidly generate high numbers of antigen-specific and functionally avid T cells (**Figure 3A**). Selection of the TAA to target can greatly alter not only the efficacy of this approach but also inform the method required for generation of the TCR. Some of the first TAAs identified were melanocyte differentiation antigens such as melanoma antigen recognized by T cells 1 (MART1), tyrosinase and GP100[309]. TCRs that are specific for these antigens can be derived from tumor infiltrating lymphocytes of melanoma patients or via TAA-specific T cell isolation from the peripheral blood. Melanoma-specific T cell responses confer relatively little risk of off-target side-effects because these antigens are only expressed within melanocytes. For TAAs that are subject to mechanisms of peripheral tolerance (such as TAAs that are expressed by a wide range of normal tissues but are present at much higher levels in tumors), allogeneic TCRs isolated from different individuals other than the patient

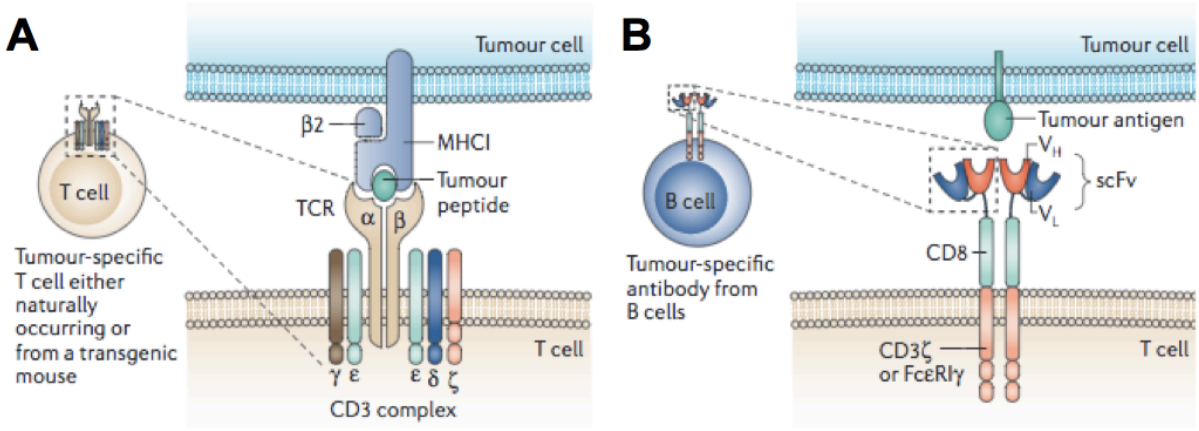


Figure 3: Molecular structure of transgenic TCRs and CARs. A: T cell receptors (TCRs) are composed of alpha and beta-chains. TCR sequences can be derived from tumor-specific T cells that are naturally occurring in humans, or from the immunization of human leukocyte antigen (HLA)-transgenic mice. B: Chimeric antigen receptors (CARs) are composed of a single-chain antibody variable fragment (scFv) extracellular domain linked through hinge and transmembrane domains to a cytoplasmic signaling region. Genes encoding the scFv are derived from a B cell that produces a tumor-specific antibody. An scFv is shown linked by a CD8 hinge to transmembrane cytoplasmic signaling regions derived from CD3ζ. CARs usually exist as a dimer, and they recognize tumor antigen directly with no requirement for HLA expression on the tumor. Adapted from Kershaw Nat Revs Cancer 2013.

themselves, or TCRs identified in transgenic, humanized mice have been used[310-312]. Virally-induced cancers, such as Merkel cell carcinoma, provide unique opportunities to employ virus-specific TCRs which avoid issues of tolerance and therefore often have higher avidity than TCRs recognizing self-antigens[313].

T cells transduced with a specific TCR, can be infused into cancer patients and may be particularly advantageous for those lacking endogenous T cell responses[310,314]. Consistent with previous discussions within this chapter, most studies employing this approach have focused on transducing MHC-I restricted TCRs into CD8 T cells. However, several models have shown that MHC-I restricted TCRs can also be effectively transferred into CD4 T cells to promote a more robust antitumor response than CD8 T cells alone[310]. For example, two TCRs specific for EBV and CMV class-I epitopes were transduced into CD4 T cells by Xue *et al*. and were able to recognize their cognate antigen. However, these cells were less avid than their CD8 counterparts. Consequently, the authors additionally co-transduced the CD8 co-receptor into these cells and saw elevated avidity. Notably, co-transduction of CD8 and the TCR did not alter the cytokine profile of transduced CD4s suggesting retention of CD4 T cell effector function[310]. In another study by Frankel *et al*, a high avidity tyrosinase-specific TCR conferred equal efficacy in mediating tumor control when transferred into CD4 or CD8 T cells in a mouse melanoma model[315]. Similarly, an HPV-specific TCR that recognizes an E7 epitope in the context of HLA-A*0201, showed potent stimulation via cytokine production in response to antigen stimulation when transduced into CD4 and CD8 T cells[314]. The use of a single TCR for induction of both CD8 and CD4 T cell responses has significant translational potential and seems largely to be dependent on the avidity of the TCR[310,314]. These results suggest that HLA class-I restricted TCR transduction into CD4 T cells can be therapeutically beneficial and therefore further investigation is warranted.

Chimeric antigen receptor (CAR) T cells

CAR T cells have revolutionized cellular therapy for liquid cancer treatment with responses rates for B cell acute lymphoblastic leukemia (B-ALL) reaching up to 90% complete remission rates[316]. These engineered receptors are comprised of 4 main elements, 1) the extracellular target binding domain which is named a single chain variable fragment; 2) a spacer domain; 3) a transmembrane domain; 4) and an

intracellular signaling/activation domain (**Figure 3B**). The genes that encode the target binding domain are derived from B cells that generate antibodies against a tumor-specific antigen. This domain is then linked to an intracellular T cell signaling domain (CD3 zeta) that upon antigen-binding, initiates intracellular signaling and mediates effector T cell function. Importantly, T cells require not only the initial antigen-induced signal, but also co-stimulation via co-receptor engagement (signal 2) and engagement of cytokines (signal 3). Signaling via the CAR alone without costimulation is insufficient and even detrimental to CAR T cell function. Therefore co-transduction of CD28 (a critical co-stimulatory molecule) leads to greatly improved effector function[309]. Efficacy of CAR T cell therapy can also be improved when CARs are transduced into both CD8 and CD4 T cells[317,318]. Studies that have transduced CARs into naïve CD4s and central memory CD8 T cells in a 1:1 ratio have yielded improve responses and lowered the cell doses required in both mouse models[317] and in adult B cell ALL patients[318].

Problems with cellular therapies

Cellular therapies are unique and can be efficacious[319], however, there are several important hurdles that have limited their wide-spread use. There are 5 critical challenges that T cell therapies must address in order to orchestrate effective anti-tumor immunity; the infused T cells must 1) traffic to the tumor, 2) recognize the tumor (but not adjacent tissues), 3) proliferate and persist, 4) counteract the immunosuppressive microenvironment, 5) and appropriately regulate activity following induction to prevent chronic inflammation or autoimmunity[319]. Attempts to improve and address these issues are reviewed in detail in a recent article by Lim & June[319]. Aside from these important biological issues, the production of these cells is costly and time consuming. Importantly, CAR T cells can only recognize TAAs that are expressed extracellularly and therefore cannot recognize ~80% of the molecules expressed by an individual cancer cell[309]. Therefore, while these agents have yielded impressive clinical utility in treating liquid tumors, their use in the treatment of solid tumors has remained limited.

CD4s and checkpoint therapy

Immune checkpoint inhibition (ICI) therapies for treating cancer have shown remarkable response rates in several cancer types and have led to several FDA approved therapies for melanoma, renal cell

carcinoma and NSCLC and most recently certain types of bladder cancer[320]. Surprisingly, the highest response rates using ICI monotherapy for solid tumors has been in MCC patients treated with pembrolizumab as a first line systemic therapy[65]. However, the identification of biomarkers predictive of response has been challenging. In melanoma, response to pembrolizumab or nivolumab (anti-PD1) can be predicted based on elevated expression of pre-existing CTLA-4^{hi} PD-1^{hi} intratumoral CD8 T cells[321] as well as elevated levels of CD8 T cells at the tumor invasive margin[216]. It is believed that CTLA4^{hi} PD-1^{hi} T cells are likely tumor-specific and therefore, having an elevated percentage of tumor-restricted T cells within the tumor microenvironment promotes an enhanced response following ICI therapy[321]. It remains to be determined whether these markers also identify responders from non-responders among MCC patients.

When investigating the effects of ICIs on CD4 T cell function, different agents alter the CD4 response in distinct ways. Ipilimumab (anti-CTLA-4) depletes intratumoral Tregs through FcγR-dependent uptake by tumor-infiltrating macrophages[157]. Targeting PD-1, on the other hand, increases the CD4 Teff:Treg ratio within tumors and primarily acts by promoting existing T cell responses and not inducing *de novo* ones. While the majority of studies have investigated the changes that occur within the tumor infiltrating lymphocyte fraction, a recent study indicated that peripheral CD4 T cell populations may also play a critical role. Using a mouse model of spontaneous breast cancer, Spietzer *et al*[322]. show that response to effective immunotherapy was correlated with an expansion of effector memory Th1 cells in the peripheral blood[322]. Adoptive transfer of these effector memory Th1 cells into untreated mice mitigated tumor development. In conjunction with these findings, melanoma patients who responded to ipilimumab and GM-CSF combination therapy had specific expansion of PD-1- CD127 lo CD4 T cells without detectable changes in CD8 T cell phenotypes within the periphery, strongly suggesting that peripheral CD4 T cells can contribute to effective immunotherapy in some settings[322].

THERAPEUTIC CANCER VACCINES AND THEIR INDUCTION OF CD4 T CELLS

Over the past few years, therapeutic cancer vaccines have seen a resurgence in interested since Dr. William Coley first stimulated the immune response against cancer via injection of inactivated

Streptococcus pyogenes and *Serratia marcescens*[323]. The main goal of a therapeutic cancer vaccine is to expand the tumor-specific T cell population from either the naïve repertoire or from a dormant/anergic state[324]. High rates of response to therapeutic cancer vaccines have been reported in the setting of premalignant disease (such as vulvar intraepithelial neoplasia) and in the adjuvant setting[324]. In patients with vulvar intraepithelial neoplasia (VIN), synthetic long peptides, fusion proteins and DNA vectors encoding the E6 and E7 oncoproteins have induced HPV16-specific T cell responses and resulted in complete or partial remissions[325-328]. In the adjuvant setting, colorectal cancer patients experienced a significant survival advantage when administered PANVAC, a recombinant pox virus expressing the tumor antigens carcinoembryonic antigen and MUC1[329]. Additionally, RNA vectors encoding 27mer

Table 2: Types of therapeutic cancer vaccines in development

Vaccine	Mechanism of Action	Examples (Clinical Trial Phase)
Antigen/Adjuvant	Specific epitopes delivered as protein fragments or peptides are used to stimulate tumor-specific T cells. Often combined with an adjuvant	HER2 for breast cancer MUC1 and CEA for colorectal cancer SF HM2, SF HM4, SF HM8 for melanoma HAPa-1 and HAPa-2 for pancreatic cancer E6/E7 for HPV induced vulvar intraepithelial neoplasia
Whole cell tumor	Uses autologous or allogeneic whole tumor cells that are attenuated or inactivated. These cells express the full cancer antigen repertoire and are often administered in combination with cytokines to stimulate tumor-specific T cells	Injectable cytogel whole-cell cancer vaccine CD19 for leukemia's and lymphomas HER2 for breast cancer CEA for lung, colorectal, gastric, breast, and pancreatic cancers CD22 for leukemia's and lymphomas
Dendritic cell	Autologous dendritic cells are obtained through leukapheresis and stimulated with autologous cancer antigens and then reinjected into the patient. DC vaccines then prime and activate tumor-specific T cells	Sipuleucel-T (Provenge; FDA approved) HER2/Neu for breast, prostate, non-small cell lung cancer Tumor lysate pulsed DCs for glioblastoma
Viral vector and DNA	DNA coding for a specific tumor antigen is transported into a viral or bacterial vector to produce cancer antigen proteins. Usually administered with adjuvants, often combined with TLR agonists.	Adenoviral vector for PSA for prostate cancer Adenoviral vector for HER2/Neu for breast cancer PROSTVAC + GM-CSF for prostate cancer 5T4 (Trovax) for RCC HSV-1 vector encoding GM-CSF and melanoma antigens HPV-detoxE7 for cervical cancer
RNA vaccine	RNA encoding tumor antigens is either electroporated or delivered in liposomes or protamines for stabilization. Usually administered with adjuvants, often combined with TLR agonists.	Personalized neoantigen vaccine for melanoma[249,250].

peptides[249] or long immunizing peptides[250] derived from melanoma patient neoantigens, showed remarkable efficacy in the adjuvant setting. Importantly, in these studies the majority of targeted neoantigens were HLA class-II restricted and generated robust CD4 T cell responses[249,250]. Targeting of CD4s via vaccination may also have the benefit of reduce co-inhibitory molecule expression on CD8 T cells, thereby reducing tumor-specific CD8 T cell exhaustion[258,299]. If activation of antigen-specific CD4 T cell help is not possible, the use of xenogenic or non-tumor antigen vaccination can provide non-specific CD4 help. For example, a synthetic helper peptide called PADRE as well as the tetanus toxoid-derived helper peptide have been shown to improve efficacy of peptide cancer vaccines in preclinical models[330,331]. However, in melanoma patients, the use of melanoma-derived helper peptides yielded higher clinical objective response rates than the use of tetanus-derived helper peptides when used in combination with CD8 peptide vaccination. This suggests that antigen-specific stimulation induces more effective immunity than non-specific stimuli. Importantly, these majority of successful therapeutic vaccination strategies used to date have only yielded responses in the non-immunosuppressive environment of premalignancy or following surgical resection. Therefore, additional work is required in order to initiate meaningful efficacy against non-resectable and malignant solid tumors.

One significant challenge limiting the efficacy of therapeutic vaccines for solid tumors it the immunosuppressive tumor microenvironment. This immunosuppressive microenvironment can polarize immune cells including CD4 T cells and macrophages away from a pro-inflammatory Th1 and M1 phenotypes and towards an immunosuppressive Treg and M2 phenotypes[324,332]. This polarization results in impairment of the CD8 and CD4 T cell response, tissue remodeling, expression of PD-L1 on tumor cells and ultimately tumor progression. M2 macrophage populations that remain after surgical resection of a tumor can inhibit otherwise effective adjuvant vaccination[333]. Myeloid derived suppressor cells (MDSCs) can also significantly impair the efficacy of vaccines. In patients with renal cell carcinoma, MDSC levels in the periphery inversely correlated with response to vaccination[334]. Therefore, novel strategies aimed at overcoming these immunosuppressive barriers will likely be required in conjunction with therapeutic vaccine delivery.

In the era of immune checkpoint inhibitors which have yielded such remarkable successes, it is important to address the role of cancer vaccines. The efficacy of immune checkpoint inhibitors such as PD-1 blockade, require the presence of pre-existing anti-tumor immune responses[216,260]. Therapeutic cancer vaccines, therefore, may serve to initiate such T cell responses and could be used in the adjuvant setting or in combination with checkpoint inhibitors. Indeed, in patients who failed vaccination alone, the addition of anti-PD-1 results in complete remission of 3 cases[249,250]. Consequently, continued investigation into combination therapies that can enhance anti-tumor immunity via multiple mechanisms is necessary, however, is it now clear that employing CD4 T cells in the context of cancer immunotherapy significantly improves efficacy and ultimately outcome.

CHAPTER 6: IDENTIFYING CD4 EPITOPES WITHIN THE MERKEL CELL POLYOMAVIRUS T-ANTIGENS

Unpublished: Not currently planning to publish this work. Perhaps could be combined with CD8 epitope data for a broader epitope study.
Contributors: Vandeven NA, Ibrani D, Laing K, Kulikauskas R, Campbell V, Iyer J, Koelle D, Nghiem P

Chapter Summary:

As described in the previous chapter, eliciting a tumor-specific CD4 T cell response is crucial in order to mount robust anti-cancer immunity, therefore, targeting CD4 T cells may be beneficial in the treatment of MCC. Characterization of tumor-specific CD4 T cells and their potential therapeutic effects, has been severely limited among many cancer types, including MCC, due to several significant barriers including the dramatically lower frequency of antigen-specific CD4s than CD8s, the increased genetic diversity of HLA class-II molecules and the difficulty in generating HLA class-II tetramers[245]. Therefore, a crucial step to isolate and study these cells is the identification of MCPyV-specific CD4 T cell epitopes so as to develop the appropriate reagents necessary to accurately identify and phenotype these cells. In this Chapter, we discuss the methods that have been used to date to identify CD4 epitopes within the MCPyV oncoproteins, including a highly immunogenic epitope encompassing the RB-binding motif that may provide an ideal therapeutic target for future therapeutic strategies.

Initial evidence that CD4 T cells can recognize the persistently expressed region of MCPyV

Within complex antigens, such as the T-antigens of MCPyV, selected regions are presented and expressed by HLA molecules for T cell recognition[335]. These regions are called 'epitopes' and the delineation of specific epitopes is critical in order to isolate cells specific to a given antigen as well as to develop various therapeutic modalities such as vaccines[336,337]. In an initial attempt to identify MCPyV-specific epitopes, 428 peptides were synthesized that span the MCPyV LT and sT region (**Figure 1**: Sigma-Aldrich). These peptides are 13mer peptides overlapping by 9 amino acids with sequences based upon a MCPyV reference strain (EU375803 GenBank). An intracellular cytokine secretion assay (ICS) was used to evaluated MCPyV peptide-specific responses. In this assay, responder cells from a patient's tumor infiltrating lymphocytes (TIL) were incubated overnight with autologous PBMC (as antigen presenting cells) and peptides. IFNγ secretion is assessed using flow cytometry to indicate reactivity in

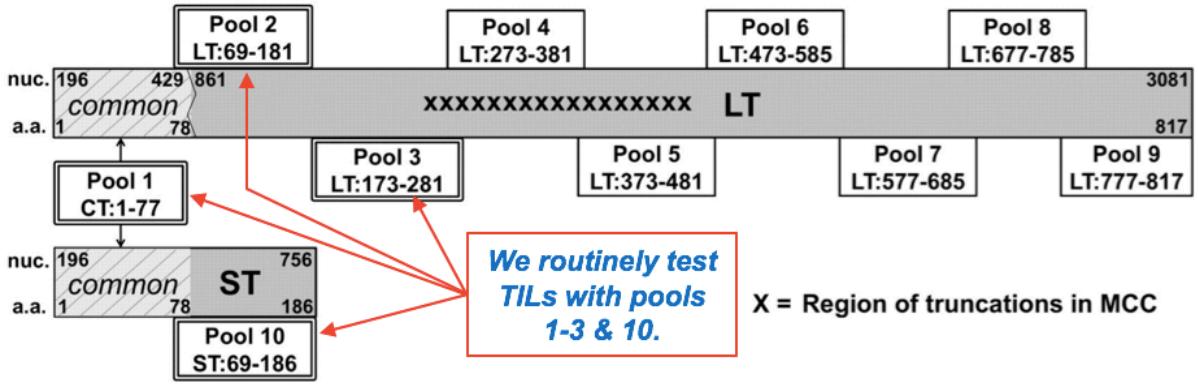


Figure 1: MCPyV synthetic peptide pools. MCPyV proteins (gray boxes) are annotated with nucleotide (nuc) and amino acid (a.a.) numbers. Peptides spanned the T-antigens of the MCPyV proteome and were grouped into pools (boxes with a.a. range indicated) on the basis of protein domains. Jagged line at nucleotide 429 represents the genomic splice site.

responses to peptide stimulation. Initial stimulation is performed using peptide pools (**Figure 1**) and cells that respond to a given peptide pool are the re-stimulated with individual peptides in a 'pool breakdown' to identify the specific 13mer(s) that the TIL specifically recognize.

Using this approach, the first MCPyV epitope identified was in fact a CD4 epitope[130]. Robust secretion of IFNγ from an MCC patient's tumor infiltrating lymphocytes (TIL) was observed in response to an epitope located within the common T region of the T-antigens (**Figure 2;** w347). In addition, two other MCPyV-specific CD4 responses were identified using this method **(Figure 2:** w406 & z1076**).** These data suggest that MCPyV-specific CD4s are biologically meaningful as they are physiologically localizing to the tumor and secreting appropriate effector cytokines. Of note, all three patients with detectable CD4 T cell responses in their TIL ultimately controlled their disease despite presenting at advanced stages.

While these preliminary findings suggest an important immunological role for CD4 T cells in MCC patients, only 3 of over 100 tested TIL from MCC patients had identifiable CD4 T cell responses[338]**.** This suggests that either IFNγ secreting CD4 T cells are below the level of detection utilizing this cytokine secretion assay, are not present, or are functionally exhausted in the majority of MCC patients. Notably, CD4 responses were evaluated in TILs, which are enriched T cell populations that have been cultured for several weeks in the presence of IL-15 (a cytokine that can bias cultures toward CD8 expansion at the cost of CD4 expansion). Evidence for this biased culture is observed in **Figure 2**, as the CD4+

populations represent 1-19% of the total lymphocyte population, which is significantly skewed from the normal CD4:CD8 ratio of roughly 2:1. Therefore, it is likely that identification of additional CD4 responses may have been limited by the detection method as the result of these CD8-skewed culture conditions.

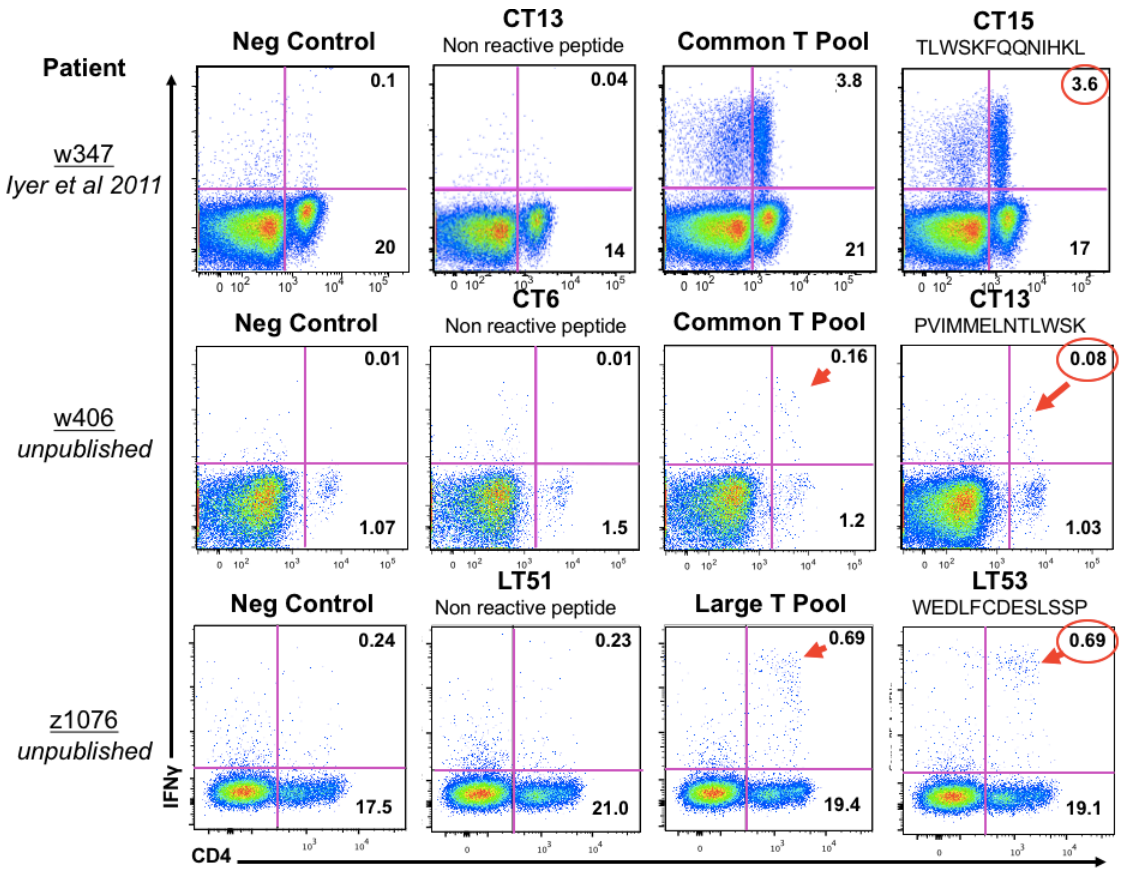


Figure 2: Characterization of MCPyV-specific CD4 epitopes from TIL cultures. Identification of IFNγ CD4 T-cell responses to T-antigen peptides by ICS assay. From left, TILs stimulated with DMSO; representative nonreactive peptide, Peptide pools and reactive peptide. Autologous PBMCs were used as APCs and IFNγ secretion was evaluated 16hrs after stimulation using flow cytometry.

Detection of MCPyV epitope using cultured ELISPOTs

After the initial identification of the first CD4 epitope (CT57-69), Iyer et al, described the use of cultured IFNγ ELISPOTs to identify additional MCPyV epitopes from PBMC[130]. In this study, PBMC from 27 MCC patients and 13 healthy donors (who never had MCC) were tested for MCPyV T cell reactivity. The use of PBMC derived responder cells that were not cultured with IL-15, avoided potential skewing away from CD4 responses. Of note, these assays used 13mer peptides for their stimulation which favors stimulation of CD8 T cells over CD4 T cells, whereas peptides of ≥15 amino acids in length are typically preferred for identifying CD4 responses[337,339]. However, using this method they found 24 MCPyV epitope within sT and

LT, 5 of which were confirmed CD4 hits (**Figure 3**) and interestingly these were all from a single healthy donor (arrows).

A few important caveats should be noted. The first is that this was a cultured ELISPOT and this assay therefore does not represent the direct *ex vivo* frequency of these antigen-specific cells. Additionally, it was later discovered that ethanol activation of the ELISPOT membranes was not performed, therefore, these results likely underestimated and/or altered responses and therefore these epitopes need validation via other methods.

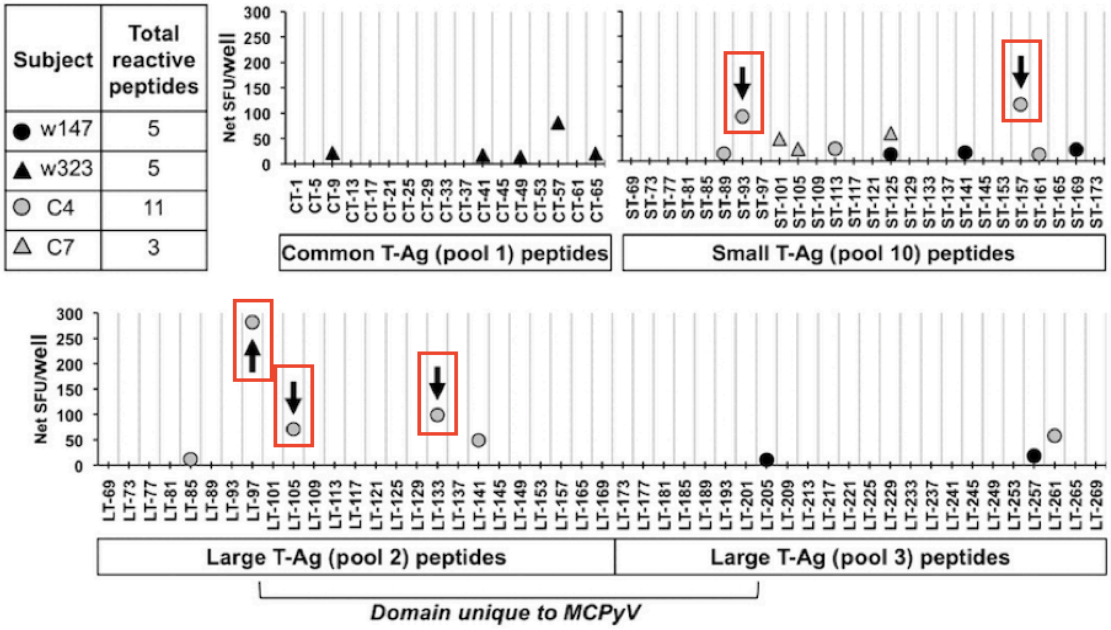


Figure 3: MCPyV epitope mapping via ELISPOT (Iyer et al 2011). Identification of immunoreactive epitopes derived from previously reactive T-Ag peptide pools by cultured IFNγ ELISPOT assay in MCC patients (black) and control subjects (gray). In subject C4, 5 peptides (arrows) were identified to be immunogenic to $CD4^+$ T cells. Adapted from *Iyer et al. 2011*

Tetramer-guided epitope mapping (TGEM) to identify MCPyV CD4 epitopes

Another epitope-mapping method that can be performed on PBMC (thereby avoiding CD8 skewing conditions) is tetramer-guided epitope mapping (TGEM), pioneered by Dr. William Kwok at the Benaroya Research Institute[337]. There are several key advantages to this approach that merit mention. While this method does require antigen-stimulation for 2 weeks to expand antigen-specific cells, at the conclusion of this assay, positive results identify not only specific epitopes but also include their HLA-restriction, and the generation of an HLA-II tetramer. Additionally, this method does not require the cells to be functional

(meaning capable of secreting cytokines). Therefore, if antigen-specific cells are 'exhausted' or dysfunctional (or simply secreting a cytokine other than IFNγ), this method will still detect them. The value of tetramers cannot be overstated. These powerful tools provide an ability to determine the frequency of antigen-specific T cells directly *ex vivo* without expansion methods (expansion in TGEM is only for the initial identification of the epitope, once the tetramer has been made, expansion can be avoided). Consequently, the number of antigen-specific cells can be tracked over time enabling characterization of T cell populations during the course of disease and/or treatment. Additionally, tetramers bind only to the surface of T cells and therefore do not require permeabilization (which is necessary for an ICS assay), enabling downstream sorting for either expansion or additional phenotypic analysis via RNA expression.

***********A brief background on HLA tetramers*********

Why must HLA molecules be tetramermized in order to label T cells? The simple answer is that the binding affinity of TCR-HLAs is inherently weak (KD ~0.1-500uM). Therefore, Altman *et al.* in 1996 described the use of HLA class-I tetramers that are composed of ~4 biotinylated HLA molecules that are multimerized via streptavidin and conjugated to fluorescent labels (**Figure 4**). The increased valency results in higher avidity allowing the tetramer to remain bound to a cell for analysis by flow cytometry[340,341]. Shortly thereafter, a group led by Dr. Kappler at the University of Colorado developed the first HLA class-II tetramer composed of the mouse HLA allele HLA-I-E[342]. Unfortunately, the development and broad use of HLA class-II tetramers for human alleles has been significantly slower and more tedious due to the low frequency of antigen-specific CD4 T cells in the periphery, the low TCR-MHC affinity and challenges in the generation of HLA monomers[343].

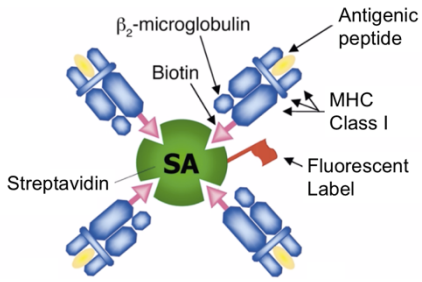


Figure 4: Schematic of fluorescent MHC class I–peptide tetramer. Adapted from Eisenbarth et al, J. Clin. Inv. 2003

Currently three methods have been developed for synthesizing HLA class-II molecules. The first covalently tethers the peptide epitope directly to the soluble HLA molecule thereby ensuring that the peptide is bound and preventing dissociation. However, this approach requires the generation of specific constructs for each individual peptide of interest. The second method uses the class-II associated

invariant chain peptide (CLIP) as a surrogate for the epitope of interest during the folding of the HLA monomer. This approach can improve folding efficiency and then the peptide of interest can be loaded exogenously by peptide-exchange reactions. The third approach is to simply generate soluble empty HLA molecules that are subsequently loaded with peptide exogenously. This is the most versatile approach as multiple peptides per HLA allele can be screened at once, however, HLA-peptide binding stability can be limiting in some cases[344]. This last approach is used by Dr. Kwok to enable high throughput screening of the entire antigen space of the MCPyV T-antigen using TGEM[337].

****

How does TGEM work?

Peripheral mononuclear blood cells (PBMC) from healthy donors are plated into 48 well plates and stimulated with 10 pools containing 5, 20mer peptides overlapping by 12 amino acids spanning across MCPyV LT and sT (**Figure 5A**). Following a two week expansion period, cells are stained with tetramer panels that are loaded with peptide pools (**Figure 5B**). Tetramer positivity indicates that one or more peptides within that pool can 1) bind that HLA-allele type and 2) T cells can recognize that HLA-peptide complex. Remaining cells from positive cells are then

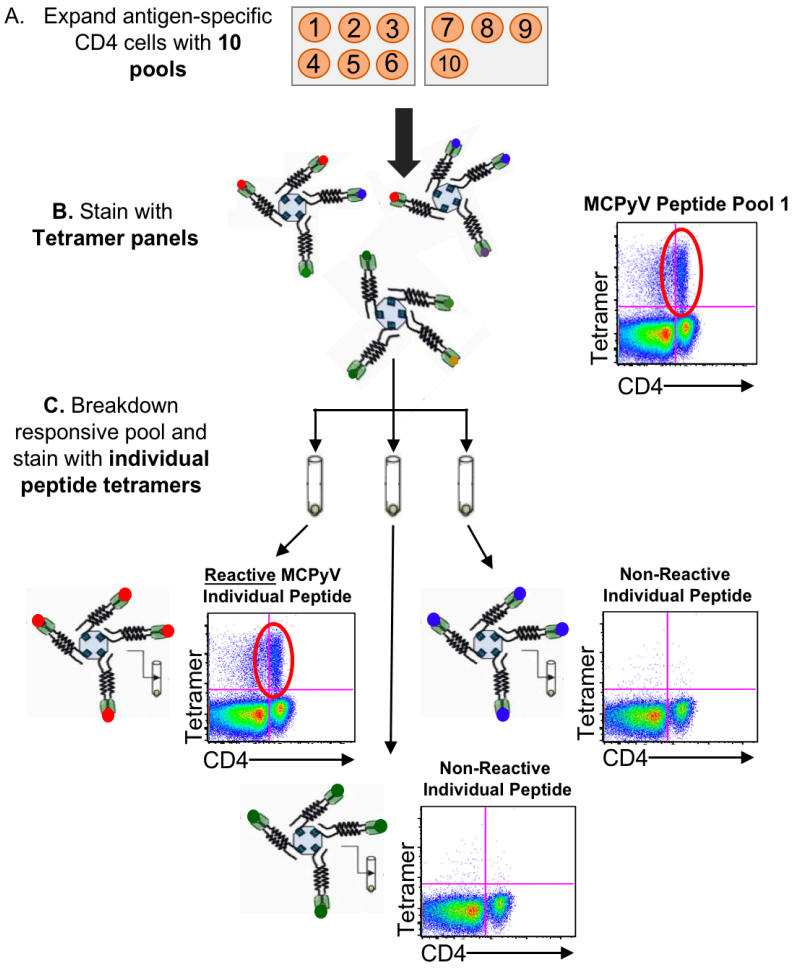


Figure 5: Tetramer-guided epitope mapping work flow. A: Whole PBMC from HLA-appropriate healthy donors was divided into 10 wells, each stimulated with a distinct pool of 20mer peptides spanning the oncogenic region of the Large and small T-antigens. Cells were expanded for 2 weeks. B: Wells are stained with CD3, CD4, live/dead dye and tetramer panels (tetramers loaded with 5 peptides from each pool). C: Wells that stain positive with a tetramer panel are restained with tetramers loaded with individual peptides from that pool. Method described by Novak *et al*. J. Immunol 2001.

stained with tetramers loaded with individual peptides to determine the fine specificity (**Figure 5C**).

Using this method, seven HLA alleles were tested (**Table 1; Figure 6**) and we identified 3 epitopes restricted to 3 discrete HLA types**.** LT-209-228 epitope (referred to as 'WED') identified using a DRB1*0401 HLA allele was also predicted to bind to DRB1*0301. We therefore synthesized an HLA-DRB1*0301-'WED' tetramer and found that 3 of 4 tested donors had tetramer binding. In summary, the use of TGEM identified 3 epitopes restricted to 4 HLA-alleles and generated 4 HLA class-II tetramers. Of note, when using these tetramers on MCC patient samples, I have successfully identified DRB1*0401-'WED' specific CD4s while the other three tetramers have had high background staining, meaning patients that are the in-appropriate HLA-type have high levels of tetramer binding. Therefore, all subsequent tetramer analyses have been done using the DRB1*0401 tetramer (described in detail in

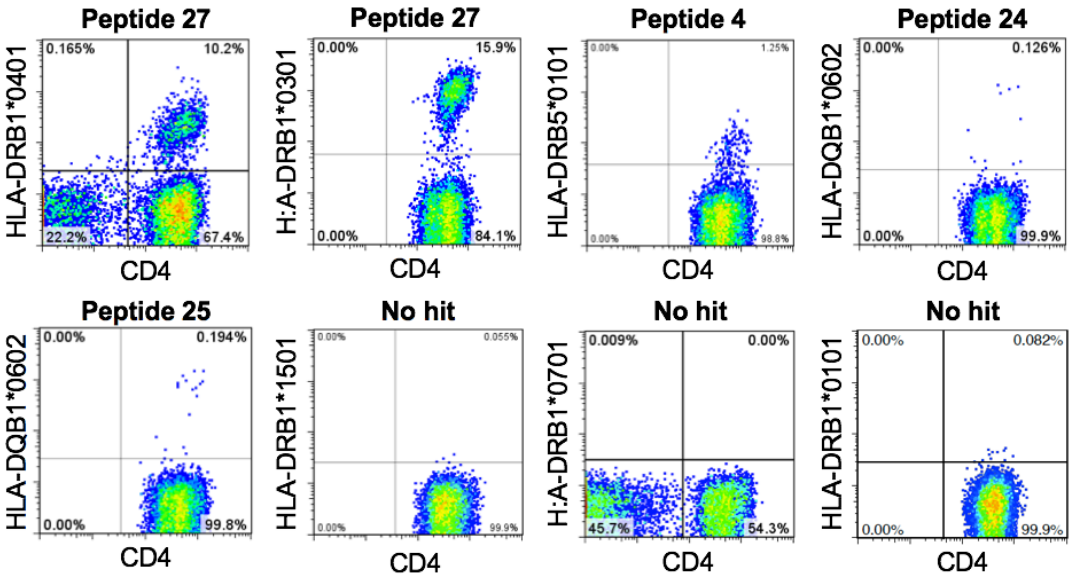


Figure 6: TGEM identified 3 epitopes presented by 5 population prevalent HLA class-II alleles. TGEM was performed as previously described testing 8 allele types. Healthy donor PBMC from HLA-appropriate individuals was expanded in the presence of peptide pools for 2 weeks. Tetramer panels were used for initial screen and subsequent staining with tetramers loaded with individual peptides from reactive pools was used to determine peptides restriction.

Table 1: HLA class-II alleles tested using TGEM

HLA	Prevalence of HLA allele in MCC cohort	# of Subjects tested	Peptide name	Sequence	MCPyV location
DRB1*0401	18%	4/4	Peptide 27	WEDLFCDESLSSPEPPSSSE	209 - 228
DRB1*0301	20%	3/4	Peptide 27	WEDLFCDESLSSPEPPSSSE	209 - 228
DRB5*0101	13%	1/4	Peptide 4	AFKRSCLKHHPDKGGNPVIM	33 - 52
DQB1*0602	18%	1/4	Peptide 24	NSGRESSTPNGTSVPRNSSR	185 - 204
DQB1*0602	18%	1/4	Peptide 25	PNGTSVPRNSSRTYGTWEDL	193 - 212
DRB1*1501	6%	0/4	No hit	N/A	N/A
DRB1*0701	20%	0/3	No hit	N/A	N/A
DRB1*0101	26%	0/1	No hit	N/A	N/A

remaining Chapters). While I do believe that these other three tetramers identify true MCPyV CD4 epitopes, this analysis was done on cultured healthy donor blood that has been highly enriched for CD4 T cells specific to the respective antigen and therefore is a rather artificial system. Additional steps to potentially optimize the peptide (i.e. try different lengths) may improve the performance of these three additional tetramers. For instance, Srinivasan *et al.* described a 23mer peptide that was 32 times more immunogenic than a 10mer peptide encompassing the core region of the epitope[345] and numerous groups have indicated that longer peptides can possess peptide-flanking residues (PFRs) which lie outside of the binding groove of the HLA molecules and stabilize binding through interaction with more distant locations[346]. Identification of critical residues required for improved binding is typically determined by alanine scanning and could be considered in the future.

Additional methods for epitope identification

While we have used TGEM, ELISPOT and ICS to identify a total of 12 CD4 epitopes to date (summarized in **Table 2**), the Immune Epitope DataBase (IEDB; http://tools.iedb.org/mhcii/) predicts that many more epitopes restricted to common HLA allele types are likely present within the MCPyV T-antigens. Therefore, in collaboration with Dr. David Koelle I am trying several additional methods. The first is an assay that measures proliferation via dilution of carboxyfluorescein diacetate succinimidyl ester

Table 2: Summary of CD4 MCPyV epitope identified to date

	Patient/Donor	Epitope	Location in genome	TIL or PBMC?	HLA Restriction	Tetramer?
ICS *(Iyer 2011; Ibrani 2015)*	**w347** *(Iyer 2011)*	TLWSKFQQNIHKL	CT-57-69	TIL	DRB1	No
	W406 *(Ibrani 2015)*	PVIMMELNTLWSK	CT-49-61	TIL	DRB1	No
	Z1076 *(ibrani 2015)*	WEDLFCDESLSSP	LT-209-221	TIL/PBMC	DQB1	No
ELISPOT *(Iyer 2011 & Zeng 2012)*	**C4**	DYMQSGYNARFCR	LT-93-105	PBMC	Unknown	No
	C4	GGFSFGKAYEYGP	LT-97-109	PBMC	Unknown	No
	C4	YEYGPNPHGANSR	LT-105-117	PBMC	Unknown	No
	C4	SSPPHSQSSSSGY	LT-133-145	PBMC	Unknown	No
	C4	FGFPPTWESFDWW	LT-157-169	PBMC	Unknown	No
TGEM	**w131** **C667** **C519**	WEDLFCDESLSSPEPPSSSE	LT-209-228	TIL/PBMC	DRB1*0401	Yes
	C533	GNIPLMKAAFKRSCLKHHPD	CT-25-44	PBMC	DRB5*0101	Yes
	C533	PNGTSVPRNSSR	LT-193-204	PBMC	DQB1*0602	Yes
CFSE	**w164**	CISCKLSRQHCSLKTLKQKN	sT-121-140	PBMC	DR?	In progress

(CFSE). The second evaluates the expression of three activation markers that are upregulated on T cells following antigen-stimulation. Both will subsequently be described as well as the accompanying preliminary data.

CFSE dilution assays

A hallmark feature of T cells is their ability to rapidly proliferate upon antigen stimulation in order to generate a robust population of effector cells[347]. Upon removal or clearance of that antigen, this effector population contracts and sustains a significantly smaller memory population[347]. We can take advantage of T cell proliferation following antigenic-stimulation and label cells with carboxyfluorescein diacetate succinimidyl ester (CFSE), a dye that becomes highly fluorescent upon uptake and conversion within a cell. This dye was originally developed for tracking lymphocytes *in vivo*[348] before becoming a standardized T cell assay mar king proliferation[349]. As labeled cells proliferate post antigen-stimulation, CFSE becomes sequentially diluted in each daughter generation. Consequently, CFSE-lo cells are identified as a population of cells that proliferated in response to antigen-stimulus suggesting that these cells are antigen-specific.

Using this technique in collaboration with Kerry Liang in the Koelle lab, we CFSE labeled PBMC from two MCC patients (w944 and w164) and stimulated cells with MCPyV 20mer peptide pools for 5 days. Cells were gated using the scheme shown in **Figure 7A** and antigen-specific cells were identified as CFSE dilute. Single cells were then sorted into 96-well plates with irradiated feeders and cultured for 2 weeks in the presence of nIL-2. Dr. Liang then did a pool break down with expanded clones using an H^3 thymidine incorporation assay. H^3 uptake is also used to measure proliferation in response to antigen-simulation, whereby expanding cells become radiolabeled as compared to non-expanding populations. Clones responded to peptide 42 (sT-121-140; **Figure 7B**).

In order to determine the HLA-restriction of this peptide, monoclonal antibodies against HLA-DR and HLA-DQ were used to block antigen presentation in an ICS assay (**Figure 8**). Following HLA block and peptide stimulation, a CIS-specific clone from w164 responded to the CIS peptide alone, however, IFNγ

secretion was inhibited in the presence of anti-HLA-DR (L243) indicating that this response is HLA-DR restricted. Further delineating the HLA-DR that this epitope is restricted to is on going.

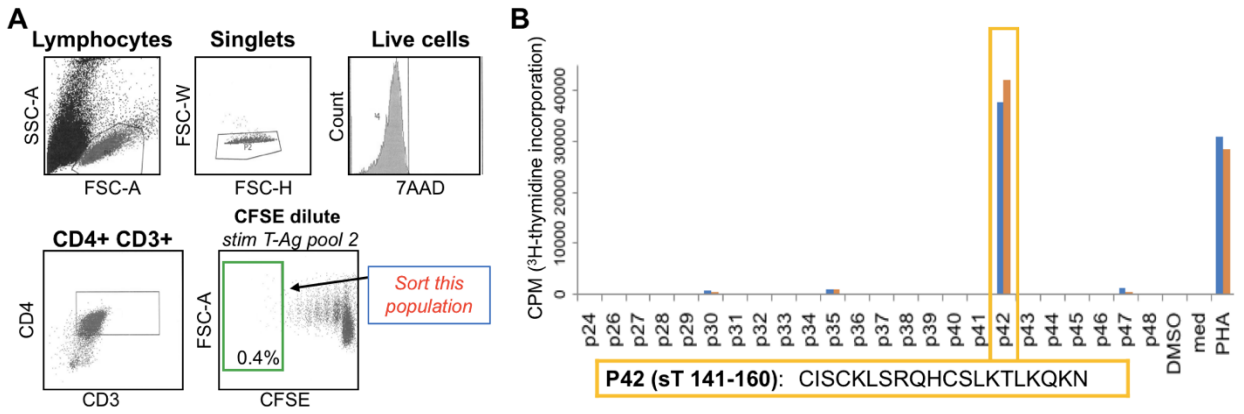


Figure 7: Novel MCPyV sT "CIS" epitope via CFSE dilution sorting. A: PBMC from an MCC patient (w164) were CFSE labeled and stimulated with MCPyV 20mer peptide pools. On day 5, CFSE dilute, CD3+CD4+ cells were sorted and expanded for 2 weeks in the presence of IL-2. **B:** Visible microclusters that expanded following 2 week incubation were re-stimulated with individual peptides from Pool 2 (initial reactive pool from CFSE dilution sort). Cells were pulsed with H^3 and assayed for proliferation in response to peptide stimulation. Unpublished.

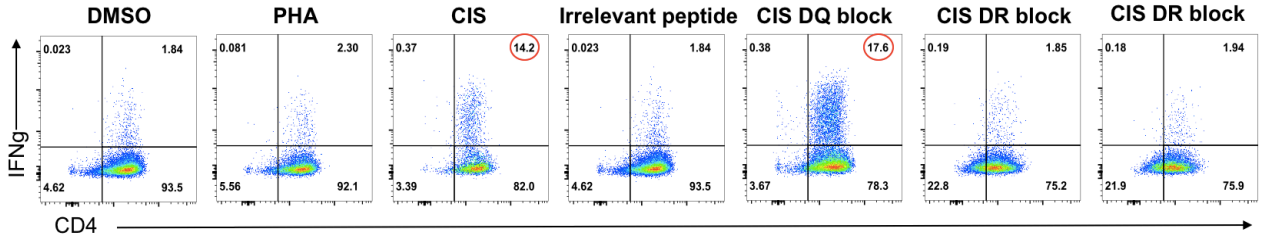


Figure 8: "CIS" epitope is HLA-DR restricted. CD4 T cell clones generated against the "CIS" epitope from MCC patient w164, were stimulated with negative control (DMSO), positive control (PHA), irrelevant peptide (influenza 20mer), and "CIS" 20mer peptide with and without HLA-blocking mAbs. IFNγ secretion was assays via ICS. Unpublished.

Triple activation marker sorting

The preliminary data from the CFSE dilution method is promising, however, that assay requires culturing cells for 5+ days which thereby prevents this assay from being useful for analyzing cells directly *ex vivo.* Culturing cells may also preferentially expand specific clonotypes, thereby narrowing the true pool of responsive TCRs. To avoid skewing of the T cell repertoire, another commonly used method is to do a shorter overnight stimulation and then sort cells based on upregulation of activation markers. CD69 is a commonly used marker that is one of the earliest to be expressed following T cell activation[350,351]. However, CD69 expression is not solely dependent on TCR signaling therefore, the level of background

signal is relatively high with this marker and so it should not be used alone[352,353]. Staining with CD69 is often done in conjunction with CD137 or CD154 which can greatly increase specificity[354]. CD154 (CD40L) is critical in mediating T helper function through interaction with CD40 which is expressed by APCs as well as CD8s. Unlike CD69, CD154 has significantly lower background staining, potentially due to rapid internalization following ligation with CD40[354]. CD137 is also upregulated early in T cell activation (under 24 hours). The Koelle lab has developed a method whereby they combine staining of all three of these activation markers to highly enrich for antigen-specific cells. Using PBMC from patient z1171 in whom we had previously detected IFNγ secretion in response to Pool 1 peptide stimulation, I tried triple marker sorting and single cell cloning (**Figure 9**). When stimulated with Pool 1 peptides spanning the common T region, 0.01% of total CD4 T cells upregulated all three markers. This is consistent with the level of IFNγ secretion observed (which was 0.018% of CD4 T cells) and in fact encouraging that it is slightly lower as this may indicate an elevated level of specificity. Notably, this is still a relatively large frequency of antigen-specific CD4 T cells for a direct *ex vivo* response in the periphery. While we were able to successfully sort these cells for single cell cloning, no cells expanded. Sorting cells from this patients' PBMC using CFSE dilution also was unsuccessful at generating clones despite detectable growth from another patient's PBMC sorted and cultured in parallel. Therefore, while this preliminary data suggests that this method may be working in the Nghiem lab, validation via clone generation and retesting is required to verify specificity.

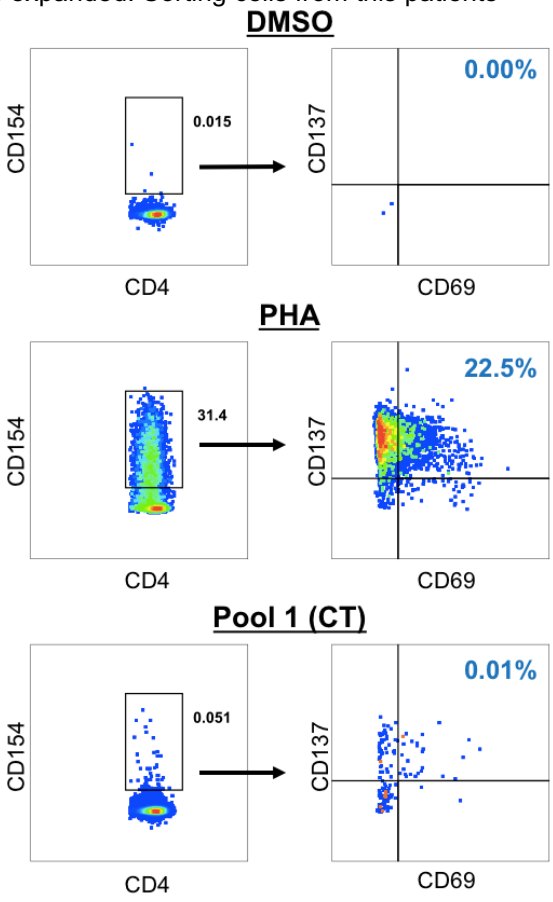


Figure 9: Triple activation marker expression for identification of rare antigen-specific CD4 T cells. PBMC from an MCC patient (z1171) was either unstimulated (DMSO) or stimulated overnight with PHA (positive control) or MCPyV peptide pool 1 containing peptides within the common T region (13mers used because initial hit was detected with a 13mer). After 16 hours, cells were stained with exclusion markers (CD14,CD19, Live/Dead), CD4, CD154, CD137 and CD69. Cells were gated on size and granularity to identify a lymphocyte population. CD14+, CD19+ and/or dead cells were excluded. CD4+Live cells were then gated on CD154 (left column). CD154+ cells were then evaluated for CD137 and CD69 double positivity (right column). Unpublished

Next Steps & Future Directions:

In summary, we have tested 5 methods for CD4 epitope identification (i.e. cultured ELISPOT, TGEM, Triple activation marker expression, ICS and CFSE dilution; **Table 3**) and identified a total of 12 CD4 epitopes within the MCPyV T-antigen oncoproteins (**Table 2).** Of these, 7 epitopes determined via TGEM, ICS and CFSE dilution have been validated as truly antigen-specific and 1 was confirmed by another group[131]. Remaining work includes HLA-restriction determine, minimal epitope identification and tetramer synthesis when possible (limited by HLA allele type).

One final reagent that is worth mentioning is the attempted synthesis of whole purified LT and sT proteins which would enable evaluation of the whole antigenic space within these T-antigens while simultaneously evaluating the ability of these epitopes to be exogenously processed and presented. These reagents could also be used to validate epitopes identified using peptides pools by testing peptide-reactive cells for reactivity against the whole protein as a non-cyclical validation of specificity. Dr. Koelle and I have been trying to generate these reagents in collaboration first with LakePharma and now with Genscript. Our initial attempts with LakePharma yielded a very small amount of Large T and no sT. The lack of sT production could be due to a transmembrane domain (TWGECFCYQCFILWFGFPPTWESF; sT-142-166). Initial production was done in HEK293 cells and there was discussion of continuing with LakePharma using a baculovirus system, however, we were unable to register them as a vendor through UW to initiate the baculovirus production. We therefore switched to Genscript and utilized CHO and 293 cells. They were also unable to generate any sT despite removal of the transmembrane domain within sT (at the nucleotide sequence level). A small amount of LT was produced which was unable to stimulated a 'WED'-specific clone suggesting that this product will be insufficient for our desired needs. Ongoing efforts should be made to improve the quality and yield of this reagent for future assays. The generation of such a reagent could be used to identify T cell responses to the entire MCPyV T-antigen-space and could be used to assess antigen-specificity in a non-cyclical argument, meaning that cells sorted based on peptide stimulation could be retested for specificity via whole protein or vice versa.

Additional patients should be tested using any of the methods described above to further probe the antigenic space of the MCPyV T-antigens. Because the majority of our work to date has used conditions that strongly favor the discovery of CD8 epitopes (i.e. use of IL-15 in culture or 13mer peptides), I am hopeful that many more CD4 epitope remain to be identified. Notably, several of the methods outlined in this chapter have led to the identification of an epitope "WEDLFCDESLSSPEPPSSSE" that can be presented by 3 population prevalent HLA class-II allele types that encompasses a critical oncogenic site within the MCPyV LT, indicating that this epitope is an ideal target for immune-based therapies in the majority of MCC patients. Data describing these exciting findings are the focus of **Chapter 7**.

TABLE 3: Methods for CD4 epitope identification and their advantages and disadvantages

Method	Advantage	Disadvantage
ELISPOT/ELISA	• Does not require purified antigen • Might detect low-avidity interactions • Technically simple	• Does not identify HLA restriction • Requires T cells to be functional • Does not permit sorting, does not identify CD8 vs CD4 restriction. • For rare cells may require expansion • Limited to one cytokine so miss cells secreting other cytokines
Tetramer guided epitope mapping (TGEM)	• HLA restriction and tetramer generation incorporated into epitope discovery • Does not require cell functionality • Low background/low ambiguity of response	• Limited to persons of appropriate HLA allele type • Expensive • Technically challenging
Triple Activation Marker	• Is not limited to specific HLA alleles • Is not limited to one particular cytokine • Can use whole antigen to probe large antigenic space	• Does not identify HLA restriction • Does require T cells to be functional • Background staining relatively high
Intracellular cytokine secretion (ICS)/IFNg capture	• Is not limited to specific HLA alleles • Can use whole antigen to probe large antigenic space • Could be used to detect multiple cytokines	• ICS requires permeabilization so limits down-stream analyses, IFNg capture avoids this limitation. • Does not identify HLA restriction • Requires T cells to be functional
CFSE dilution	• Is not limited to specific HLA alleles • Is not limited to one particular cytokine • Can use whole antigen to probe large antigenic space	• Does not identify HLA restriction • Does require T cells to be functional • Background staining relatively high • Requires 5 day expansion

CHAPTER 7: A POPULATION PREVALENT AND PROMISCUOUS MERKEL CELL POLYOMAVIRUS CD4 EPITOPE; AN IDEAL THERAPEUTIC TARGET FOR MERKEL CELL CARCINOMA?

Vandeven N, Yang J, Liang K, James E, Campbell V, Crispin D, McIntosh M, Kwok W, Koelle D, Nghiem P. [Manuscript in preparation]

Chapter Summary:

Merkel cell carcinoma (MCC) is a deadly, virus-associated skin cancer with a 5-year mortality rate of 46%. The Merkel cell polyomavirus (MCPyV) is clonally integrated into 80% of MCCs and persistent expression of MCPyV T-antigen oncoproteins is required for tumor survival and growth, providing ideal targets for the immune system. This notion is strongly supported by the finding that robust intratumoral CD8 infiltration is associated with 100% survival among MCC patients. Additionally, strikingly high response rates to agents blocking the PD-1 pathway have been reported in patients with advanced MCC. However, still roughly half of patients do not benefit from these modalities, indicating an urgent need to identify biomarkers predictive of response and immune evasion mechanisms that underlie PD-1 blockade resistance.

Because of the link between CD8 T cell infiltration and MCC survival, we hypothesize that CD4 help is necessary to enhance the CD8 response. Lack of CD4 help has been proposed as a immune evasion mechanism that limits anti-tumor effects of CD8 T cells. The study and use of tumor-specific CD4s has been hindered by the ability to isolate and characterize these cells. Here we identified a novel MCPyV CD4 epitope, WEDLFCDESLSSPEPPSSSE ('WED') which can be presented by at least three population prevalent HLA class-II alleles and lies within the Large T-antigen (LT-209-228) encompassing the LxCxE motif. The LxCxE motif is required for T-antigen binding to the tumor suppressor retinoblastoma protein (Rb), one of the main oncogenic mechanisms in MCC. Consequently, expression of the 'WED' epitope is required for tumor growth and persistence, and sequence analysis indicates that this region is highly conserved among MCC tumors. 'WED'-specific CD4 T cell clonotypes can be expanded from healthy donors and MCC patients and secrete IFNγ in response to stimulation with 'WED' peptide, LT protein and MCC tumor cell lines. Furthermore, we show that 'WED'-specific CD4 T cells are capable of homing to and infiltrating MCC tumors and exhibit strikingly diverse T cell receptor repertoires. Taken together, we have identified a highly conserved, population-prevalent CD4 epitope and generated HLA class-II

tetramers, enabling for the first-time detailed study of the MCPyV-specific CD4 T cell response in a ~80% percentage of MCC patients. Importantly, these data provide a robust foundation to develop CD4-based cellular therapies and/or a therapeutic cancer vaccine against MCPyV, both of which could be used to rescue patients who do not respond to PD-1 blockade.

INTRODUCTION:

Merkel cell carcinoma (MCC) is a rare but deadly skin cancer, with a relative mortality rate of 46% making it approximately three times as deadly as malignant melanoma on a per case basis[221]. With an incidence of ~2,500 new cases per year in the US, this incidence is projected to climb to ~3,500 by 2025 (**Chapter 2**). In 2008, Chang and Moore discovered that ~80% of MCC are etiologically linked to the Merkel cell polyomavirus (MCPyV)[57]. Like other human polyomaviruses, MCPyV is a small (~5kb), dsDNA virus that encodes several T-antigens including large T (LT) and small T (sT). Importantly, truncation within the C-terminal region of LT is critical for oncogenesis, while retention of the N-terminal region promotes cell cycle progression, predominantly mediated through the highly conserved LxCxE motif. This motif binds the retinoblastoma protein (Rb) and dysregulates E2F-facilitated transcription thereby driving cellular proliferation[103,355]. This Rb-binding motif is highly conserved among all human polyomaviruses as well as other dsDNA viruses including Herpeseviridae, Papillomaviridae, Adenoviridae suggesting that induction of cellular proliferation via this mechanism is critical for viral propagation more broadly[356-359]. Notably, knockdown of MCPyV T-antigens results in cell cycle arrest and death in MCPyV-positive MCC cell lines and persistent expression of T-antigens is observed in MCC tumors indicating that these viral oncoproteins may be ideal targets for immune therapy[58,111].

Indeed, the importance of immune cell function in MCC is highlighted by the fact that MCC patients with robust CD8 T cell intratumoral infiltration have 100% disease-specific survival. However, this robust infiltration profile is only observed in 4-18% of patients[91,92] and MCPyV-specific CD8 T cells express elevated levels of exhaustion markers PD-1 and Tim-3 relative to other virus-specific T cells[134]. Importantly, recent clinical trials utilizing agents targeting PD-1 axis blockade have yielded remarkable responses rates of 50% in the first line[65,66] indicating the potential of immune based therapies in treating this cancer. However, roughly half of patients do not respond and no biomarkers have been identified to date that can predict response.

CD4 T cell inclusion within immune-based therapies such as CAR T cells[317] and the targeting of CD4 T-cells by therapeutic cancer vaccines[249,250] have yielded significantly higher response rates than when

targeting CD8 T cells alone. These findings suggest that CD4 T cells can significantly improve cancer immune-based therapies and therefore their role and importance should be further investigated in the context of response to PD-1 checkpoint blockade therapies. Consequently, using tetramer-guided epitope mapping (TGEM), we sought to simultaneously identify MCPyV-specific CD4 epitopes, delineate their HLA-restriction and develop HLA class-II tetramers with the goal of enabling characterization of the CD4 T cell response against MCPyV. We have identified a novel CD4 epitope (WEDLFCDESLSSPEPPSSSE; 'WED') encompassing the critical LxCxE motif of MCPyV. Consequently, this epitope is persistently expressed, highly conserved among MCC tumors, and resistant to immunoediting, potentially providing an ideal target for immune therapies including a therapeutic cancer vaccine which could be used to modulate responses to PD-1 blockade.

METHODS:

Human subject samples and cell lines

This study was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board IRB #6585 and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Subjects were HLA class-II typed via polymerase chain reaction (PCR) at Bloodworks Northwest (Seattle, WA). All samples were clinically annotated with long-term patient follow-up data.

Peripheral blood mononuclear cells (PBMC)

Heparinized blood was obtained from MCC patients and healthy donors, and PBMC were cryopreserved after routine Ficoll preparation at a dedicated specimen processing facility at FHCRC or the Benaroya Research Institute (BRI).

Patient Tumors

When available, fresh MCC tumor material from core and/or punch biopsy samples were processed and tumor infiltrating lymphocytes (TIL) cultured for 2 weeks before analysis as described[130]. For excised

tumors of larger volume (>1 cm^3), the remaining tissue was digested as described[134], and single-cell suspensions were cryopreserved.

Patient derived EBV-transformed lymphocyte cell lines (LCL)

LCL lines were derived from MCC patient's PBMC and maintained in LCL medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 1% penicillin-streptomycin, $2x10^{-5}$ M 2-mercaptoethanol, 1 mM pyruvate) as described[360].

EPITOPE DETERMINATION

Tetramer generation

The generation of HLA-DR tetramers was performed as previously described[337] in the BRI Tetramer Core Facility. Briefly, tetramer panels composed of either HLA-DRB1*0401 or HLA-DRB1*0301 were loaded with one of 10 pools, each containing 5 different 20mer peptides. These 20mer peptides encompassed the small T and truncated Large T-antigens with a 12 amino acid (a.a.) overlap between adjacent peptides (Genscript; **Supplementary Table 1**). Biotinylated HLA-DRB1*0401 monomers were incubated with the 10 peptide pools for 48 h at 37°C. Subsequently, tetramers were formed by incubating class-II molecules with PE-labeled streptavidin (BioSource). Peptide restriction within a responsive pool was determined by generating HLA-DRB1*0401 or HLA-DRB1*0301 monomers incubated with individual peptides and subsequently streptavidin to create single-peptide tetramers.

Tetramer guided epitope mapping (TGEM)

The use of TGEM for identification of novel CD4 epitopes has been previously described[361,362]. Briefly, PBMC from HLA-DRB1*0401-positive healthy donors were stimulated with 10 ug/ml of peptide and cultured for 7 days at 37°C in the presence of rIL-2 in T-cell medium (TCM) containing RPMI, 8% human serum, 200 nM, L-glutamine and 100 U/mL penicillin–streptomycin. Tetramer staining was carried out between days 14 and 20 of the culture. After the initial round of tetramer screening using 10 tetramer panels (loaded with 10 peptide pools), cells from tetramer-positive wells were subjected to a second

screening (fine typing) using tetramers loaded with individual peptides from within the corresponding peptide pool.

Intracellular cytokine secretion assay (ICS)

Responder cells (MCC patient TIL or 'WED'-specific CD4 T cell clones) stimulated with antigen (1 ug/ml of peptides or 1:100 dilution of cellular lysates) in the presence of anti-CD28 and anti-CD49d monoclonal antibodies (BD Biosciences), and brefeldin A (Sigma-Aldrich) were incubated with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen)-labeled antigen-presenting cells (APC) for 12 to 18 hours. Control stimuli included a negative control, dimethyl sulfoxide (DMSO) and a positive control phytohaemagglutinin (PHA). Cells were subsequently stained with LIVE/DEAD Violet (Invitrogen), followed by monoclonal antibody staining using CD4-A700 (clone OKT4; eBioscience). Cells were then permeabilized and stained with anti-IFNγ-PE (BD Biosciences). Data acquired on a FACS RUO cytometer (Becton Dickinson) using BD-FACS Diva software (v6.1.1) were analyzed with FlowJo (10.0.8r1). Data are reported as percentage of viable cells of phenotypic interest identified as CFSE-negative, CD4+ responder cells in the lymphocyte forward/side scatter region.

HLA-DQ-restriction determination

LCLs derived from patients with partially matched HLA-DQB1 alleles were used as APCs and incubated with 'WED'-specific clones and 'WED' peptides. IFNγ secretion was evaluated using an ICS assay following the protocol previously described.

CREATION OF ''WED''-SPECIFIC T-CELL CLONES*.*

IFNγ capture

Four million peripheral blood mononuclear cells (PBMC) from an MCC patient were plated into a 24-well plate and pulsed with a high concentration of 'WEDLFCDESLSSPEPPSSSE' 20mer peptide ('WED'; LT-209-228; 20 ug/ml) for 1 hour at 37°C in 5% CO_2. After 1 hour, PBMC were diluted with 2 ml of T cell media to a final peptide concentration 1 ug/ml. rIL-7 was added (20 ng/ml; R&D) and cells were incubated overnight. Cells were expanded for two weeks in the presence of rIL-2 (20 U/ml; R&D). Media was

removed every other day and replaced with fresh media containing rIL-7 and rIL-2. Following 'WED'-specific T cell expansion, 'WED'-specific T cells were detected using an IFNγ secretion assay (Miltenyi Biotech), per manufacturers' instructions. Briefly, expanded cells were re-stimulated overnight with 1ug/ml of 'WED'-peptide, DMSO negative control or PHA positive control. Cells were washed and resuspended in 80 ul of RPMI per 10^7 cells. IFNγ catch reagent (20 ul) was added to each condition, mixed and incubated for 5 min on ice. Warm medium was added to dilute cells to a volume of 10 mls and then cells were incubated for an additional 45 mins at 37°C. Unbound catch reagent was washed away and 20 ul of IFNγ detection antibody (PE) and 1 ul of CD4-A700 (clone OKT4; eBioscience) were added into a total volume of 100 ul and samples were incubated on ice for 25 mins. Following another wash, 20 ul of anti-PE-microbeads were added into a total volume of 100 ul and incubated on ice for 15 mins. Excess beads were washed off and cell pellets were resuspended in 500 ul buffer (phosphate-buffered saline, pH 7.2, 0.5% Bovine serum albumin and 2 mM EDTA) and added to a magnetic column (MS column; Miltenyi Biotech). After rinsing the column three times with 500 ul of cold buffer, columns are removed from magnet source and cells are eluted with 1 ml of cold buffer. Following a wash with cold buffer, 50 ul of a 1:1000 dilution of LIVE/DEAD Violet (Invitrogen) stain was added and cells were incubated on ice for 25 minutes. Two washes were performed and IFNγ+ CD4+ responder cells in the lymphocyte forward/side scatter region were sorted on a BD Aria III cell sorter.

Tetramer sorting

PBMC or TIL (5-20 million) from 3 MCC patients and 2 healthy donors were thawed and washed with PBS. Cells were incubated with 100 nM dasatinib (SelleckChem) for 10min at 37C. Cells were washed and resuspended in 50 ul of RPMI, with 2 ul DRB1*0401-'WED'-PE tetramer and incubated for 1hr in the dark at RT. Cells were washed with 3 ml cold buffer (1% BSA in PBS) and pelleted. Cells were resuspended in 500 ul buffer and enriched for PE-positive cells using the StemCell Technologies Easy Sep anti-PE kit. Following enrichment, samples were resuspended in 95 ul of Fc block (StemCell Technologies EasySep anti-PE kit) and stained with CD4-488 (Biolegend), CD8-APC (Invitrogen), CD14-PacBlue (Biolegend), CD19-PacBlue (Biolegend) and 50 ul of 1:2000 diluted aqua LIVE/DEAD Violet (Invitrogen). Following a 25 minutes incubation on ice, cells were washed 2x with plain RPMI and

resuspended in 150 ul of 2% human serum in RPMI for single cell sorting on a BD Aria III cell sorter. Cells of interest were viable and identified as CD4+ tetramer+ responder cells in the lymphocyte forward/side scatter region which were sorted directly into 96-well plates containing 100 ul TCM.

Single cell clone generation

Following IFNγ capture or tetramer sorting of single cells into 96-well plates, allogeneic irradiated feeders (150,000 cells per well), and PHA (Remel) were added as described[363]. After 24 hours, rIL-15 (20 ng/mL; R&D Systems) and natural IL-2 (Hemagen Diagnostics) were added. After 2 weeks, microcultures with visible growth were screened for specificity via IFNγ ELISA or tetramer staining as described under "T-cell functional assays". Confirmed positive wells were further expanded in the presence of irradiated feeder cells, rIL-2 (50 IU/ml; R&D Systems), and OKT3 (30ng/ml; Miltenyi Biotec) as described[130] plus rIL15 (20 ng/mL).

T-CELL FUNCTIONAL ASSAYS

T-cell clones generated by HLA-DRB1*0401-'WED' tetramer sorting, were further tested for minimal epitope determination, specificity and exogenous processing determination via cytokine release assays.

Minimal epitope determination and exogenous processing

To determine the minimal epitope recognized by 'WED'-specific T cell clones 10, 11mer peptides overlapping by 10 a.a. that spanned 'WED' were synthesized (Genscript). An ICS assay was performed as described above to identify responsive 11mers. In order to determine whether 'WED'-specific CD4 T cell clones could respond to LT presented by MCC cell lines, cell lysates of two MCPyV-positive cell lines WaGa, MKL-1 (gift of Dr. Juergen Becker, German Cancer Research Center, 2015; authenticated by Becker lab via short-tandem repeat analysis in 2014 as described by Reid et al.[364]) and one MCPyV-negative MCC cell line (MCC-13) were generated through three cycles of freeze-thaw using dry ice and a water bath heated at 37℃. Lysed cells were centrifuged and supernatants harvested. Supernatants were incubated with autologous PBMC and 'WED'-specific CD4 T cell clones overnight and an ICS assay was used to evaluate IFNγ secretion.

EC_{50} determination via ELISA

Secreted IFNγ was measured after co-incubating 'WED'-specific T cell clones with a plus antigenic peptide ('WED'-WT; 'WED'-S220phos; 'WED'-S220A) at 10-fold dilutions to final concentrations of 10^{-6} to 10^{-14} g/ml in 200 mL TCM for 16 hours. IFNγ in cell culture supernatants was assayed by ELISA according to the manufacturer's recommendations (Human IFNγ ELISA Ready-SET-Go Kit; Affymetrix). To calculate EC_{50} (the amount of peptide leading to 50% of maximum IFNγ secretion), IFNγ secretion by each T-cell clone was analyzed via nonlinear regression using Prism version 7.0 (Graph-Pad).

Large T-Ag production and site directed mutagenesis

Large T-Ag (LTAg) fusion protein (pDEST103-GFP-LTAg) was created using Gateway recombination cloning technology (ThermoFisher Scientific) to insert LTAg from pCMVMCV156[137] into pDEST103-GFP[365]. Site directed mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit to generate S220A and E216K mutants with the following primers; S220A-Forward 5'-GCGATGAATCACTTTCCGCTCCTGAGCCTC-3'; S220A-Reverse 5'-AGAAGAGATCCTCCCAGGTGCC-3'; E216K-Forward 5'-CGACAAGTCACTTCCTCCCCTGAG-3'; E216K-Reverse 5'-CAGAACAGATCCTCCCAGGTGCCATC-3'. Mutagenesis was sequence-confirmed using T7 and BGHR primers (Genewiz). COS-7 cells (ATCC, CRL-1651, 2005) were plated at 75,000 cells/0.5ml/well in 12-well plates in DMEM + 10% FBS, 200 nmol/L L-glutamine and 100U/mL penicillin–streptomycin. Twenty-four hours later, wells were transfected using FuGENE HD (Promega) at a 6:1 ratio of transfection reagent to DNA with 1ug of either pDEST103-GFP-tLTAg, pDEST103-GFP-tLTAg-S220A, pDEST103-GFP-tLTAg-E216K or no plasmid. Seventy-two hours after transfection, transfection efficiency was measured via GFP expression using flow cytometry (data not shown) and GFP-positive cells were sorted. Lysates of GFP-positive, sorted cells were generated through three cycles of freeze-thaw using dry ice and a water bath heated at 37℃. Lysed cells were centrifuged and supernatants harvested and added to target wells in duplicate. Lysates, autologous PBMC and 'WED'-specific clones were incubated overnight and IFNγ was evaluated using ICS as previously described.

T-cell receptor beta sequencing and analysis

Dextramer sorting 'WED'-specific cells

At least 3 million cells from fresh tumor digest were stained with a HLA-DRB1*0401/'WED'-PE dextramer (Immundex), and monoclonal antibodies against CD4-A488 (Biolegend), CD8 APC (Life Technologies), Pacific Blue-CD14 (Biolegend), Pacific Blue-CD19 (Biolegend) and LIVE/DEAD Violet (Invitrogen). Live, dextramer-positive, CD4+, CD14-, CD19-, CD8- cells were sorted via FACS Aria III (BD) and flash frozen. Samples were submitted to Adaptive Biotechnologies for genomic DNA extraction, TRBV sequencing, and normalization. All TRVB sequences detected in 2 cells (estimated number of genomes 2) were categorized as dextramer-positive clonotypes.

T-cell receptor clonality

For dextramer-sorted cells, Shannon entropy was calculated on the estimated number of genomes(≥2) of all productive TRB and normalized by dividing by the $\log_2$ of unique productive sequences in each sample. Clonality was calculated as 1 – normalized entropy. For whole tumors, clonality was calculated in the same method, using all TRB sequences in the sample to calculate normalized entropy.

Statistical Analysis

Analyses were completed Prism software, version 6 with a statistical significance threshold of 5%.

RESULTS:

TGEM identifies CD4 T cells recognizing a unique epitope presented by both HLA-DRB1*0401 and HLA-DRB1*0301

Tetramer-guided epitope mapping (TGEM) developed by Dr. William Kwok[337], is a tetramer-based epitope identification platform that can simultaneously identify MCPyV-specific CD4 T cell epitopes and determine the HLA class-II restriction (**Figure 1A**). Peripheral mononuclear blood cells (PBMC) were obtained from 4 HLA-DRB1*0401 positive healthy donors. Cells were plated in 48-well plates and stimulated with 10 pools containing 5 different, 20mer peptides overlapping by 12 a.a. spanning across MCPyV LT and sT (**Suppl Table 1; Figure 1E**). After 2 weeks of culture, cells were stained with pooled

peptide HLA-DRB1*0401 tetramers and screened for tetramer binding (**Figure 1B**). All of the tested HLA-DRB1*0401 donors demonstrated tetramer binding with pool 6 peptides (peptides 26-30, spanning LT-201-252). Cells from positive wells were then screened with 5 individual tetramers each loaded with one of the 5 peptides from pool 6. Binding of CD4 T cells was observed with HLA-DRB1*0401 tetramers loaded with peptide 27 with the sequence WEDLFCDESLSSPEPPSSSE ('WED'; spanning LT- 209-228) from all four donors (**Figure 1C**). The 'WED' epitope was also predicted to bind to HLA-DRB1*0301 with intermediate affinity (IC_{50} = 235.8 nM) according to the neural network-based alignment algorithm for MHC class-II peptide binding prediction[366]. Therefore, HLA-DRB1*0301 tetramers loaded with 'WED' were generated and donor PBMC from 4 HLA-DRB1*0301 positive healthy donors was stained following two weeks of culture with 'WED' peptide. HLA-DRB1*0301-'WED' tetramer positivity was observed in 3 of 4

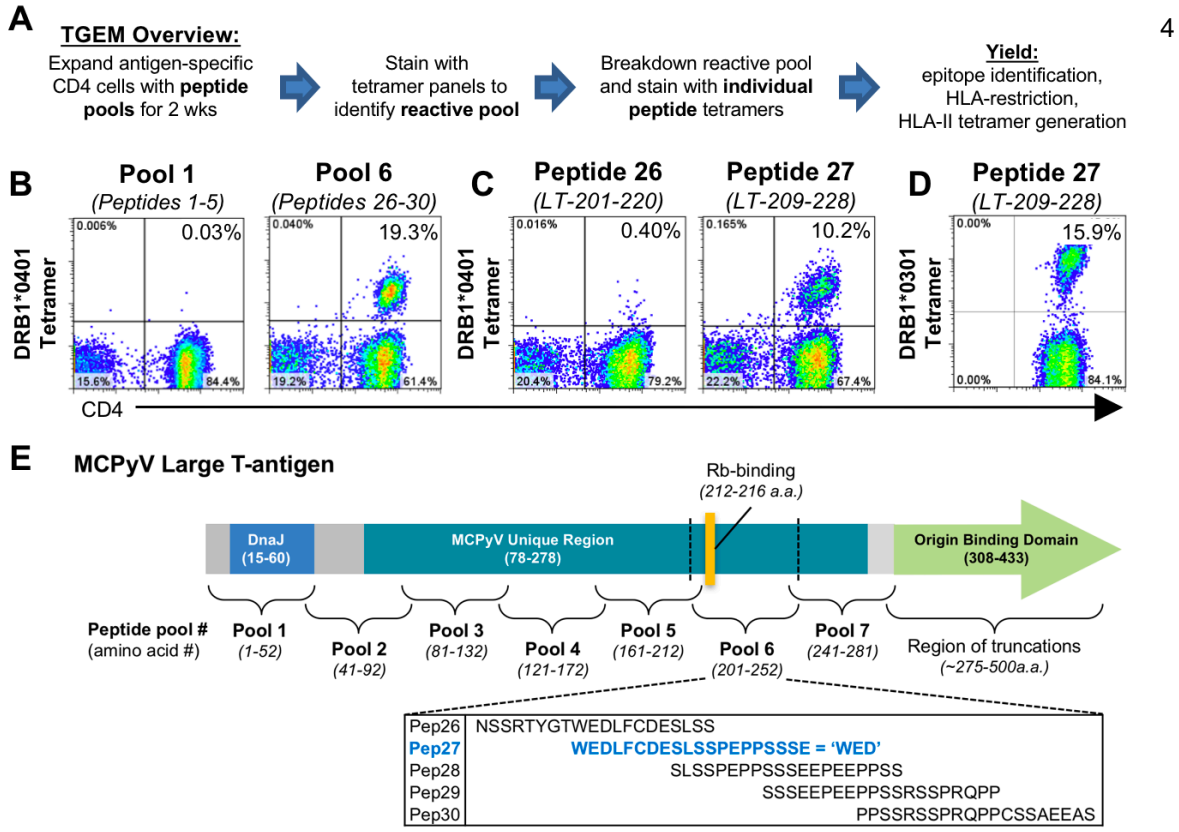


Figure 1: CD4 T cells can recognize an epitope within MCPyV LT that can be presented by both HLA-DRB1*0401 and HLA-DRB1*0301. A: Schematic of TGEM workflow. B: Peripheral blood mononuclear cells (PBMC) from a healthy donor was cultured for 2 weeks with peptide pools containing 5 peptides. HLA-DRB1*0401 tetramers loaded with peptides pools were generated to stain cultured cells. C: Cells from pool 6 were stained with tetramers loaded with individual peptides 26-30 from pool 6 (LT-201-252). D: PBMC from 4 healthy donors were cultured for two weeks with 'WED' and stained with HLA-DRB1*0301-'WED' tetramer bound CD4 T cells from 3/4 healthy donors (representative plot shown). E: Schematic of the MCPyV Large T-antigen and Rb binding site. Peptide pools are denoted with amino acid location in parentheticals. Pools 8, 9 and 10 encompass MCPyV sT and are not pictured here. Pool 6 peptide sequences depicted with 'WED' peptide highlighted in blue.

tested healthy donor PBMC (**Figure 1D).** These results suggest that 'WED' is a CD4 epitope that can be promiscuously presented by both HLA-DRB1*0401 and HLA-DRB1*0301 alleles.

'WED' can be presented in the context of a third HLA class-II allele

Interestingly, we first observed reactivity against the 'WED' epitope using an intracellular cytokine secretion assay (ICS)[338]. ICS assays evaluating IFNγ secretion from MCC tumor infiltrating lymphocytes (TIL) in response to MCPyV peptide stimulation are routinely used within our group to identify CD8 T cell epitopes as previously described[130,338]. Because of this, we used shorter peptides (13mers) instead of 20mers that optimize detection of CD8 responses. Despite this, TIL from one MCC patient showed CD4 reactivity to a pool of 13mer peptides spanning LT from residues 173-281 (**Figure 2A**). Upon pool breakdown, CD4 T cell reactivity was found to be restricted to "WEDLFCDESLSSP" (**Figure 2A)** encompassing a portion of the 'WED' epitope that was identified using TGEM. Importantly, this patient does not express either HLA-DRB1*0401 or HLA-DRB1*0301 and therefore, this response had to be restricted to yet a third HLA class-II allele type. To determine the HLA class-II restriction, PBMC were expanded in the presence of 'WED' peptide for two weeks and sorted using an IFNγ capture assay to generate single cell clones[367]. These 'WED'-specific T cell clones were then re-stimulated with autologous PBMC and 'WED' peptide in the presence of HLA-DR, HLA-DQ or HLA-DP monoclonal blocking antibodies[368] (**Figure 2B**). HLA-DQ blocking abolished the robust IFNγ response from stimulated 'WED'-specific clones, while HLA-DR and DP blocking antibodies had no effect on IFNγ secretion, indicating an HLA-DQ allele restricted presentation. The patient in which this response was identified has the HLA-DQB1*0301/HLA-DQB1*0501 genotype. Therefore, to determine the specific HLA-DQ allele, immortalized lymphoblastoid cell lines (LCLs) from 3 different patients with or without HLA-DQB1*0301 and HLA-DQB1*0501 (as depicted in **Figure 2C**) were used as APCs. 'WED'-specific clones were incubated with these LCL lines in the presence of 'WED' peptide with and without HLA-DQ blocking antibody. APCs derived from patients w683 and w420 who only share the HLA-DQB1*0301 allele, both reproduced strong IFNγ signaling that was lost upon incubation with the HLA-DQ blocking antibody. Patient w678 who is negative for both HLA-DQB1*0301 and HLA-DQB1*0501 did not invoke a response even without HLA-DQ blocking. Collectively, these results indicate that the 'WED' epitope can also be

presented in the context of HLA-DQB1*0301 in addition to HLA-DRB1*0301 and HLA-DRB1*0401. Current methods to produce HLA class-II monomers are not capable of generating HLA-DQB1*0301 tetramers. Therefore, the remaining experiments focus upon the HLA-DRB1*0401-restricted response, while additional assays for HLA-DQB1*0301- and HLA-DRB1*0301-restricted responses are ongoing.

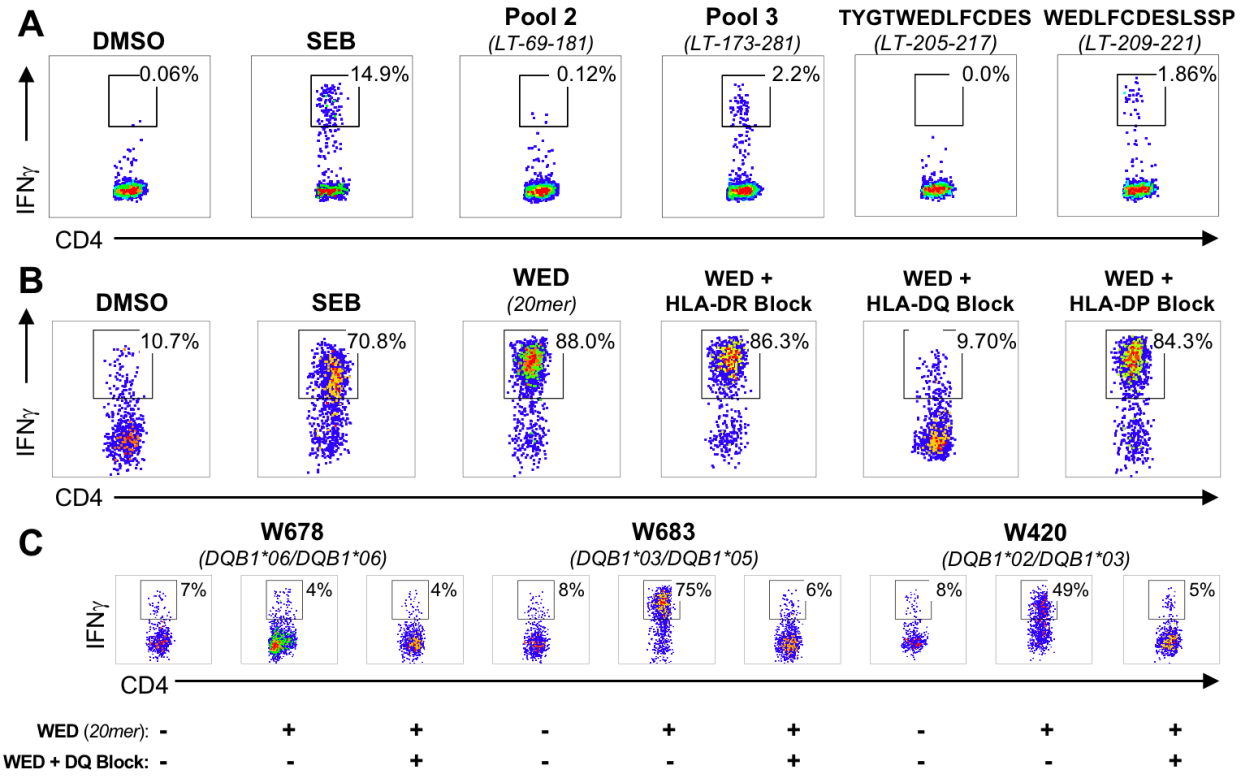


Figure 2: 'WED' epitope is also presented by HLA-DQB1*0301. Panel **A** depicts the initial ICS screening of MCC patient TIL incubated with MCPyV T-antigen 13mer peptide pools. Pool 3 was broken down into individual 13mer peptides. Panel **B** shows 'WED'-specific clones plus or minus HLA-DR, HLA-DQ and HLA-DP monoclonal blocking antibodies to determine the HLA allele family restriction. In Panel **C,** immortalized B cell lines (LCLs) from three patients (w678, w683 and w420) with discordant HLA-DQ alleles were used to determine HLA-DQ restriction.

CD4 T cells respond functionally to 'WED' and the minimal epitope encompasses the LxCxE motif

Next, we sought to verify that cells capable of binding 'WED'-DRB1*0401 tetramer, could functionally respond to stimulation with this peptide. To test this, DRB1*0401-'WED'-tetramer-positive CD4 T cells were sorted and single cell clones were generated as previously described[137] (clonotype TCR sequences listed in **Supplemental Table 3**). 'WED'-specific clones were then incubated overnight with HLA-DRB1*0401-positive PBMCs and IFNγ secretion was evaluated in response to a negative control (DMSO), positive control (PHA), an irrelevant peptide (an HLA-DRB1*0401 restricted influenza epitope)

or the 'WED' 20mer. MCPyV-restricted clones secreted IFNγ in response to PHA and 'WED' but not to DMSO or the irrelevant peptide, indicating that these clones not only bind tetramer but functionally respond via cytokine secretion to this specific epitope (**Figure 3A**).

CD4 T cell epitopes can range in size from ~9-22 amino acids in length[369], therefore we sought to determine the core sequence necessary for T cell recognition within the 'WED' 20mer epitope. 11-mer peptides overlapping by 10 a.a. spanning across the 'WED' 20mer were generated **(Supplementary Table 2)** and 'WED'-specific CD4 clones were then stimulated overnight with 11mer peptides and HLA-DRB1*0401 LCLs. Functional reactivity was assessed by IFNγ secretion (**Figure 3B & 3C**). 'WED'-specific clones responded to 11mers 2 to 4 (spanning LT-210-222) which share 9 a.a. residues LFCDESLSS (LT-212-220) (**Figure 3B**). Stimulation with this 9mer sequence also elicited IFNγ secretion, though to a markedly lower degree (**Figure 3B**). This suggests that while this 9mer sequence may be the core sequence required for recognition by these MCPyV-responsive CD4 T cell clones, adjacent flanking residues may stabilize and improve strength of TCR stimulation and/or HLA binding. Importantly, this epitope encompasses the LxCxE binding motif that is critical for MCPyV-LT

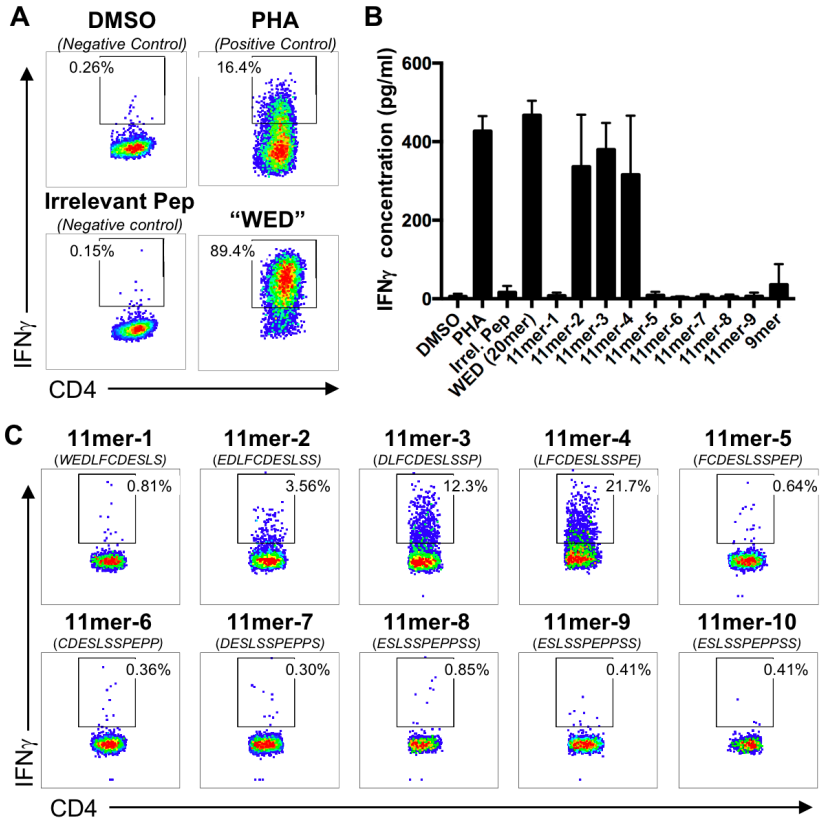


Figure 3: Functional response and minimal epitope determination of 'WED'-specific CD4 T cells. A: Representative flow plots from 1 of 5 tested individuals. 'WED'-specific CD4 T cells were incubated with HLA-DRB1*0401 LCLs and stimulated with mock (DMSO vehicle control), positive control (PHA), irrelevant peptide (an influenza epitope) or 'WED'. **B**: Minimal epitope validation was performed using 3 clones from 3 individuals. IFNγ secretion in response to 11mer peptides was evaluated using an IFNγ ELISA. **C**: A 'WED'-specific clone was incubated with 11mers (1-10) overlapping by 10 a.a. spanning the 'WED' 20mer sequence and IFNγ secretion was evaluated using an ICS assay.

binding to Rb. Therefore, persistent expression of this region is required for MCC tumor development and growth[103] and highly conserved in MCCs. Indeed, of the 99 MCPyV truncated Large-T-antigen sequences deposited in GenBank, only 1 has a single nucleotide polymorphism within LT-212-220 (S219F; Accession number KJ128376.1) suggesting that this epitope is an ideal, highly conserved target for immune recognition against MCPyV in the context of MCC.

CD4 T cells can recognize 'WED' within the context of MCC tumors

It has recently been described that phosphorylation of serine residue 220 within MCPyV LT is required for binding to Rb[3] (**Figure 4A**). Consequently, this residue must be phosphorylated within MCC tumors to mediate oncogenic function, although not formally demonstrated. Because S220 falls within the 'WED' minimal epitope (LT-212-220), it is critical to evaluate whether phosphorylation of this residue disrupts antigenicity of this region. To test this, 'WED'-specific T cell clones were stimulated with peptide dilutions (1×10^{-5} – 1×10^{-14} g/ml) of either wild type 'WED' or a 'WED' peptide that was phosphorylated at S220

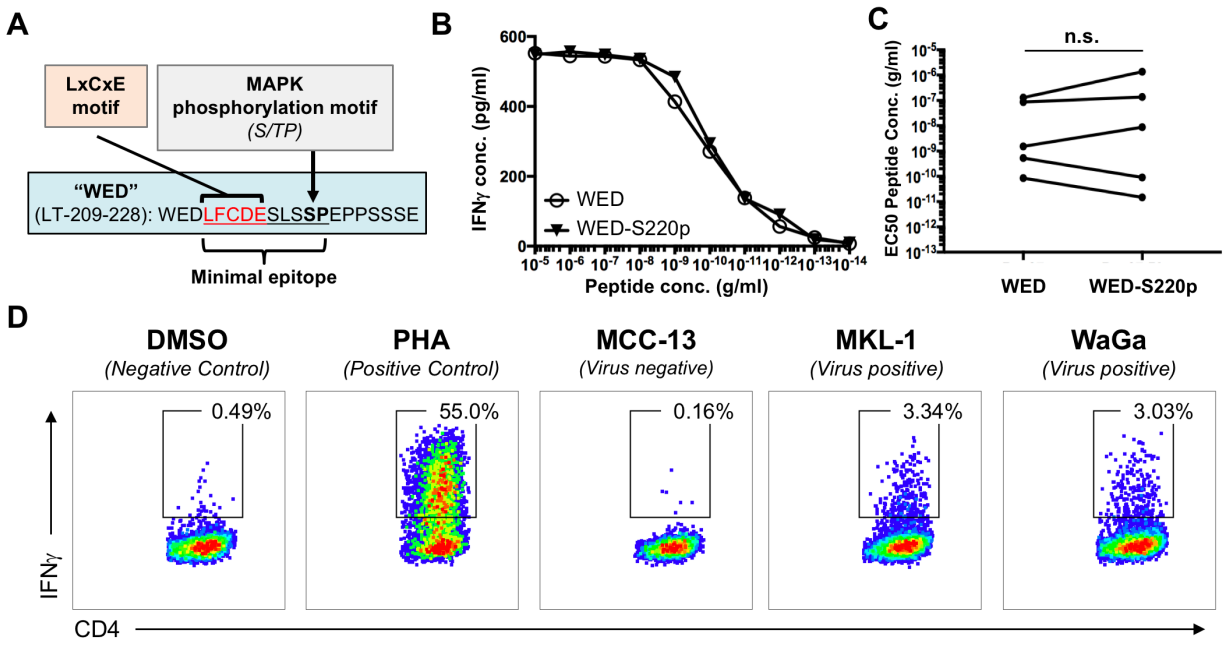
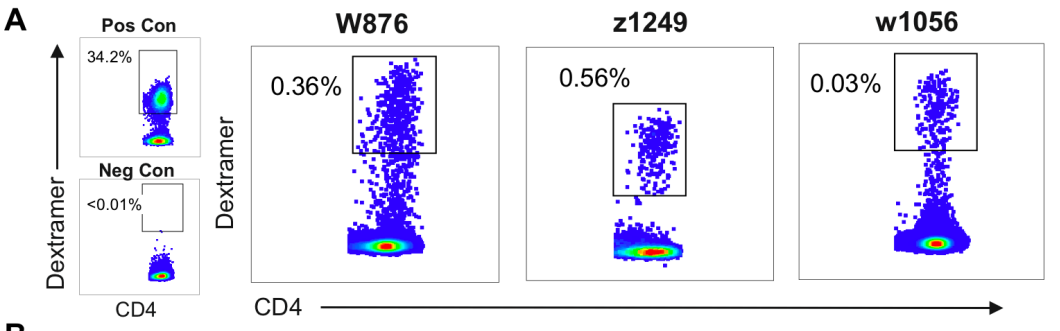


Figure 4: 'WED'-specific CD4 T cell clones can recognize LT as presented in the context of MCC tumors. A: Schematic of 'WED' peptide features[3]. **B**: A representative dose response curve is depicted of a 'WED'-specific clone stimulated with 'WED' or 'WED'-S220p peptides. IFNγ secretion was measured by ELISA. **C**: Dose response curves were used to calculate the EC_{50} of 5 distinct 'WED'-specific CD4 T cell clones when stimulated with either 'WED' or 'WED'-S220P peptides. **D**: 'WED'-specific clones were incubated with DRB1*0401 positive PBMC and either MCPyV-negative cell line (MCC-13) or two MCPyV+ cell lines (MKL-1 and WaGa). Intracellular IFNγ was measured using an ICS assay. Flow plots are representative data of 4 distinct clonotypes.

(denoted as 'WED'-S220p; **Figure 4B**). EC_{50}'s were calculated for 5 clones that were generated from 5 different donors (3 MCC patients and 2 healthy controls; **Figure 4C**). Phosphorylation of S220 did not significantly impair 'WED'-specific CD4 T cell clones from functionally responding to this epitope suggesting that this region should be immunogenic within MCC tumors. To test this notion and to evaluate whether the 'WED' 20mer peptide can be naturally processed from LT protein by antigen processing machinery within professional antigen presenting cells (APC), we lysed two virus-positive MCC cell lines (MKL-1 and WaGa) expressing LT and one virus-negative MCC cell line (MCC-13) and incubated lysates with autologous PBMCs and 'WED'-specific clones. 'WED'-specific CD4 T cell clones responded via IFNγ secretion to the two virus-positive cell lines (MKL-1 and WaGa), but were unresponsive to the virus-negative cell line (MCC-13; **Figure 4D**). These data indicate that LT protein expressed in the context of virus-positive MCC cell lines can be exogenously processed and presented, strongly suggesting that 'WED' is likely to be presenting within the context of MCC tumors *in vivo*.

'WED'-specific CD4 T cells can infiltrate MCC tumors and have diverse TCR repertoires

We next wanted to determine whether 'WED'-specific cells could infiltrate MCC tumors. Importantly, several reports have indicated that the use of dextran-based HLA multimers increases the sensitivity of antigen-specific T cell detection, particularly in the case of HLA class-II reagents[370-372]. Therefore, using a HLA-DRB1*0401-'WED' dextramer, we stained tumor digests from three MCC patients. The percentage of 'WED'-specific cells of the total CD4 fraction ranged from 0.03-0.56% (**Figure 5A**). Notably, T cell receptors (TCRs) are composed of two chains, an alpha and a beta, and are necessarily diverse to enable protection against a wide array of pathogens[373]. Epitope-specific T cell populations can be highly clonal (low number of distinct TCR clonotypes) or highly diverse (many discrete TCR clonotypes). This TCR diversity is the result of imprecise rearrangements and insertions between the V and J segments of the TCR alpha chain and the V, D and J segments within the TCR beta chain, in particular the CDR3 region. Therefore, to determine the TCR diversity within 'WED'-specific intratumoral populations, we performed next generation sequencing of the T cell receptor beta locus (TRB) from dextramer-sorted cells isolated from these tumor digests as previously described[137]. Analysis of the complementary determining region 3 (CDR3) sequences indicated that 366 unique TRB sequences were identified across the three



	w876	z1249	w1056
# of cells sorted	529	380	195
# of unique clonotypes	118	126	122
# of shared clonotypes	2	0	2
Clonality of sample	0.111	0.16	0.137
Frequency of predominant clonotype	6.3%	12.5%	8.2%

Figure 5: 'WED'-specific CD4 T cells infiltrate MCC tumors and are highly diverse. **A:** Fresh tumor digest samples were acquired from three patients who are HLA appropriate and stained with DRB1*0401-'WED' dextramer. A cellular clone generated against the 'WED' epitope was spiked into PBMC as a positive control. Tumor infiltrating lymphocytes from an HLA inappropriate patient were used as a negative control. Cells that stained with exclusion markers were excluded and singlet lymphocytes that were CD3+CD4+dextramer+ cells were sorted. **B**: TRBV sequencing was performed and pie slices depict the frequency of an individual clonotype within the bulk sorted 'WED'-specific CD4 population. Exploded slices indicate two TRBV sequences that are shared between w876 and w1056. These clonotypes share identical CDR3 regions on the amino acid level but had disparate nucleic acid sequences.

tumor digests (**Figure 5B**). Of the 366, 2 clonotypes were shared between patients 'w876' and 'w1056' (indicated by the exploded pie slices; **Figure 5B**). These shared clonotypes were identical between patients at the amino acid level, but not at the nucleic acid level, suggesting that these clonotypes were truly shared TCRs and not contaminating sequences. In order to quantify the clonal diversity of each tumor's 'WED'-specific CD4 T cell population, clonality scores were calculated (see Materials and Methods for details). Clonality scores can range from 0-1 with 0 indicating a completely heterogeneous population with all unique TCR sequences while a score of 1 denotes a population composed of a single expanded clonotype. The clonality of each dextramer-sorted sample ranged from 0.111-0.137 indicating that the TRB repertoires of infiltrating T cells within these MCC tumors are extremely diverse.

S220A mutation within 'WED' may reduce oncogenicity while retaining immunogenicity

The data presented thus far suggests that the 'WED' epitope could be a therapeutically useful target either via generation of a cellular therapy targeting this epitope or via inclusion within a therapeutic cancer vaccine. Therapeutic cancer vaccines have been historically unsuccessful, however, recent success has been observed in the treatment of HPV-induced lesions[374] and melanoma[249,250]. Fascinatingly, many of these studies have indicated that CD4 T cells can preferentially respond to these effective vaccination strategies as compared to CD8 T cells[249,250,375], suggesting that CD4 stimulation is essential for these clinical responses. Importantly, there are many potential vaccine types that could be utilized including DNA or RNA-based vector vaccines, long peptides and protein-based vaccines. There is the potential risk of inducing cellular transformation when vaccinating with DNA/RNA or protein vaccines that encode oncoproteins such as the MCPyV T-antigens. This problem has been address in HPV-vaccines by mutating the LxCxE motif rendering the E7 oncoprotein non-oncogenic[376]. Notably, in this HPV study, they did not describe a prominent epitope that spanned the LxCxE motif within the HPV E7 oncoprotein and therefore, mutation of this region did not apparently alter immunogenicity. However, here we have reported a strongly immunogenic CD4 epitope that spans the oncogenic LxCxE motif. Therefore, we sought to test whether immunogenicity could be retained with two mutations within the 'WED' epitope that are reported to inhibit oncogenic activity *in vitro*. These include an E216K mutation directly within the LxCxE motif[103] and an S220A mutation which prevents phosphorylation required for Rb binding[3].

To test whether 'WED'-specific clones could respond to either of these two mutants, COS-7 cells were transfected with a pcDNA3 vector encoding a GFP-tagged truncated LT[137] with or without either the E216K or S220A mutant. GFP-positive cells were sorted, lysed and subsequently incubated with autologous PBMC and 'WED'-specific clones. 'WED'-specific clones responded to PHA stimulation (positive control), 'WED' and to lysates of COS-7 cells transfected with MCPyV-LT **(Figure 6A).** The S220A mutation did not impair stimulation of 'WED'-specific clones while the E216K mutation rendered this region non-immunogenic. This suggests that S220A mutation could provide a means to impair the oncogenic function of LT while maintaining its immunogenicity. To further test the strength of response of 'WED'-specific clones against 'WED'-S220A, clones were stimulated with 10-fold dilutions of 'WED' and

'WED'-S220A peptides to calculate EC_{50}'s. Surprisingly, stimulation with the mutant peptide S220A lowered the EC_{50} for all tested clones, indicating increased functional avidity in response to this mutated peptide (**Figure 6B & C)**. While this did not reach significance, this was highly unexpected and suggests that this specific mutation may be capable of ablating Rb binding while retaining and potentially even improving immunogenicity.

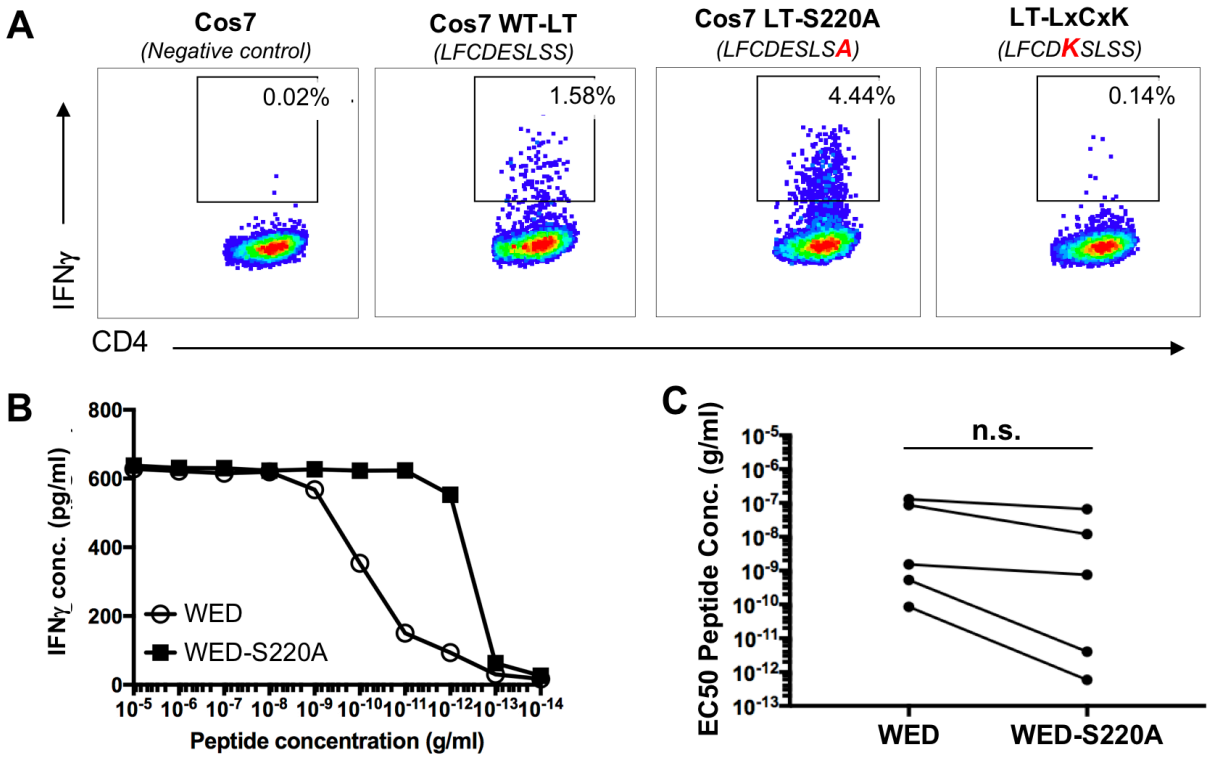


Figure 6: 'WED'-specific CD4 T cell clones can recognize mutant S220A but not E216K mutant. A: 'WED'-specific clones were incubated with DRB1*0401 positive PBMC and COS-7 cell lysates either untransfected, transfected with wild-type LT, S220A mutant LT or E216K mutant LT. Intracellular IFNγ was measured on flow cytometry. Flow plots are representative data of 4 clones. **B:** Dose response curves of a 'WED'-specific clone stimulated with 'WED' or 'WED'-S220A peptides. Clone was incubated with HLA-DRB1*0401-positive LCLs, and peptides. IFNγ secretion was measured by ELISA. **C:** Dose response curves were used to calculate the EC_{50} of 5 distinct 'WED'-specific CD4 T cell clones when stimulated with either 'WED' or 'WED'-S220A peptides.

Discussion

Here we report a highly immunogenic, promiscuous CD4 epitope within the MCPyV Large T-antigen spanning LT-209-228 (''WED''). This epitope is presented within the context of at least three discrete HLA types which are expressed within 24% (HLA-DRB1*0301), 17% (HLA-DRB1*0401) and 45% (HLA-DQB1*0301) of the Caucasian population respectively (Allele Frequency Net Database;

http://www.allelefrequencies.net). Among the HLA class-II typed patients within our cohort, 80% express at least one of these three alleles, suggesting that this epitope is likely expressed and presented within the majority of MCC tumors. Further supporting this notion, the 'WED' epitope encompasses the LxCxE motif which is a critical binding site of the tumor suppressor Rb[3,103]. Rb binding is required for oncogenesis in MCC indicating that conservation and expression of this region are required for MCC tumor growth and persistence. Additionally, 'WED'-specific T cells infiltrated all three tested MCC tumors at high frequencies with highly diverse TCR repertoires. Collectively, these data indicate that this epitope is highly immunogenic and expressed within MCC tumors representing an ideal therapeutic target for immune-based strategies.

Historically, tumor immunology research has largely focused upon studying and improving CD8 T cell responses because CD8 T cells can directly lyse and kill target cancer cells expressing HLA class-I. However, numerous lines of evidence now indicate that CD4 T cells are critical for effective antitumor immunity[250,255,377]. Many reports have indicated that CD4 T cells can mediate antitumor function through either direct interaction with HLA class-II expressing tumors, such as some melanomas[305,306], or indirectly through IFNγ-dependent mechanisms[378,379]. Previously, 8 CD4 epitopes within MCPyV (including 'WED') were identified based upon detection of IFNγ secretion using either ICS or cultured ELISPOT assays[130,131,338]. While these techniques can successfully identify immunogenic regions, these methods require T cell functionality which is often compromised in the setting of cancer[297]. Conversely, HLA tetramers enable direct *ex vivo* analysis of antigen-specific T cells without the need for expansion, antigenic stimulation or the ability to secrete cytokines[380]. Excitingly, in this study we generated two MCPyV-specific HLA class-II tetramers. Furthermore, recent studies have indicated that the use of more highly multimerized HLA reagents such as HLA dextramers, have higher mean fluorescent intensities and reduced background as compared to tetramers, thereby enabling more accurate characterization of antigen-specific cells[344,381]. The reagents generated in this study, therefore provide powerful tools to enable direct *ex vivo* sorting and down-stream characterization of the phenotype and function of MCPyV-specific CD4 T cells for future studies.

Evaluation of the TCR repertoire diversity of 'WED'-specific CD4 T cells from three tumors indicated a surprising degree of diversity and low clonality. A total of 366 distinct TCR beta (TRB) chain sequences, with only two clonotypes that were shared at the amino acid sequence level but were unique at the nucleotide sequence level. There are conflicting reports as to whether increased TCR diversity is protective or detrimental. In melanoma patients treated with anti-PD-1 therapy, increased intratumoral clonality (decreased diversity) prior to treatment was associated with improved outcome suggesting that pre-existing T cell clonotypes that were re-activated during treatment mediated protection[216]. However, this diversity assessment was not antigen-specific or restricted to CD4 T cells. Conversely, in the setting of viral infections, increased TCR diversity of antigen-specific repertoires has been associated with improved response to VZV vaccination[382] and enhanced control of latent CMV infections[383]. Additionally, highly diverse TCR repertoires may prevent immune escape[384] and enable selection of higher avidity TCRs[383,385]. Therefore, these diverse 'WED'-specific TCR repertoires within MCC tumors may provide a large pool of clonotypes capable of mediating antitumor efficacy following immune-based therapies such as checkpoint blockade or therapeutic vaccination. Additional characterization of the 'WED'-specific TCR repertoire in patients who do and do not respond to PD-1 blockade therapy, could indicate whether TCR clonality correlates with outcome. If patients who respond to PD-1 blockade have highly clonal 'WED'-specific TCR repertoires, this would suggest that narrow TCR responses may confer protection, which could be therapeutically recapitulated using transgenic T cell based approaches.

Aside from potentially developing a cellular transgenic T cell therapy targeting the 'WED' epitope, this region could also be immunogenic within a cancer vaccine for MCC. To date, two groups have described potential MCPyV therapeutic vaccine strategies and are separately detailed here. Zeng *et al*. used a pcDNA3 vector encoding truncated LT (LT-1-258) which was intramuscularly injected into C57BL/6 mice prior to tumor challenge with a subcutaneous B16/F10 melanoma cell line transduced with MCPyV LT. IFNγ secretion from CD4 T cells was observed following vaccination, while CD8 T cell responses were largely undetectable. Vaccinated mice never developed tumors and had 100% survival for the duration of follow-up (45 days), as compared to empty vector vaccinated mice that developed tumors within two weeks of inoculation and had reduced survival[131]. While this model suggests that MCPyV vaccination may

have immunogenic and protective effects due to activation of CD4 T cells, this is a highly artificial model and likely does not accurately reflect human disease. In a second model by Gerer et al.[386], dendritic cells (DCs) were electroporated with mRNA encoding truncated LT and *in vitro* immunogenicity testing indicated that this approach induced MCPyV-specific human T cell responses from peripheral blood of both healthy donors and MCC patients. DC vaccination is an appealing vaccination strategy as these cells can process and present antigen while simultaneously expressing the necessary co-stimulatory factors and cytokines to initiate a robust T cell response and prevent tolerance induction[387]. While DC vaccinations have proven safe and effective in the treatment of some malignancies such as renal cell carcinoma and prostate cancer[388], this approach is labor intensive, expensive and patient specific. Consequently, vaccination platforms that utilize 'off-the-shelf' reagents would be more broadly appealing.

The data presented here indicate that a vaccination platform encoding MCPyV LT-S220A may provide such a reagent due to several characteristics. In a recent review article summarizing the recent progress made with therapeutic cancer vaccines, Galaine *et al*.[260] describes four key attributes of a potentially good tumor antigen for vaccination: 1) The antigen should be shared among individuals with the disease or within a population and not patient-specific; 2) Tumor antigens should be critical in oncogenesis to avoid immune escape; 3) Epitopes should be highly promiscuous so as to be useful in a wide percentage of the population; 4) Epitopes should preferentially stimulate Th1 responses. Here we provide strong data suggesting that 'WED'-S220A fulfills the first three of these criteria. More specifically, the epitope is located within the MCPyV large T-antigen which is a shared viral antigen expressed in 80% of MCCs. 'WED' encompasses the LxCxE motif, which binds to Rb, promotes cell cycle progression and is therefore a critical oncogenic driver in LT. Indeed, mutation within this motif (E216K) or at serine 220 prevent Rb binding as assessed by co-immunoprecipitation[3,103] and knockdown of the LT in MCPyV-positive cell lines results in growth inhibition which can be rescued by ectopic expression of the wild-type sequence but not by the E216K or S220A mutants[3,112]. Here we show that of these two mutations, only S220A retains the ability to stimulate 'WED'-specific T cell clones suggesting that this mutation would be more efficacious in stimulating an immune response. Furthermore, the epitope is promiscuous, as we have shown that it can be presented in the context of at least 3 population prevalent HLA class-II allele types expressed by

~80% of our MCC cohort. As a result of these findings, we believe that this epitope represents a potentially ideal candidate for therapeutic vaccination either in the context of a detoxified DNA vaccine or as a synthetic peptide.

Notably, there are several important limitations of these studies. The two mutations within 'WED' have only been reported by single institutions and therefore require further validation to ensure that oncogenic activity is truly lost. Furthermore, we have only tested whether these mutants can induce immunogenic responses from clones that were generated against the wild-type, unphosphorylated 'WED' peptide. Therefore, it is possible that vaccination with these mutants could induce T cell responses in patients. Future work will evaluate the transforming potential of these mutants and determine the best method of MCPyV-vaccine administration with the ultimate goal of improving patient care and outcomes.

SUPPLEMENTAL FILES:

Supplementary Table 1: 20mer peptides spanning the MCPyV T antigen space			
Pool	Peptide	Sequence	A.A. Location Large T Antigen
1	P1	MDLVLNRKEREALCKLLEIA	1 - 20
	P2	EREALCKLLEIAPNCYGNIP	9 - 28
	P3	LEIAPNCYGNIPLMKAAFKR	17 - 36
	P4	GNIPLMKAAFKRSCLKHHPD	25 - 44
	P5	AFKRSCLKHHPDKGGNPVIM	33 - 52
2	P6	HHPDKGGNPVIMMELNTLWS	41 - 60
	P7	PVIMMELNTLWSKFQQNIHK	49 - 68
	P8	TLWSKFQQNIHKLRSDFSMF	57 - 76
	P9	NIHKLRSDFSMFDEVDEAPI	65 - 84
	P10	FSMFDEVDEAPIYGTTKFKE	73 - 92
3	P11	EAPIYGTTKFKEWWRSGGFS	81 - 100
	P12	KFKEWWRSGGFSFGKAYEYG	89 - 108
	P13	GGFSFGKAYEYGPNPHGANS	97 - 116
	P14	YEYGPNPHGANSRSRKPSSN	105 - 124
	P15	GANSRSRKPSSNASRGAPSG	113 - 132
4	P16	PSSNASRGAPSGSSPPHSQS	121 - 140
	P17	APSGSSPPHSQSSSSGYGSF	129 - 148
	P18	HSQSSSSGYGSFSASQASDS	137 - 156
	P19	YGSFSASQASDSQSRGPDIP	145 - 164
	P20	ASDSQSRGPDIPPEHHEEPT	153 - 172
5	P21	PDIPPEHHEEPTSSSGSSSR	161 - 180
	P22	EEPTSSSGSSSREETTNSGR	169 - 188
	P23	SSSREETTNSGRESSTPNGT	177 - 196
	P24	NSGRESSTPNGTSVPRNSSR	185 - 204
	P25	PNGTSVPRNSSRTYGTWEDL	193 - 212
6	P26	NSSRTYGTWEDLFCDESLSS	201 - 220
	P27	**WEDLFCDESLSSPEPPSSSE**	**209 - 228**
	P28	SLSSPEPPSSSEEPEEPPSS	217 - 236
	P29	SSSEEPEEPPSSRSSPRQPP	225 - 244
	P30	PPSSRSSPRQPPCSSAEEAS	233 - 252
7	P31	RQPPCSSAEEASSSQFTDEE	241 - 260
	P32	EEASSSQFTDEEYISSSFTT	249 - 268
	P33	TDEEYISSSFTTPKTPPPFS	257 - 276
	P34	SFTTPKTPPPFSRKRKF	265 - 284
	Peptide	**Sequence**	**A.A. Location Small T Antigen**
7	P35	NIHKLRSDFSMFDEVSTKFP	1 - 20
8	P37	FSMFDEVSTKFPWEEYGTLK	9 - 28
	P38	TKFPWEEYGTLKDYMQSGYN	17 - 36
	P39	GTLKDYMQSGYNARFCRGPG	25 - 44
	P40	SGYNARFCRGPGCMLKQLRD	33 - 52
	P41	RGPGCMLKQLRDSKCACISC	41 - 60
9	P42	QLRDSKCACISCKLSRQHCS	49 - 68
	P43	CISCKLSRQHCSLKTLKQKN	57 - 76
	P44	QHCSLKTLKQKNCLTWGECF	65 - 84
	P45	KQKNCLTWGECFCYQCFILW	73 - 92
	P46	GECFCYQCFILWFGFPPTWE	81 - 100
10	P47	FILWFGFPPTWESFDWWQKT	89 - 108
	P48	PTWESFDWWQKTLEETDYCL	97 - 116
	P49	WQKTLEETDYCLLHLHLF	105 - 124

Supplementary Table 2: 11mer peptides for minimal epitope determination	
Name	**Sequence**
“WED”	WEDLFCDESLSSPEPPSSSE
11mer-1	WEDLFCDESLS
11mer-2	EDLFCDESLSS
11mer-3	DLFCDESLSSP
11mer-4	LFCDESLSSPE
11mer-5	FCDESLSSPEP
11mer-6	CDESLSSPEPP
11mer-7	DESLSSPEPPS
11mer-8	ESLSSPEPPSS
11mer-9	SLSSPEPPSSS
11mer-10	LSSPEPPSSSE

Supplementary Table 3: TCR sequences of MCPyV-WED-specific CD4 T cell clones					
Subject	**Source**	**CDR3 Alpha**	**TRAV**	**CDR3 Beta**	**TRBV**
w164	MCC patient	CATAKGGSEKLVF	TRAV17	CASSLRGTGGIEQYF	TRBV27
z1107	MCC patient	CGAHSGGYQKVTF	TRAV30	CSVRPGLARSSYNEQFF	TRBV29-1
w131	MCC patient	CAGALKGAQKLVF	TRAV27	CASSSPRTGGFTYNSPLHF	TRBV9
DA11152	Healthy Donor	CATVKGGSRLTF	TRAV17	CASSRRGAGEKLFF	TRBV27
wb688	Healthy Donor	CATVRGGSRLTF	TRAV17	CASSLRGGGEKLFF	TRBV27

CHAPTER 8: NOVEL METHOD FOR IDENTIFICATION OF RARE ANTIGEN-SPECIFIC T CELLS

Contributors: Vandeven N, U'Ren L, Hayes E, Stilwell J, Kaldjian E, Nghiem P.

Chapter Summary: Significant progress has been made identifying MCPyV CD4 epitopes (as described in **Chapter 6**) and in the generation of HLA class-II tetramers to isolate MCPyV-specific CD4 T cells (as described in **Chapter 6 & 7**). However, tetramer staining is typically not bimodal, but rather smeared, making delineation of true tetramer-positive cells from non-specific background staining challenging. This smeared staining pattern is the result of several factors including the inherent range of TCR avidity within an antigen-specific T cell populations as well as non-specific staining due to immune complex formation or tetramer binding by cellular debris. Visualization of tetramer localization can enable differentiation of true antigen-specific cells from non-specific staining, a feature which is of particular importance when identifying dimmer, low avidity cells. To obtain morphologic data, we have adapted a digital scanning microscope originally developed to identify rare circulating tumor cells to successfully detect rare MCPyV-specific CD4 T cells. The use of this platform has implications more broadly for the characterization of rare antigen-specific cells.

Why are CD4 T cells so challenging to study?

Over the last several years, important progress has been made delineating epitopes within MCPyV that CD4 T cells can recognize (described in **Chapter 7**). However, the low frequency of these antigen-specific cells has still limited characterization of their phenotype and function. The frequency range for antigen-specific CD4 T cells is highly varied depending upon the antigen[354,389,390], and in many cases can be below the detection of standard methods such as flow cytometry (**Figure 1**). The development of magnetic bead-based enrichment protocols in combination with flow-cytometry has enabled study of cell populations with frequencies as low at 1 per 10^7 T cells. These methods use fluorescently conjugated HLA tetramers which specifically label cells of the correct antigen-specificity. Magnetic beads coupled to an appropriate anti-fluorochrome antibody are then used to magnetically enrich tetramer-positive,

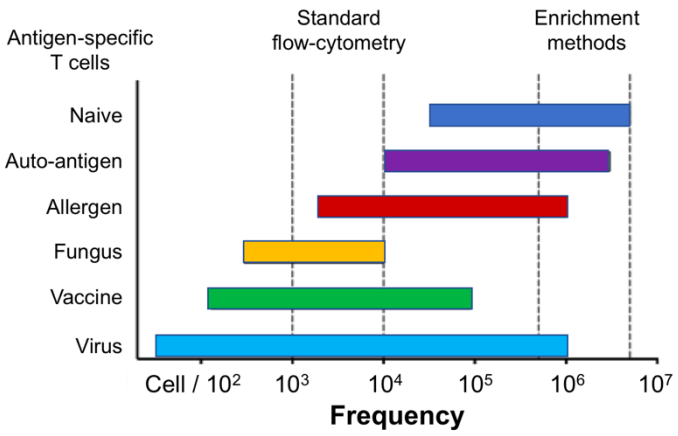


Figure 1: Frequencies of antigen-specific CD4 and CD8 T cells within human peripheral T cell repertoire determined directly by ex vivo enrichment methods. Dashed lines indicated the detection limit range of standard flow cytometry and magnetic enrichment methods, respectively. Adapted from Bachar *et al.* cytometry 2013.

antigen-specific cells [391-393]. However, several limitations exist using this approach. Firstly, the amount of starting material required for these enrichment protocols is on the order of 40 million PBMC at a minimum. When studying antigen-specific T cells from patients with severe disease (such as cancer patients), samples of this size are rarely obtainable. The requirement for such large samples size is largely due to the low frequency of these cells, but is also an artifact of the significant cell loss that occurs using these enrichment methods[394]. Secondly, because T cells can have a wide range of TCR avidity, tetramer staining can often be dim or smeared making it challenging on flow cytometry to differentiate true antigen-specific cells from background signal. Indeed, we show that when single cells are sorted and cloned from MCPyV-tetramer populations many of these clones (~20-50%) will not re-stain with tetramer and/or will not respond to the antigenic peptides via IFNγ secretion. These data suggest that not all cells are truly antigen-specific upon the initial sorting process. In keeping with this idea, we have also found that when attempting to sort two different antigen-specific cell populations simultaneously, cross-contamination can occur in the two sort tubes. Therefore, we sought to develop a method that is as sensitive as flow cytometry, but that does not require any enrichment (preventing cell loss), the enables more accurate detection of truly tetramer positive cells and reduces cross-contamination.

METHODS:

Human subjects and samples

This study was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Subjects were HLA class-II typed via polymerase chain reaction (PCR) at

BloodWorks Northwest (Seattle, WA). All samples were clinically annotated with long-term patient follow-up data.

PBMC

Heparinized blood was obtained from MCC patients and healthy donors, and peripheral blood mononuclear cells (PBMC) were cryopreserved after routine Ficoll preparation at a dedicated specimen processing facility at FHCRC, the Benaroya Research Institute (BRI) or obtained from BloodWorks Northwest.

Tetramer staining protocol

At least 5 million PBMC were CD4 T cell enriched using EasySep CD4 negative selection kit (# 19052 StemCell Technologies). Cells were then washed in 1% BSA in PBS and then incubated with 100nM dasatinib (SelleckChem) for 10 minutes at 37°C. Cells were again washed with 1% BSA and resuspended in 50 ul of anti-human CD32 FcR blocker (StemCell Technologies). HLA class-II tetramers composed of HLA-DRB1*0401 and the 20mer peptide WEDLFCDESLSSPEPPSSSE ('WED') derived from a portion of the MCPyV Large T-antigen (LT-209-228) were used to label MCPyV-specific cells (described in **Chapter 7**). These reagents are conjugated to phycoerythrin (PE) for fluorescent detection. Tetramer staining was performed at 37°C for 1 hour. Subsequently, surface marker monoclonal antibodies against CD3-A488 (Clone SK7; Biolegend), CD4-Qdot800 (Clone S3.5; LifeTechnologies), CD8-BV421 (Clone RPA-T8; Biolegend), CD14-BV421 (Clone M5E2; Biolegend), CD15-BV421 (Clone W6D3; Biolegend), CD19-BV421 (Clone SJ25C1; Biolegend), CD20-BV421 (Clone 2H8; Biolegend), and DAPI.

Automated image capture and analysis

Stained cells were resuspended in 0.1% BSA in PBS at 10^6 per 100 ul and added to 2.5 ml of PBS into a custom chamber-well slide (CyteSlide). Centrifugation of slides for 5 minutes at 100xG distributes cells into a monolayer for imaging. CyteSlides were placed onto the CyteFinder digital scanning microscope (DSM) to acquire fluorescent images. The CyteFinder can acquired 6-channel fluorescent images of low

magnification (10 × objective) fields of view for each CyteSlide covering the entire well. High-resolution images (40 × objective) of revisited points can be 'stacked' by 1-um steps within the Z plane. Images are analyzed for the presence of signal above background for CD4, CD3, and MCPyV-tetramer. Using image analysis software that employs an adaptive auto-threshold algorithm (RareCyte), candidate cells are determined by the algorithm CD4+CD3+tetramer+ and dump negative (CD8/CD14/CD15/CD19/CD20/ dead). Candidate cells are presented to the operator for manual confirmation as potential MCPyV-specific CD4 T cells.

RESULTS:

Digital scanning microscopy for ultra-rare cell detection

While flow cytometric analysis can provide a tremendous amount of data pertaining to cell phenotype and function, one significant cellular characteristic that is not captured is morphology. Consequently, antibody complexes or cellular debris that can adhere to cells (and therefore not be gated out based upon size or granularity) can be fluorescently labeled and lead to false positive cell detection. Excitingly, a recently developed digital scanning microscope (DSM) from RareCyte Inc. enables acquisition of morphological data allowing visual distinction of cellular debris and cells and provides rapidly acquired, high resolution images of cells stained with up to 6 fluorophores. This platform was initially developed for identifying rare circulating tumor cells[395] and circulating fetal cells[396]. We therefore sought to adapt this platform to enable detection of rare, antigen-specific T cells. This method, like flow cytometry, uses fluorescently labeled tetramers and antibodies against intracellular or surface markers to identify desired cell populations (**Figure 2A**). Following cell staining, 10^6 cells are plated into chamber well slides (**Figure 2B**) and centrifugation results in a cellular monolayer distributed across the glass chamber well slide. Wells are then scanned by the RareCyte DSM (**Figure 2C**) and identification of candidate target cells are algorithmically selected based on the desired fluorescent parameters (**Figure 2D**). Candidate cells are then reviewed by the operator and visually validated. Cells of interest can then be acquired with a 40 um hydraulically controls needle for downstream phenotypic or functional analysis[396] (**Figure 2E).**

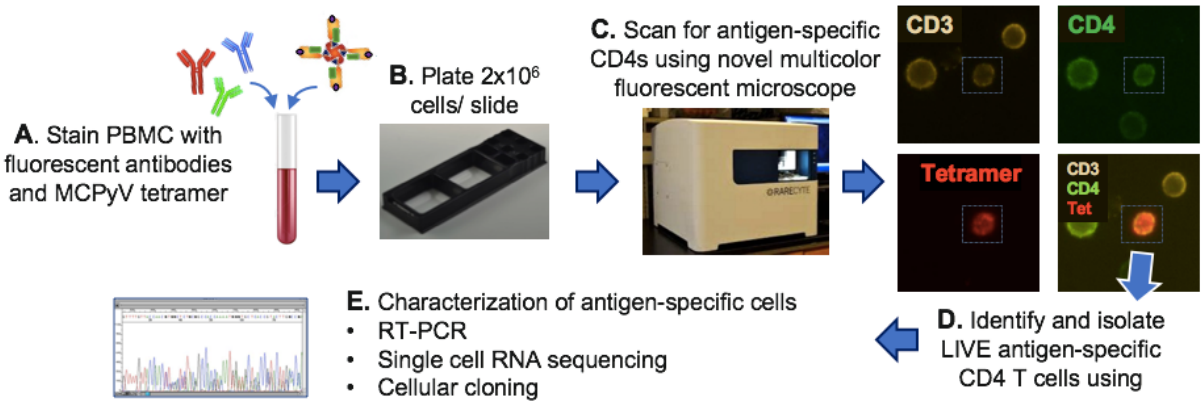


Figure 2: Fluorescent microscope with hydraulically controlled needle for isolation of rare cells. PBMC are stained with MCPyV-specific tetramers and surface marker antibodies and 1 million cells per well are plated into a 2-well chamber slide. Laser interrogation of slides presents high definition (40x) live cell imaging in each color in real time. Software identifies candidate cells based on fluorescent markers and the user validates selected cells which can then be isolated individually using 40um needle and deposited into PCR tubes for further sequencing analysis.

High sensitivity of digital scanning microscopy (DSM)

In order to assess the sensitivity of this novel platform, we used cellular clones generated against the Merkel cell polyomavirus (MCPyV) for which we have developed an HLA-DRB1*0401 tetramer (MCPyV-DRB1*0401). Clone cells were first labeled with carboxyfluorescein succinimidyl ester (CFSE) and subsequently clone cells and PBMC (from a non-HLA-DRB1*0401-positive individual) were separately labeled with the MCPyV-DRB1*0401 tetramer, exclusion markers (CD14, CD15, CD19, CD20, CD8), CD3, CD4, and a live/dead stain. Following antibody and tetramer staining, cells were washed and PBMC were resuspended at 10^6 cells per 100 uL of PBS and 100 ul of cells were loaded into 2 wells of a chamber well slide. Clone cells were resuspended in 1 ml of PBS and counted. Using a hemocytometer, 17 clone cells were counted per uL of PBS and 1 ul of stained cells was added into one well of a chamber well slide. The slide was then scanned and candidate cells were selected as exclusion marker negative, CD4+ and tetramer+. Using visual validation of candidate cells, 16 cells were identified as antigen-specific (**Figure 3**). When evaluating cells labeled with CFSE (should identify all clone cells), all 16 tetramer-positive cells were verified and an additional cell was identified as CFSE+ and tetramer low. These data suggest that this platform is highly sensitive and able to detect low frequency antigen-specific cells.

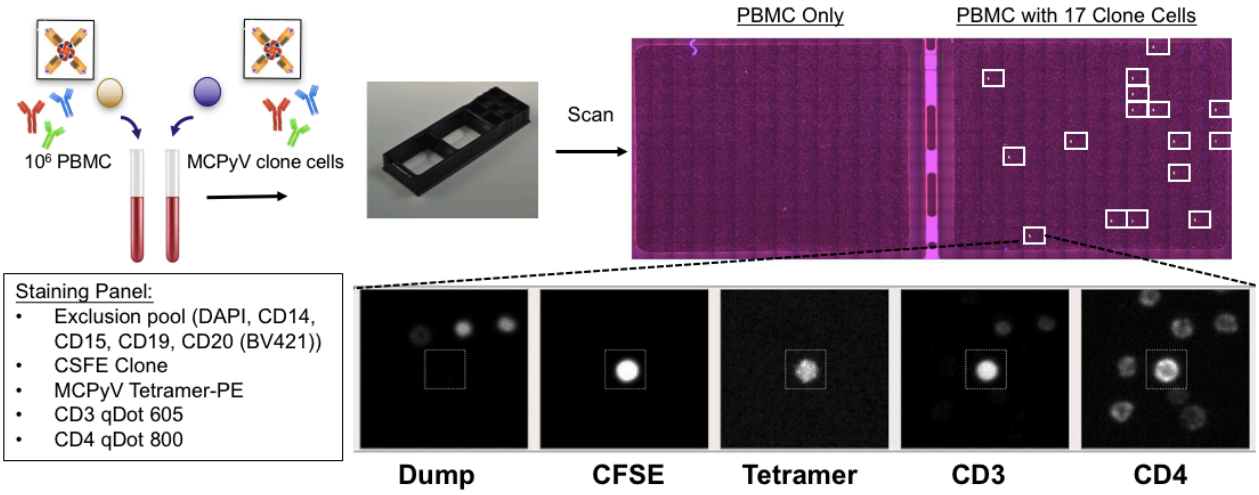


Figure 3: High sensitivity of RareCyte DSM platform. 'WED'-specific CD4 clone was CFSE labeled and washed. PBMC and 'WED'-specific clone were tetramer and surface marker stained. Following a final wash, 17 clone cells were spiked in PBMC. Cells were plated into chamber well slide with one well containing no spiked in clone and the other with 17 spiked in clone cells. Candidate cell selection parameters were defined as CD4+, Tet+ and exclusion marker negative. Candidate cells were visually reviewed and verified via CFSE staining.

Morphological assessment adds confidence to cell identification

When identifying rare antigen-specific cells, standard flow cytometry relies on fluorescent labeling and size exclusion gating strategies to identify cell populations. While this is highly effective and specific on a population level, when isolating very rare cells, even one or two inaccurately identified cells can skew phenotypic characterization. To determine the number of cells that were truly antigen-specific using flow cytometry, we tetramer stained tumor infiltrating lymphocytes (TIL) from a patient with Merkel cell carcinoma (**Figure 4A**) and performed single cell sorting of tetramer positive cells. Sorted cells were then expanded and re-stained with tetramer to verify antigen-specificity (**Figure 4A**). Eight of the 13 expanded clones were tetramer positive following expansion, suggesting that a significant fraction of sorted cells were not antigen-specific (5 of 13; 38%). Therefore, using DSM, we sought to identify a morphological feature that could delineate between true- and false-positive antigen-specific cells. PBMC from a healthy donor was stained with tetramer and acquired 10x images of candidate antigen-specific cells (**Figure 4B**). We observed two patterns of tetramer staining. In the majority of candidate cells, tetramer staining is diffuse, membrane-specific and co-localizes with the co-receptors CD3 and CD4 (**Figure 4B**). This pattern reflects the appropriate biological interaction of an HLA tetramer binding to T cell receptors

(TCRs) expressed on the cell surface. Enhanced images at 40x magnification can also be acquired (**Figure 4C**) to further evaluate this cellular morphology. The second pattern that we observed, was a punctate expression of tetramer (**Figure 4B;** bottom row). Cells with this second pattern were still viable, CD3+ and CD4+, however, tetramer staining was not co-localizing with CD3 and CD4 and was in fact largely extracellular. These data strongly suggest that extracellular debris staining with tetramer can falsely label cells as antigen-specific. The extracellular nature of this staining combined with a lack of CD3 and CD4 co-localization suggest that this is also not TCR capping and internalization as has been described[397]. Therefore, the added morphologic data acquired using the RareCyte platform significantly enhances our ability to differentiate between true- and false-positive antigen-specific T cells

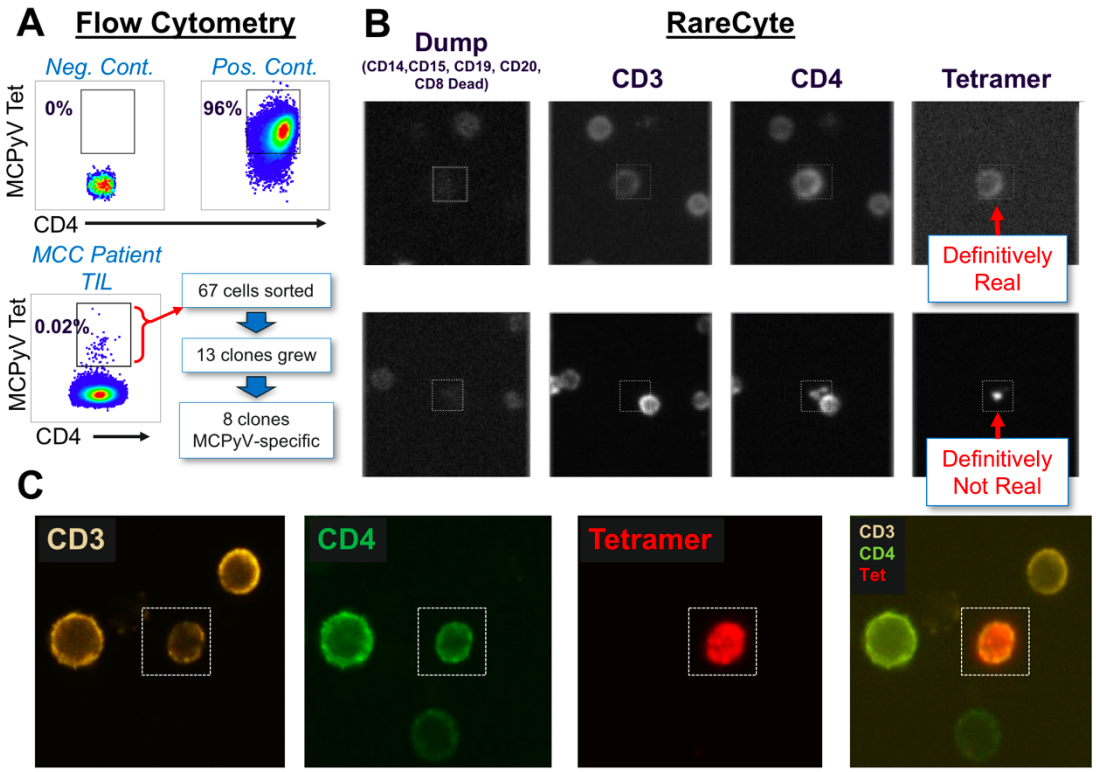


Figure 4: RareCyte digital scanning microscope (DSM) provides valuable morphologic data. A: Flow cytometric analysis of MCPyV-specific CD4 T cells was performed on tumor infiltrating lymphocytes from an MCC patient. Tetramer-positive CD4 T cells were sorted and expanded for 4 weeks. Tetramer re-staining was then performed to validate antigen-specificity. **B**: PBMC from a healthy donor was MCPyV-tetramer stained and candidate cells were selected based upon exclusion marker negativity, CD4 and tetramer positivity. Candidate cells were then visually validated using morphologic data. **C:** 40x image analysis can be collected to evaluate tetramer, CD3 and CD4 co-localization.

DISCUSSION

In this study, we describe the use of a novel digital scanning microscope (DSM) developed by RareCyte Inc. that enables sensitive identification of rare, antigen-specific T cells. Flow cytometry is the standard method used to detect rare, antigen specific populations of varying frequencies, however, it has several significant limitions. These include: 1) the need for enrichment protocols, a process that results in significant cell loss[394]; 2) An inability to clearly differentiate between background tetramer staining and true-positive, but low avidity T cells; 3) Cross-contamination of sorted cells when attempting to sort population of varying specificities at once. Using DSM, we negate the requirement for pre-enrichment, the added morphologic data enables differentation of true-positive antigen-specific cells and cells are being individually picked and visualized, thereby preventing cross-contamination.

The identification and isolation of rare antigen-specific T cells has many wide-ranging potential applications. In the era of immune checkpoint blockade for treating malignancies, there have been major breakthroughs with response rates >50% in some solid tumors[65,398]. However, a significant fraction of patients are still not responding and severe adverse events can occur, some of which have been fatal[399]. Therefore, the development of biological assays to delineate patients who will and will not-respond to checkpoint inhibitors is a pressing priority. Most studies attempting to predict response to checkpoint therapy to date have evaluated T cell changes within the tumor. Through this analysis, pre-existing intratumoral expression of PD-L1 and CD8 T cell infiltration have been associated with improved response[216], however, specific T cell populations have not been identified as the main effectors of response. Furthermore, in many circumstances, the ability to obtain sufficient tumor tissue to support adequate T cell phenotyping is simply not possible. Therefore, investigation into T cell subsets within the periphery that may predict checkpoint response would be highly beneficial. Recent studies have provided encouraging results on this front. Spietzer *et al.* indicated that a small peripheral Th1-like CD4 T cell population is associated with response to CTLA-4 and GM-CSF therapy in melanoma patients[322]. In a second study, melanoma patients treated with pembrolizumab (anti-PD-1) that had reinvigoration of peripheral exhausted CD8 T cells (expressing PD-1, CTLA-4, 2B4 and low perforin and granzyme B) had improved outcomes as compared to patients who had reinvigoration of CD8 effector cells (expressing

elevated perforin and granzyme B) post treatment[400]. These findings, indicate that detailed examination of peripheral blood subsets may indeed provide a predictive set of biomarkers for checkpoint therapy response. The use of the RareCyte DSM would enable identification and isolation of circulating tumor-specific CD8 and CD4 T cells using smaller blood volumes than is required for flow cytometry which therefore may provide a more feasible and rapid method for evaluating the phenotype and function of these peripheral cells.

Furthermore, the ability to accurately capture low avidity tumor-specific T cells may also be of importance. While there is a general consensus that more functionally avid T cells (T cells that have higher effector function in response to low antigen concentration) have higher efficacy and ability to eliminate cancer cells and viral infection[401,402], there is evidence to suggest that high avidity T cells are also the most prone to exhaustion and clonal deletion in the setting of chronic antigen stimulation[403,404]. Therefore these cells may be less protective overtime in cancer patients. Indeed T cells expression high affinity TCRs are more likely to plateau[405,406] or even attenuate[407-409] in their ability to respond. This is particularly true when T cell responses are strong such as in the setting of viral infections and virally induced cancers such as Merkel cell carcinoma. Therefore, the ability to accurately isolate and study low avidity, antigen-specific T cells, could enable development of therapeutic options that include T cells with a range of avidities.

CHAPTER 9: AN UNUSUALLY IMMUNOGENIC SUBSET OF MCC, PATIENTS WITHOUT DETECTABLE PRIMARY SKIN LESIONS

Accepted pending revisions: Vandeven N, Lewis CW, Makarov V, Riaz N, Paulson K, Bestick A, Doumani R, Marx T, Takagishi S, Chan TA, Choi J Nghiem P. Merkel cell carcinoma patients presenting without a primary lesion have elevated markers of immunity, higher tumor mutation burden and improved survival. Clin Can Res.

Chapter Summary:

Purpose: Patients presenting with nodal Merkel cell carcinoma without an identifiable (unknown) primary lesion (MCC-UP) are nearly twice as likely to survive compared to similarly staged patients with known primary lesions (MCC-KP). The basis of this previously reported finding is unclear.

Experimental Design: Survival analyses and markers of immunity were evaluated in 123 patients with advanced MCC. Whole exome sequence data was analyzed from 16 tumors.

Results: As in prior studies, patients with nodal MCC-UP had strikingly improved MCC-specific survival as compared to MCC-KP patients (HR 0.297, $p < 0.001$). Surprisingly, patients presenting with distant metastatic MCC-UP also had significantly improved survival (HR 0.296, $p = 0.038$). None of the 72 patients with MCC-UP were immunosuppressed as compared to 12 of the 51 (24%) patients with MCC-KP ($p < 0.001$). Merkel polyomavirus oncoprotein antibody median titer was higher in MCC-UP patients (26,229) than MCC-KP patients (3,492; $p < 0.001$). Additionally, the median number of nonsynonymous exome mutations in MCC-UP tumors (688 mutations) was markedly higher than MCC-KP tumors (10 mutations, $p = 0.016$).

Conclusions: This is the first study to our knowledge to explore potential underlying immune-mediated mechanisms of MCC-UP presentation. In this cohort, MCC-UP patients were never immune suppressed, had higher oncoprotein antibody titers, and higher tumor mutational burdens. Additionally, we show that nodal tumors identified in MCC-UP patients did indeed arise from primary skin lesions as they contained abundant UV-signature mutations. These findings suggest that stronger underlying immunity against MCC contributes to primary lesion elimination and improved survival

TRANSLATIONAL RELEVANCE

Numerous reports show that Merkel cell carcinoma (MCC) patients presenting with nodal disease without detectable (unknown) primary skin lesions have ~50% improved survival as compared to similarly staged patients with skin lesions. This finding will be incorporated into the new staging system for MCC (active as of January 1st, 2018). Here we also show a significant survival difference among MCC-unknown primary (MCC-UP) patients presenting with distant metastatic disease. Additionally, this is the first report to our knowledge to explore potential mechanisms underlying MCC-UP presentation. Here we found that MCC-UP patients have higher levels of tumor-specific antibodies and higher tumor mutational burdens suggesting enhanced tumor immunogenicity and immune-mediated clearance of primary skin lesions. In the era of immune checkpoint blockade therapy, it may be that MCC-UP patients respond differently to these immune-based agents and therefore should be examined in future studies.

Introduction

Merkel cell carcinoma (MCC) is a highly aggressive skin cancer with a relative mortality of 46%[221], making this disease ~3 times as deadly as malignant melanoma on a per case basis. While rare (~2,000 new cases per year in the US), the incidence has dramatically risen over the past 25 years due to improved detection methods and increased prevalence of risk-factors for MCC[69,70,97]. Among patients presenting with palpable or scan-detectable regional lymph nodes at the time of MCC diagnosis (macroscopic nodal disease; stage IIIB), one-third to half of patients do not have a detectable skin primary. Several studies have documented that among stage IIIB patients with MCC, those presenting with an unknown primary tumor (MCC-UP) have significantly improved survival as compared to stage IIIB patients with known primary tumors (MCC-KP)[127,410-413]. The magnitude of this survival benefit ranges from 60%-70% decreased chance of death if no primary lesion is present[127,410,412].

Several reports postulate that regression of the primary lesion may be attributable to immune-mediated mechanisms[61,127,412], however, limited evidence has been published to support this notion. Importantly, despite two etiologically distinct mechanisms[414] to MCC development (viral versus ultraviolet carcinogenesis), nearly all MCCs are highly immunogenic. In the majority of cases (80%), the Merkel cell polyomavirus (MCPyV) is clonally integrated in MCC tumors and persistent expression of the immunogenic MCPyV large and small T-antigens drive oncogenesis in these virus-positive tumors[57]. The 20% of MCCs that are MCPyV-negative are induced via UV-mediated mutagenesis and harbor very high mutational burdens with UV-signatures[61,62,414]. In multiple malignancies, high mutational burdens have been associated with immunogenicity and response to immunotherapy, likely through generation of neoepitopes[175]. Importantly, both virus-positive and -negative MCCs have shown remarkable response rates to immune checkpoint inhibitor therapy, providing the strongest evidence that both virus-positive and -negative MCCs are immunogenic and responsive to immune mediated regression[65].

In this study, we report significantly improved survival of patients presenting with both virus-positive and –negative MCC-UP and we probe the relationship between immunity and MCC-UP presentation. We

demonstrate that MCC-UP patients have enhanced immune function and significantly higher tumor mutation burdens than MCC-KP patients.

METHODS

Patient selection criteria

All studies were performed in accordance with Helsinki principles and approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center (IRB # 6585). All patients included in this study provided informed consent for enrollment in this IRB-approved database.

In our repository of 1,099 MCC patients, 407 were enrolled within 180 days of diagnosis of histologically confirmed MCC between June 1st, 2006 and December 9th, 2015 **(Fig. 1)**. The median overall survival was significantly reduced and disease-specific death was increased in patients referred to UW more than 180 days after initial diagnosis, therefore to prevent selection bias, patients enrolled > 180 days after diagnosis were excluded from analysis. Additionally, we have previously reported improved

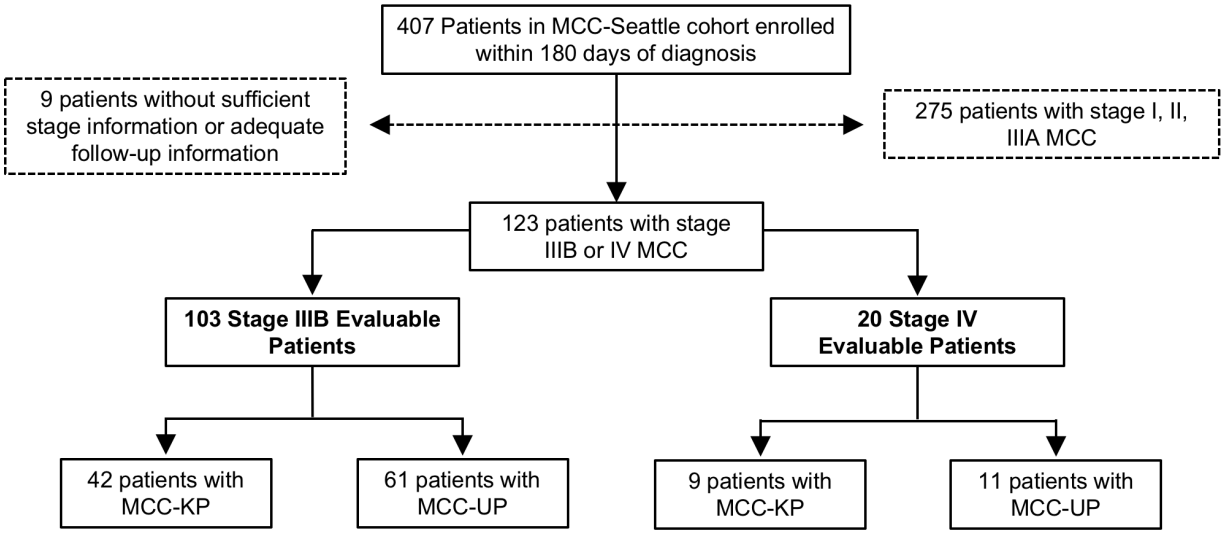


Figure 1. Enrollment criteria for patients with stage IIIB or IV MCC. Patients included in the analysis were enrolled within 180 days of their diagnosis of stage IIIB or IV MCC as defined by AJCC 7th ed. criteria. All patients had clinical information on the presence or absence of a primary lesion and the time points necessary to calculate survival. 123 patients met all selection criteria with breakdowns as shown in terms of stage and primary lesion

outcomes among MCC-UP patients from a separate de-identified Kaiser Permanente Northern California cohort of patients. There is 1 patient (<1%) that we are aware of that was included in both cohorts, and

while additional overlap is possible as patients were de-identified from the Kaiser Permanente group, we estimate that this number does not exceed 5 (~4%). Staging was performed as per AJCC 7th edition guidelines[221]. The analysis was then restricted to 123 patients diagnosed with regional nodal (stage IIIB) and distant metastatic (stage IV) MCC and who had a primary status, diagnosis date, and date of last follow-up. As per guidelines, patients were classified as stage IIIB if they presented with clinically evident (via scan or physical exam) nodal involvement from skin-draining nodal basins without evidence of distant disease. Patients were classified as stage IV if they presented with clinically evident nodal disease in non-skin draining lymph nodes or with visceral metastatic disease. All patients received at least two comprehensive skin exams, including one at the Seattle Cancer Care Alliance and at least one or more at outside facilities in order to determine primary status presentation.

Serological evaluation, viral status, sample preparation and tumor whole exome sequencing

Serological testing for antibodies against the MCPyV T-antigen oncoproteins was performed on 103 patients as previously described [129] at the University of Washington Clinical Immunology Laboratory and these results are shown in **Table 1**. Only patients with virus-positive tumors produce these antibodies[107,129,415], therefore all tumors from patients who tested serologically positive (n = 57) were considered virus-positive. The remaining 46 patients tested were serologically negative, however, because roughly half of seronegative MCC patients do in fact have virus-positive tumors[107,129], additional testing was done on patients with available tumor samples (n = 21). Viral status was evaluated in these patients using qPCR detection of viral DNA and immunohistochemical staining (IHC) using the CM2B4 (SC136172; Santa Cruz Biotechnology[103] and Ab3 antibodies (a generous gift from James DeCaprio, Dana-Farber Cancer Institute[416] targeting the MCPyV large T-antigen as previously described[415] (**Table 1)**.

A previous study by Goh *et al*.[414] performed whole exome sequencing on 16 tumors (10 from MCC-UP and 6 from MCC-KP) enrolled in our cohort and determination of the number of somatic nonsynonymous mutations was performed as previously described. UV and age-related mutational signatures were defined according to Alexandrov *et al*[417]. C to T transitions that are characteristic of UV-induced mutational signatures were counted as follows. The fastq files were aligned with ELAND, and somatic

mutations were called using previously published algorithms[414]. Each mutation, such as a C>T, was called accordingly. C>T's that occur on neighboring nucleotides were noted as CC>TT transitions. Aside from UV- and age-associated mutational signatures, several other signatures were identified, however, none were consistently represented across samples and therefore these were condensed into 'other' as described previously[414].

Table 1. Patient demographic details by stage and primary status

	Stage IIIB			Stage IV		
	MCC-KP (n = 42)	MCC-UP (n = 61)		MCC-KP (n = 9)	MCC-UP (n = 11)	
Characteristic	**No. (%)**	**No. (%)**	**P**	**No. (%)**	**No. (%)**	**P**
Sex						
Male (n=85)	30 (71.4)	40 (65.6)	0.668	8 (88.9)	7 (63.6)	0.319
Female (n=38)	12 (28.6)	21 (34.4)		1 (11.1)	4 (36.4)	
Age at Dx						
≥65 (n=67)	23 (54.8)	32 (52.5)	0.843	4 (44.4)	8 (72.7)	0.362
<65 (n=56)	19 (45.2)	29 (47.5)		5 (55.6)	3 (27.3)	
Immune Suppressed**						
Yes (n=12)	11 (26.2)	0 (0.0)	**<0.001**	1 (11.1)	0 (0.0)	0.450
No (n=111)	31 (73.8)	61 (100.0)		8 (88.9)	11 (100.0)	
MCPyV Viral Status						
Positive (n=68)	22 (95.6)	36 (85.7)	0.406	4 (80.0)	6 (75.0)	1.000
Negative (n=10)	1 (8.7)	6 (14.3)		1 (20.0)	2 (25.0)	
*Unknown (n=45)	19	19		4	3	
MCPyV Oncoprotein Serostatus						
Positive (n=57)	17 (48.5)	32 (59.3)	0.385	3 (60.0)	5 (55.6)	1.000
Negative (n=46)	18 (51.5)	22 (40.1)		2 (40.0)	4 (44.4)	
*Unknown (n=18)	7	7		4	2	
Received Radiation Therapy?						
Yes (n=106)	37 (90.2)	58 (98.3)	0.156	4 (44.4)	7 (70.0)	0.370
No (n=13)	4 (9.8)	1 (1.7)		5 (55.6)	3 (30.0)	
*Unknown (n=4)	1	2		0	1	
Received Chemotherapy?						
Yes (n=31)	6 (14.3)	13 (22.4)	0.439	5 (55.6)	7 (63.6)	1.000
No (n=89)	36 (85.7)	45 (77.6)		4 (44.4)	4 (36.4)	
*Unknown (n=3)	0	3				

*Unknown values were excluded from percentage calculations

** Causes of immunosuppression were HIV (n=4), Chronic lymphocytic leukemia (n=3), solid organ transplant (n=2), mycosis fungoides (n=1), methotrexate treatment (n=1)

Statistical analysis

Analyses were completed using STATA software, version 11.0 and Prism software, version 6 with a statistical significance threshold of 5%. Comparisons of ordinal variables between MCC-KP and MCC-UP groups were performed using the Mann-Whitney test. Comparison of categorical variables in **Table 1** were performed using the Fisher's exact test. MCC-specific survival was defined as the length of time between the date of diagnosis (defined as date of first biopsy confirming MCC) and the date of death caused by MCC. Fine and Gray's proportional sub-hazards model was used to evaluate competing-risks and calculate MCC-specific survival significance and hazard ratios in both the univariate and multivariate setting. The competing-risk was death by all causes except MCC. Overall and recurrence-free survival

were defined as the length of time between the date of diagnosis (defined as date of first biopsy confirming MCC) and the date of death by any cause or the development of recurrent disease. Overall and recurrence-free survival was analyzed using a Cox-proportional hazards model. Patients for all survival analyses were censored by date of last contact. Multivariate analyses for stage IIIB patients controlled for age at diagnosis, sex, MCPyV oncoprotein antibody serological status and having received radiation therapy or chemotherapy. For stage IV patients (n = 20), multivariate analysis was limited to age at diagnosis and sex because of the small samples size and the fact that not all characteristics could be assessed on all 20 patients.

RESULTS

Characteristics of MCC-UP and MCC-KP patients

Among the 123 evaluable patients who were diagnosed with stage IIIB and stage IV MCC, 51 (41%) presented with MCC-KP and 72 (59%) presented with MCC-UP (**Table 1).** These 123 patients were followed for a collective 471.5 person-years and a median of 1.5 years per patient following diagnosis. When evaluating potential demographic characteristics associated with MCC-UP and MCC-KP presentation, we found no statistically significant difference in sex, age at diagnosis, MCPyV oncoprotein serological status, MCPyV viral status, treatment with radiation therapy, or treatment with chemotherapy between MCC-UP and MCC-KP patients **(Table 1).**

Differentiation of regional versus distant metastatic MCC without a primary

The definition of regional (stage III) versus distant (stage IV) disease in MCC-UP patients who present with only nodal involvement (i.e. no visceral metastasis) has not been clearly established to the best of our knowledge. In this study, we defined MCC-UP patients presenting with nodal disease within skin-draining lymph node basins as stage IIIB (regional), while patients presenting with deeper, non-skin-draining nodal disease were classified as stage IV (distant; **Figure 2A).** Notably, skin-draining lymph nodes could potentially be sites of distant metastases, however, in the absence of a detectable primary tumor it is impossible to determine whether these lesions represent regional or distant disease. Using this classification, among MCC-UP patients presenting with only nodal disease, stage IIIB patients had

significantly improved MCC-specific survival (HR=3.98; p=0.003) relative to stage IV MCC-UP patients **(Figure 2B),** suggesting this dichotomy identified a meaningful difference in risk.

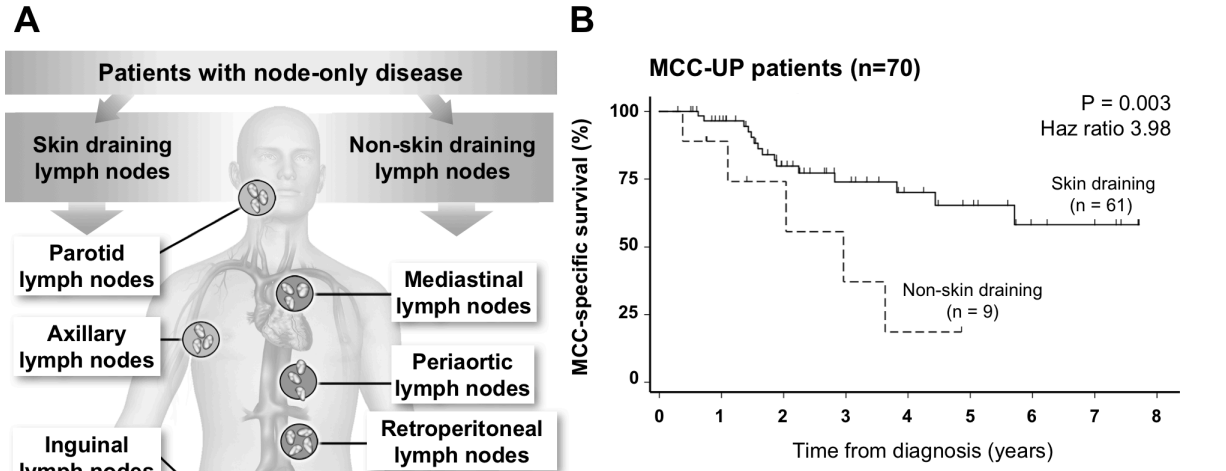


Figure 2. Patients with skin-draining lymph nodes have improved survival compared with patients with nodal disease in non-skin-draining nodes. Panel **A** depicts representative skin-draining lymph nodes that were classified as stage IIIB and non-skin draining lymph nodes that were classified as stage IV. Panel **B** depicts MCC-specific survival for patients presenting with node-only disease. Sixty one patients were classified as stage IIIB with skin-draining lymph nodes and unknown primary lesions while 9 patients were classified as stage IV with non-skin draining lymph nodes and no primary lesions (2 stage IV MCC-UP patients were excluded due to presentation with visceral metastasis).

Patients with regional nodal (stage IIIB) MCC-UP have improved survival

To determine survival differences between MCC-UP and MCC-KP patients, Kaplan-Meier curves were used to evaluate overall, MCC-specific and recurrence-free survival for stage IIIB **(Figure 3A-C).** Among living stage IIIB patients, the median follow-up time was 2.2 years for MCC-UP and 1.4 years for MCC-KP patients. This difference in follow-up time is largely due to a significant difference in survival between these two groups. Indeed, the MCC-specific survival among stage IIIB patients was dramatically improved for MCC-UP patients as compared to MCC-KP patients at 2 years (80% vs 45%) and 5-years (66% vs 30%; p <0.001; **Figure 3A**) with an overall reduced risk of death by MCC of 70% (HR = 0.297, P < 0.001; **Table 2**). Similarly, overall (**Figure 3B**) and recurrence-free survival (**Figure 3C**) were also significantly improved for MCC-UP patients. Specifically, stage IIIB MCC-UP patients had a 70% reduction in the risk of death from any cause or MCC (HR = 0.300; p < 0.001) and a 64% reduced risk of

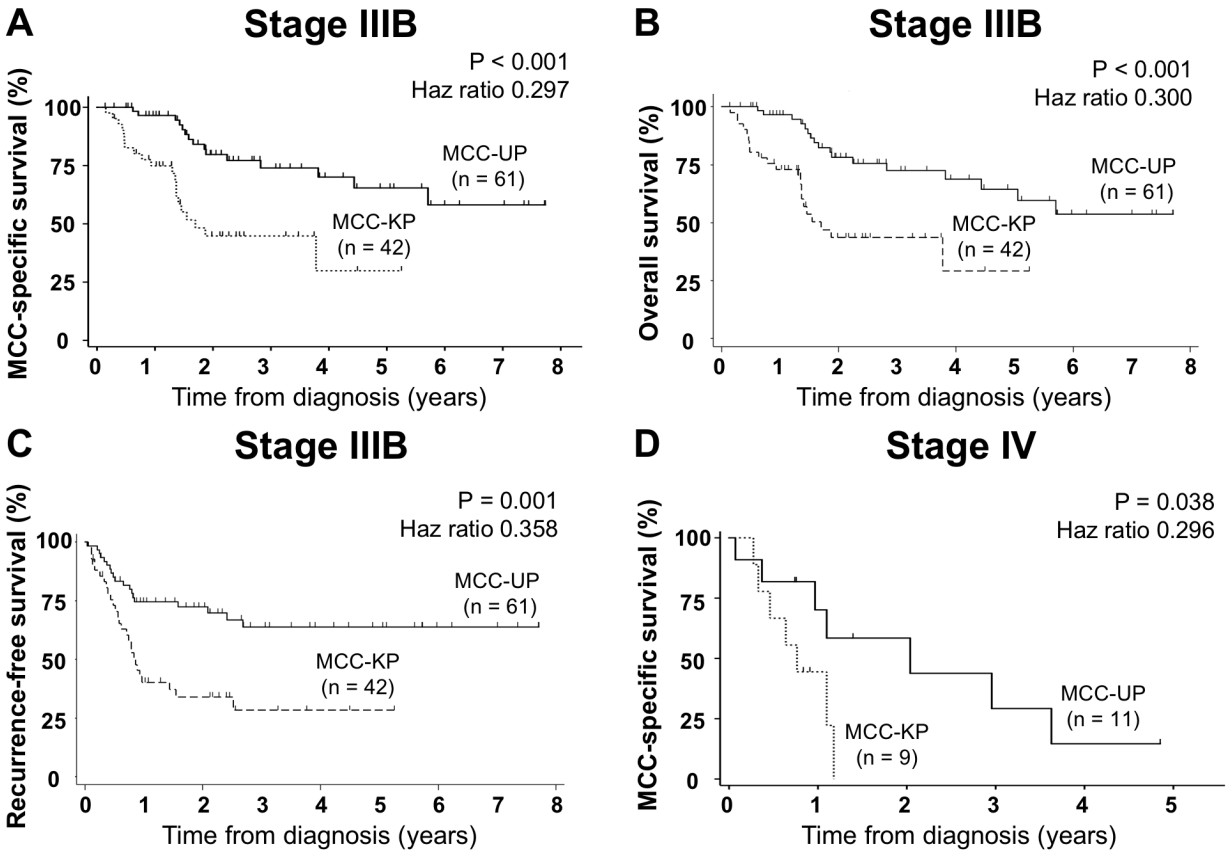


Figure 3. MCC-UP status predicts better survival among patients with either stage IIIB or IV disease. Panel **A** depicts MCC-specific survival, **B** illustrates overall survival and **C** indicates recurrence-free survival for 103 patients with stage IIIB MCC by unknown primary status. Panel **D** describes MCC-specific survival for 20 patients with stage IV MCC. MCC-specific survival analyses was completed using Fine and Gray's proportional sub-hazards model to evaluate competing risks for MCC-specific survival analyses. For overall and recurrence-free survival analyses, we used a Cox proportional hazard model.

recurrence when compared to MCC-KP (HR = 0.358; p = 0.001; **Table 2**). This clinically and statistically significant improvement in survival among MCC-UP patients persisted on multivariate analyses controlling for age at diagnosis, sex, MCPyV-oncoprotein serological status, treatment with radiation therapy, and treatment with chemotherapy (**Table 2**).

Table 2: Merkel cell carcinoma survival and unknown primary status

	Univariate analysis			Multivariate analysis		
Stage IIIB (n = 103)	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
MCC-specific survival	0.297	0.152 to 0.582	<0.001	0.257	0.117 to 0.542	< 0.001
Overall survival	0.300	0.156 to 0.578	<0.001	0.287	0.137 to 0.592	0.001
Recurrence-free survival	0.358	0.199 to 0.646	0.001	0.363	0.188 to 0.700	0.002
Stage IV (n = 20)						
MCC-specific survival	0.296	0.093 to 0.935	0.038	0.219	0.049 to 0.968	0.045
Overall survival	0.296	0.093 to 0.935	0.038	0.190	0.038 to 0.945	0.042
Recurrence-free survival	0.618	0.243 to 1.570	0.312	0.667	0.200 to 3.383	0.512

Patients with distant metastatic MCC-UP also have improved MCC-specific survival

A dramatic survival difference was also observed in patients with distant metastatic MCC without a primary lesion, with MCC-UP having improved MCC-specific survival as compared to MCC-KP patients at 2 years (59% vs 0%; p = 0.038; **Figure 3D**). A 5-year follow-up time point was not reached. The median follow-up time for stage IV MCC-UP was 1.5 years as compared to 0.8 years for MCC-KP. On multivariate competitive-risks regression also accounting for age at diagnosis and sex, presenting with stage IV MCC-UP was associated with a remarkable 79% decreased risk of MCC-specific death when compared with presenting with stage IV MCC-KP (HR = 0.219; p = 0.045; **Table 2**). MCC-UP patients also had significantly improved overall survival despite a similar rate of recurrence (**Supplemental Fig. 1**).

Patients with MCC-UP have intact immune function and higher oncoprotein antibody titers

Within our cohort, 12 patients presented with profound immune suppression (i.e. HIV, CLL, organ transplant). Among those without immune suppression, 72 of the 111 patients (65%) presented with MCC-UP at diagnosis whereas among those with immune suppression, 0 of 12 (0%; p < 0.001) presented without a primary lesion (**Figure 4A).** Given the variable nature of human disease, we were unable to control for the relative degree of immune suppression between various immune-suppressed patients and could not determine the relative impact of various forms of immune suppression on survival. However, in

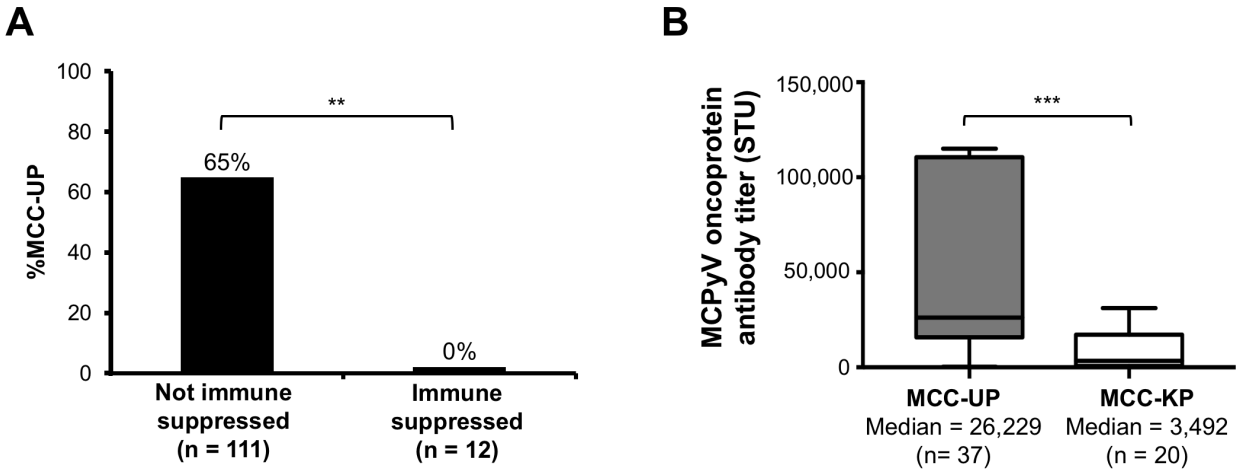


Figure 4. Patients with MCC-UP have intact immune function including robust oncoprotein antibody titers. Panel **A:** Among the 123 patients with stage IIIB and IV MCC, no MCC patients with MCC-UP presented with immune suppression (**p < 0.001) whereas 65% of non-immune suppressed patients presented with MCC-UP. Panel **B:** MCC-UP patients presented with a significantly higher oncoprotein antibody titer (median 26,229 STU) as compared to MCC-KP patients (median 3,492 STU; ***p < 0.001). The median oncoprotein titers are indicated by the horizontal black lines and the P value was calculated using the Mann-Whitney test.

order to verify that the disproportionately higher number of MCC-KP patients presenting with immune suppression was not the underlying cause of the reduced survival we observed, survival analyses were also performed excluding all cases of immune suppression (n= 92; **Supplemental Fig. 2).** Survival analyses for stage IIIB patients excluding those with immunosuppression retained statistical significance on univariate and multivariate analysis (**Supplementary Fig. 2**; **Supplementary Table 1**). For stage IV patients, overall and MCC-specific survival retained statistical significance on univariate analysis but became only marginally non-significant on multivariate analysis (n = 19; MCC-specific survival: p = 0.071; overall survival: p = 0.069; **Supplementary Table 1**). Overall, these data strongly suggest that immune competence correlates with MCC-UP presentation and immunosuppression does not appear to explain the difference in prognosis between MCC-UP and MCC-KP patients.

An additional marker of an MCPyV-specific immune response is the presence of antibodies specific to the MCPyV oncoproteins which can be detected in most virus-positive MCC patients (but are almost never present in healthy controls)[107]. Among MCC patients who produce MCPyV oncoprotein antibodies (n = 57), MCC-UP patients had significantly higher median antibody titers (26,229) compared to seropositive MCC-KP patients (3,492, p < 0.001; **Figure 4B**), suggesting that MCC-UP patients experienced more robust humoral immune responses than MCC-KP patients.

MCC-UP patients have a higher tumor mutational burden than patients with MCC-KP

It has been documented that higher mutational loads within tumors (including melanoma, colorectal, and several types of lung cancer) are associated with an increased prevalence of tumor-associated neoantigens, enhanced immunogenicity and ultimately improved response to immune-based therapies[175]. We hypothesized that the improved survival advantage observed among MCC-UP patients may be correlated with higher tumor mutation burdens resulting in increased neoantigen presentation and immunogenicity as compared to tumors from MCC-KP patients. Previously, whole exome sequencing (WES) was performed on 16 tumors, which included 10 MCC-UP and 6 MCC-KP patients enrolled in our cohort[414]. Analysis of these cases revealed that MCC-UP tumors harbor a significantly higher median number of nonsynonymous mutations (688/tumor) than MCC-KP tumors (10/tumor, p = 0.016; **Figure**

5A). As anticipated, virus-negative tumors (filled in symbols) overall harbor significantly higher mutation burdens than virus-positive tumors (open symbols). When evaluating mutation burden among virus-positive cases independently, patients presenting with MCC-UP have higher mutational loads than MCC-KP tumors (25 vs 7 nSSNV's per tumor respectively; p = 0.029). This trend was also observed among virus-negative tumors with MCC-UP tumors having a median of 1,041 nSSNV's per tumor as compared to MCC-KP tumors with a median of 310 nSSNV's per tumor. While this comparison in virus-negative tumors did not achieve statistical significance, potentially due to low sample numbers, the 3-fold difference observed between these two subgroups strongly suggests that this is a meaningful distinction.

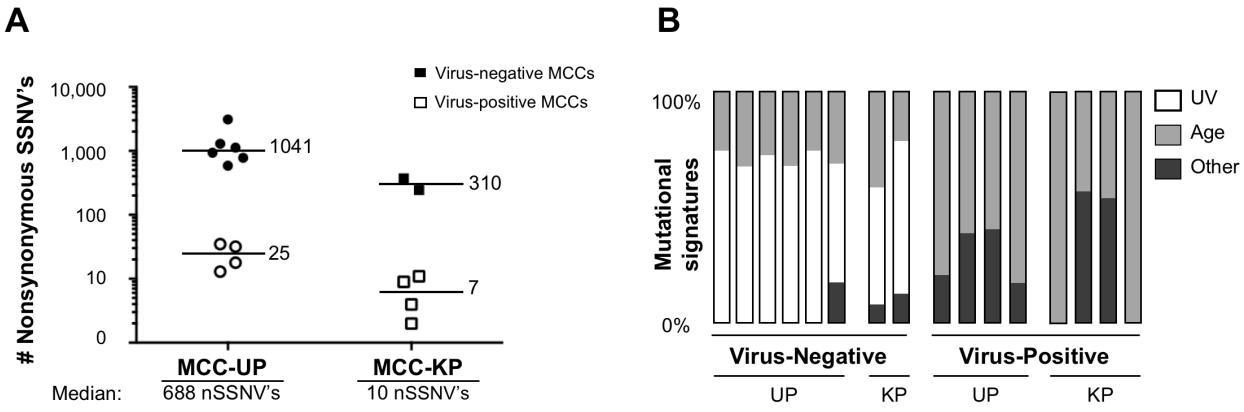


Figure 5. Relationships of mutational burden, MCPyV status and unknown primary status in MCC. Panel **A:** Number of nonsynonymous single somatic nucleotide variations (nSSNV's) among virus-negative cases MCC-UP (n=10) and MCC-KP (n=6). Median values for virus-positive and -negative subgroups are denoted adjacent to horizontal black lines. The median number of nSSNV's for MCC-UP and MCC-KP patients are denoted below the X-axis. The Mann-Whitney test was performed to characterize the difference between the median values (p =0.016). Panel **B**: Relative frequency of an ultraviolet light or age-induced mutational signature grouped by viral and primary status. This panel is modified from data presented in Goh et al.

DISCUSSION

Here we report that among patients presenting with nodal disease, those with MCC with an unknown primary (MCC-UP) had a striking 70% reduced risk of death from MCC as compared to MCC-KP patients. We show that unknown primary status is also relevant for outcomes among patients presenting with distant metastatic (stage IV) disease. Additionally, we examined the relationship between MCC-UP presentation and immune function. MCC-UP patients never presented with immune suppression, had elevated MCPyV oncoprotein antibody titers and presented with a strikingly higher median number of tumor-associated nonsynonymous exome mutations as compared to patients presenting with MCC-KP.

Mutational analyses further revealed UV-signature mutations in virus-negative tumors even among patients presenting with MCC-UP, indicating that these nodal lesions did arise from primary skin disease. These findings collectively suggest that enhanced immune function may underlie the development of MCC-UP through elimination of the primary skin lesion.

Our findings indicating improved survival among nodal MCC-UP patients are highly consistent with several previous reports which also indicate a 60-70% reduced risk of death from MCC[127,410,412]. Other reports have speculated that regression of the primary lesion may be immune-mediated[61,127,412], however, there has been little evidence to support this theory. Therefore, we investigated differences in immune function and tumor immunogenicity between MCC-UP and MCC-KP patients. We found a statistically significant difference in the incidence of immunosuppression among MCC-UP and MCC-KP patients. This suggests that immune function is protective in MCC and may be contributing to regression of the primary lesion. While we saw no examples of MCC-UP arising in immunosuppressed patients (among 72 patients), there are in fact isolated cases in the literature. These include 2 reported cases of MCC-UP occurring in patients who received organ transplantation, and 3 with HIV[411,413]. These five cases were reported among a total of 90 that were drawn from largely independent case reports and therefore likely reflect a publication bias that might tend to over emphasize this less common scenario in which MCC-UP can develop in patients with suppressed immune function[411,413].

Additionally, our finding that MCC-UP patients have higher oncoprotein antibody titers at the time of diagnosis may reflect a more robust immune response against MCC[129]. Notably, serological status was included as a parameter in our multivariate survival analyses and overall oncoprotein seropositivity was not found to be statistically different between MCC-UP and MCC-KP patients, indicating that simply the presence of an antibody response is not associated with MCC-UP presentation. Rather, the magnitude of the response as reflected by the antibody titer is associated with MCC-UP presentation, suggesting that these antibodies reflect augmented immunity.

Indicating that the tumors themselves may be more immunogenic in MCC-UP patients, we found that these tumors harbor significantly higher mutational burdens than MCC-KP tumors. High mutational burdens have been shown to elicit robust immune responses against neoantigens in several tumor types[175]. Therefore, higher mutational loads among MCC-UP tumors may reflect enhanced neoantigen presentation, thereby enabling immune-mediated clearance of the primary lesions and improving survival. Notably, higher mutational loads among MCC-UP tumors relative to MCC-KP tumors were observed among both virus-negative and virus-positive subsets of MCC, though statistical significance was only achieved within the virus-positive group. This was surprising in the setting of virus-positive tumors because they have a much lower mutational burden (median 11 per tumor) than virus-negative tumors (864.5 per tumor). This finding suggests that the presence of even these small numbers of neoantigens within the virus-positive MCCs (median 25 for MCC-UP and 7 for MCC-KP) may significantly enhance immune activity even for these MCC tumors known to express highly antigenic viral oncoproteins. Future investigation into differences in T cell infiltration and function between tumors from MCC-UP and MCC-KP patients could provide additional insight into the immunological underpinnings of unknown primary presentation.

Our study also has important implications relating to the origin of MCC-UP tumors. It has been proposed that nodal disease observed in MCC-UP patients originated within the nodal basin instead of on the skin[418]. Here we provide strong evidence that virus-negative MCC-UP tumors are skin derived based on the finding that when these tumors present in a lymph node they have high-levels of UV-signature mutations[417] (namely C to T transitions: **Fig. 5B).**

Notably, MCC is not the only cancer in which unknown primary presentation is associated with improved survival. A recent systematic review of melanoma presenting with an unknown primary (MUP) reported a reduced risk of disease-specific death among stage III and stage IV disease (17% and 15% reduction respectively)[419]. Like MCC, it is postulated that MUP presentation is immune mediated. While there is currently limited evidence to link immune function and MUP presentation, one study indicated that MUP patients were 1.9-fold more likely to either present or develop vitiligo during follow-up than patients

with a known primary site[420]. This suggests that a specific anti-melanocytic immune response is correlated with clearance of the primary tumor[420]. Importantly, the markedly reduced relative risk of dying from MCC observed among MCC-UP (70%) as compared to MUP (17%) suggests that MCC may be a more immune-responsive disease. This notion is supported by the higher response rates to checkpoint inhibition observed in MCC[1,65].

Importantly, we do not believe that the survival advantage observed among MCC-UP patients is attributable to differences in initial treatment including immune-based therapies. In all but one case, initial treatment was via standard therapies (surgery, radiation and/or chemotherapy) and these parameters are included within our multivariate analyses that indicated no significant difference in initial treatment between MCC-UP and MCC-KP patients. Notably, we did not include recurrent disease treatment modalities within our multivariate analysis because the probability of developing a recurrence is significantly affected by the initial presentation of a primary lesion (i.e. MCC-KP patients were significantly more likely to recur). Of note, 17 patients within our cohort who developed recurrent disease received various immune-based therapies (**Supplementary Table 2**). However, there was no association between receiving immunotherapy and presentation with a primary lesion (24.2% of MCC-UP and 23.7% of MCC-KP patients received immunotherapy for their subsequent recurrence). To date, the most effective immunotherapies for treating MCC are PD-1 checkpoint inhibitors[1,65] and of the 6 patients treated with these agents, all 6 presented with a known primary lesion. Therefore, any benefit that immunotherapy had on improving survival in this cohort would potentially reduce the survival advantage associated with MCC-UP presentation.

Our study had several limitations. Because of the retrospective nature of this study, some patients' records were not complete or could not be obtained. Notably, there was likely referral and self-selection bias due to the tertiary, highly specialized nature of our multidisciplinary program. As a result, our cohort has a slightly higher proportion of MCC-UP (59% of stage IIIB) as compared to other cohorts (32%-55%)[127,410,421]. The classification of MCC-UP status was based upon at least two comprehensive skin exams, including one by the initially diagnosing physician and one at the referral or tertiary site. It is

possible, however, that diagnoses of other skin cancers were in fact missed cases of MCC. Based upon our prior experience with reviewing pathology records and pathological evaluation of other tumor biopsies at the time of MCC diagnosis, we estimate that misdiagnosis of other skins cancers as MCC occurs in fewer than 5% of cases. Importantly, the survival data for stage IIIB patients in our cohort closely resembles previously published reports[127,410,411,421,422], indicating that the survival difference observed between MCC-UP and MCC-KP patients is likely not attributable to recruitment bias or consistent misdiagnoses of other skin lesions within this cohort.

Additionally, although MCC is increasing in incidence it remains an uncommon disease. Therefore, while our study size of 123 is large for advanced MCC, only 20 patients presented with stage IV disease, limiting conclusions that can be drawn from this small subgroup. Most notably, when evaluating the presence of visceral disease among stage IV patients, 3 of 11 (27%) MCC-UP patients presented with visceral involvement, while 7 of 9 (78%) MCC-KP presented with visceral disease. Therefore, we cannot conclude whether presentation with an UP versus a KP affects survival when accounting for the presence of visceral disease because of the small sample size. Ideally, unknown primary status would be evaluated among patients with stage IV node-only disease separately from stage IV patients with visceral disease, however, the size of our study prevents this distinction. A reasonable interpretation is that KP disease is more likely to spread and persist successfully in key organs, however, further evaluation of these findings in a larger cohort is necessary.

Importantly, there are several clinically relevant implications of these findings. Multiple independent groups have corroborated that patients presenting with nodal MCC-UP have significantly improved survival. Therefore, unknown primary status is now being used to prognostically stratify patients in the recently released AJCC 8th edition staging system to more accurately reflect their improved outcomes[61].

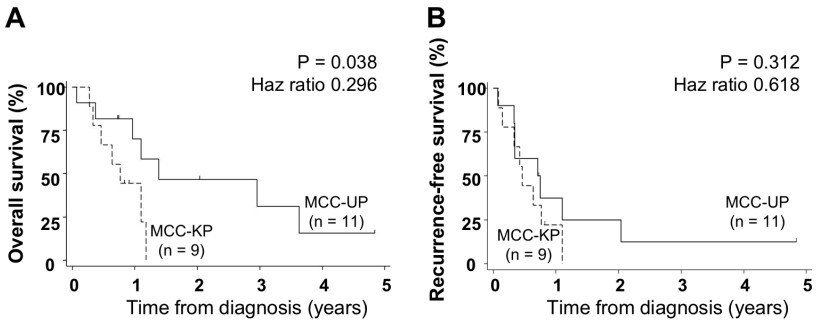
Our results also support additional changes for future staging revisions. Firstly, we show that there is a statistically significant survival difference between patients presenting with nodal involvement of skin-draining basins only as compared to those presenting with non-skin draining nodes. We therefore

propose that MCC-UP patients presenting with only skin-draining nodal involvement should be classified as regional (stage III) while those with involvement of non-skin draining nodes should be classified as distant metastatic (stage IV) disease. Secondly, further investigation into the survival advantage observed among stage IV MCC-UP patients may improve prognostic accuracy for patients with distant metastatic disease.

Lastly, it is possible these findings may have implications for the appropriate management of patients presenting with MCC-UP. While there are limited therapeutic options for late stage MCC patients, the use and availability of immune-based therapies is rapidly increasing. Checkpoint inhibitors, including anti-PD-1, have remarkable efficacy in treating both virus-positive and -negative MCC[65]. The likely link between immune function and unknown primary status suggests that unknown primary status and response to immune therapies should be examined in future studies.

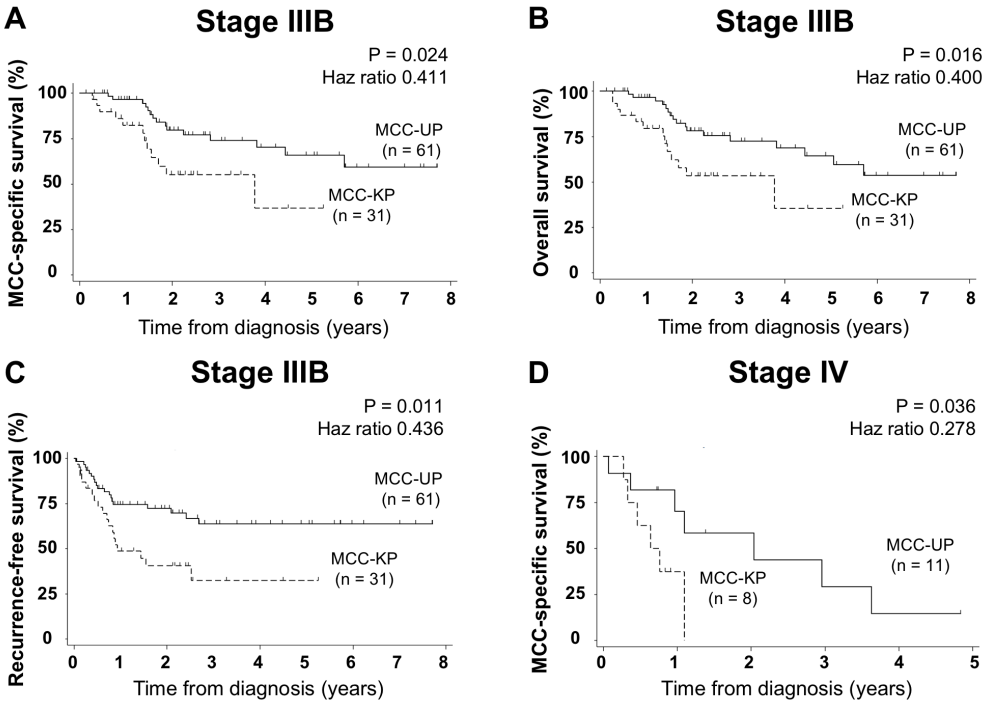
SUPPLEMENTAL FILES

Supplementary Figure 1



Supplementary Figure 1. MCC-UP status predicts better overall but not recurrence-free survival among patients with stage IV disease. Panel A depicts overall survival and panel B depicts recurrence-free survival among 20 stage IV MCC patients. Overall and recurrence-free survival were performed using a Cox-proportional hazards model.

Supplementary Figure 2



Supplemental Figure 2. MCC-UP status predicts better survival among <u>non-immunosuppressed patients</u> with either stage IIIB or IV disease. Panel A depicts MCC-specific survival B illustrates overall survival and C indicated recurrence-free survival for 92 patients with stage IIIB MCC by unknown primary status. Panel D describes MCC-specific survival for 19 patients with stage IV MCC. MCC-specific survival analyses was completed using a competing-risks regression model with the competing risk being defined as death by all causes except MCC. Overall and recurrence-free survival was performed using the Cox-proportional hazards model.

Supplementary Table 1: Patients with unknown primary lesions and without immunosuppression have improved survival among patients with stage IIIB and stage IV MCC						
	Univariate analysis			Multivariate analysis		
Stage IIIB (n = 92)	**HR**	**95% CI**	**P**	**HR**	**95% CI**	**P**
MCC-specific survival	0.411	0.191 to 0.888	0.024	0.362	0.154 to 0.849	0.021
Overall survival	0.400	0.190 to 0.841	0.016	0.407	0.179 to 0.926	0.032
Recurrence-free survival	0.436	0.230 to 0.826	0.011	0.422	0.205 to 0.865	0.018
Stage IV (n = 19)						
MCC-specific survival	0.278	0.084 to 0.922	0.036	0.230	0.046 to 1.137	0.071
Overall survival	0.278	0.084 to 0.922	0.036	0.198	0.035 to 1.136	0.069
Recurrence-free survival	0.685	0.027 to 1.772	0.436	0.801	0.229 to 2.802	0.728

Supplementary Table 2: Summary of patients receiving immunotherapies		
Non-checkpoint inhibitor immunotherapies	**# of UP patients**	**# of KP patients**
Anti-CD137 (4-1BB)	2	3
TLR4 agonist (GLA)	0	2
IFNbeta injection	0	1
IL-12 DNA electroporation	2	0
Anti-CD56	2	0
Autologous T cell therapy	1	0
IL-2	1	0
Checkpoint inhibitor immunotherapies	**# of UP patients**	**# of KP patients**
Anti-PD-L1 (avelumab)	0	2
Anti-CTLA4 and anti-PD-1 (ipilimumab and nivolumab)	0	1
Anti-PD-1 (nivolumab/pembrolizumab)	0	3
Other therapy (excluding radiation, surgery, chemo)	**# of UP patients**	**# of KP patients**
Tyrosine kinase inhibitor (votrient)	1	1
Somatostatin analog (sandostatin)	4	0
topoisomerase inhibitors (Topotecan and irinotecan)	3	0

CHAPTER 10: CONCLUSIONS AND FUTURE DIRECTIONS!

Vandeven, NA

Summary of Research Findings

The first virally-driven cancer was described in 1911 by Francis Peyton Rous. His seminal experiments indicated that cell-free extracts could transmit avian cancer between hens of the same brood[423]. Since that time, seven oncogenic viruses have been described and their mechanisms of oncogenesis are discussed within **Chapter 1**[424]. Broadly speaking, pathogen-driven oncogenesis is either induced indirectly through chronic inflammatory processes or directly through integration and expression of oncogenes[9]. Protection against infection or pathogen clearance prior to cancer development are obvious approaches to reduce pathogen-driven cancer incidence. However, several of these pathogens are typically asymptomatic and ubiquitous[104,105]. One such virus is the Merkel cell polyomavirus (MCPyV) which is the causative agent of ~80% of Merkel cell carcinomas (MCC), a rare but deadly skin cancer[57]. MCPyV is highly prevalent (60-81% of adults are infected[425]), typically asymptomatic and very rarely causes cancer, making preventative vaccination against this polyomavirus of little therapeutic value. In the unlikely event that MCPyV causes cancer, mortality rates are three times that of malignant melanoma[221]. MCPyV initiates cancer formation directly through clonal integration into the host cell genome and the subsequent expression of two oncoproteins the small and the Large T-antigens (sT & LT)[57]. Oncogenic features of these two T-antigens are described in **Chapters 1 & 3.** While currently only ~2,500 new cases of MCC are diagnosis per year in the US, the incidence rate of MCC is rising with the advancing age of the 'baby boomer' generation and rates are projected to reach ~3,500 per year by 2025 as described in **Chapter 2**.

Immunity and MCC

Since the discovery of MCPyV almost a decade ago, significant progress has been made in understanding the etiology of this disease and MCC's complex interplay with the immune system (details discussed in **Chapter 3**). The importance of immune function in MCC was first noted with the finding that patients with overt immunosuppressed are 10-30 times more likely to develop MCC than non-

immunosuppressed patients[26,84,99,120]. Subsequently, infiltration of CD8 T cells as well as a Th1-like inflammatory response were found to be associated with improved outcome among MCC patients[91]. Since these early studies, the significance of the CD8 T cell response against MCC has only been further validated with the finding that intratumoral infiltration of MCPyV-specific CD8 T cells is associated with improved outcome[91,92], particularly when these T cells have high functional avidity (i.e. they can elicit effector function at low concentrations of cognate antigen)[137]. Despite these data, the use of autologous MCPyV-specific CD8 T cell therapy had surprisingly little efficacy against MCC in four initially treated patients[154,426]. Fortunately, agents targeting the PD-1 pathway have yielded strikingly high response rates in MCC as compared with other solid tumors, and markedly improved outcomes compared to cytotoxic chemotherapy, the prior treatment of choice for advanced MCC. Despite the durable benefit observed in many patients, roughly half of MCC patients do not respond, indicating an urgent need to 1) identify biomarkers predictive of response, 2) identify immune evasion mechanisms that underlie PD-1 blockade resistance, and 3) explore novel immune therapies that can rescue non-responders. Topics pursued in this dissertation pertain to all three of these goals and seek to provide a basis for ongoing efforts to improve our ability to treat this disease.

Downregulation of E-selectin

While robust CD8 T cell infiltration into MCC tumors is associated with 100% survival, only 4-18% of patients experience this endogenous response suggesting that most MCC tumors actively block T cell entry. In order for T cells to extravasate from tumor vasculature into tumor tissue, ligation between adhesion molecules such as cutaneous lymphocyte antigen (CLA) expressed on the T cells and E-selectin expressed by endothelial cells must occur[147]. Squamous cell carcinomas (SCCs) have been shown to downregulate expression of E-selectin within tumor vasculature and thereby prevent T cell entry[222]. In order to determine whether a similar mechanism was occurring in MCC tumors, we performed immunohistochemical staining against E-selectin on MCC tumors as described in **Chapter 4**[149]. We found that the majority (52%) of MCCs had downregulation of E-selectin and that this was associated with poor intratumoral CD8 lymphocyte infiltration and worse outcome. Downregulation of E-selectin can be induced by local nitric oxide production, often produced by myeloid derived suppressor cells (MDSCs) or

M2-type macrophages[148]. Nitrosylation of proteins is a hallmark sign of local nitric oxide production and was therefore used as a surrogate to measure NO production within MCC tumors[223]. We found that increased nitrotyrosine within MCC tumors was associated with low E-selectin expression and decreased CD8 lymphocyte infiltration[149]. This suggests that local NO production by intratumoral MDSCs or M2 macrophages may be facilitating the observed T cell exclusion. In order to further investigate tumoral macrophage phenotypes and function, we are collaborating with Drs. Robert Pierce and Jean Campbell at the Fred Hutch Cancer Research Center to develop multispectral immunohistochemistry (mIHC) panels that will allow us to stain 6 markers simultaneously within fixed tumor tissue. Panels in development are listed in **Table 1** and will enable delineation of M1 and M2 macrophage phenotypes and help determine whether these phenotypes correlate with T cell homing and infiltration. In addition, we will evaluate changes in E-selectin expression within MCC tumors pre- and post- PD-1 blockade therapy to assess the role of this mechanism in response to these therapeutic agents.

Table 1. multiplexed-IHC panels in development

CD8 focus	Antigen Presentation	Mphage phenotype	CD4 T helper type	T cell Homing
CK20/CD56	CD163/68	CD163	CD4	CD4
CD3	CD80/86	CD33	PD-1	E-selectin
CD4	PD-L1	CD14	FOXP3	CD8
CD8	CIITA	CD206	GATA-3	CK20/CD56
PD-1	HLA-DR	NOS2	TBET	CD31
PD-L1	pan HLA class-I	Arginase	BCL-6	CLA

Impaired antigen presentation in MCC may prevent adequate T cell stimulation and recruitment

An additional component required for appropriate T cell homing and infiltration is the expression of pro-inflammatory chemokines, secreted predominantly by activated dendritic cells (DCs) and macrophages. These cells become licensed through interactions with CD4 T cells recognizing cognate antigen and simultaneous ligation of CD40 and CD40L[257]. Uptake of antigen by dendritic cells and macrophages, requires phagocytosis which can be inhibited by CD47, a signal-regulatory protein that binds to the receptor SIRPα expressed on the surface of macrophages and dendritic cells. CD47 is often called a 'don't eat me' signal[427] and can be expressed by tumor cells, including MCCs (described in **Chapter 3**). A recent study by Xu *et al.* indicated that CD47 blockade increases phagocytosis and can induce potent antitumor T cell activity that is STING and type-I IFN dependent[428]. We have recently found that increased

expressing of CD47 in MCCs is associated with poor outcome among MCC patients[2] and that treatment of MCC cell lines with TTI-621, a SIRPα agonist capable of inhibiting the CD47 pathway, results in increased phagocytosis of virus-positive and virus-negative cells lines[2] (**Figure 1**). These data suggest that this agent may be able to facilitate tumor degradation and subsequent antigen presentation, thereby stimulating antitumor T cell responses. A clinical trial (NCT02890368) treating patients with TTI-621 is currently enrolling patients with advanced and refractory percutaneously-accessible solid tumors including MCC patients. Of note, an additional anti-phagocytic molecule CD200 has been shown to be upregulated on MCC tumor cells as described in **Chapter 3**[96]. Future studies will investigate the efficacy of agents targeting these two anti-phagocytic pathways and their effects on tumor immune responses including DC and T cell activation.

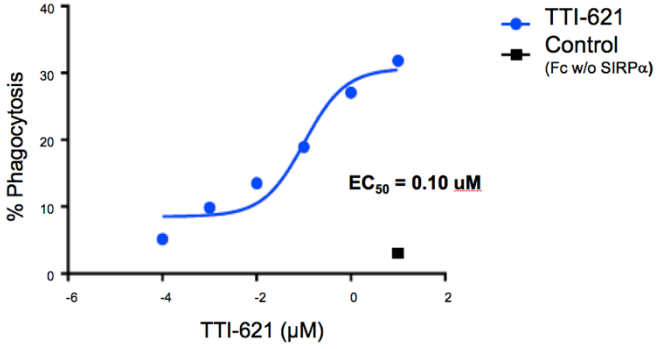


Figure 1: Targeting CD47 with TTI-621 promotes increased phagocytosis of MCC cell line. An MCPyV-positive cell line was incubated with isotype control or TTI-621 and phagocytosis was calculated as the percentage of macrophages that were also positive for the tumor cell label by flow cytometry (Vandeven SITC 2016[2]).

Additional aspects of DC activation including stimulation by type I IFNs induced via the stimulator of interferon genes (STING) pathway which may also be impaired within MCC tumors. The STING pathway senses cytosolic DNA released by dying or digested tumor cells which can bind to cyclic GMP-AMP synthase (cGAS), resulting in the generation of cyclic GMP-AMP (cGAMP; **Figure 2**)[429-432]. cGAMP then binds STING which is expressed by numerous cell types including endothelial and epithelial cells as well as macrophages and dendritic cells. Ultimately, STING activation results in type I IFN production and expression of IFN-stimulated genes (**Figure 2C)**[433]. Fascinatingly, several tumor viral oncogenes, including E7 from human papillomavirus and E1A from adenovirus, can inhibit the cGAS-STING pathway thereby evading type-I IFN induction and reducing DC activation[434]. Disruption of this pathway is mediated by viral expression of the LxCxE motif, a motif that is shared among many DNA viruses and is also essential in binding the tumor suppressor protein retinoblastoma (Rb)[434]. The MCPyV Large T-antigen also contains an LxCxE motif and therefore it is *hypothesized that MCPyV LT can also abrogate*

the STING pathway via this motif which may result in impaired DC activation, chemokine expression, antigen-presentation and ultimately T cell activation. Experiments to determine the role of the STING pathway and the potential for MCPyV to block this signaling are ongoing. Investigation into this pathway is of significant interest moving forward as agents have been developed that can target the STING pathway which may be beneficially helpful in MCC.

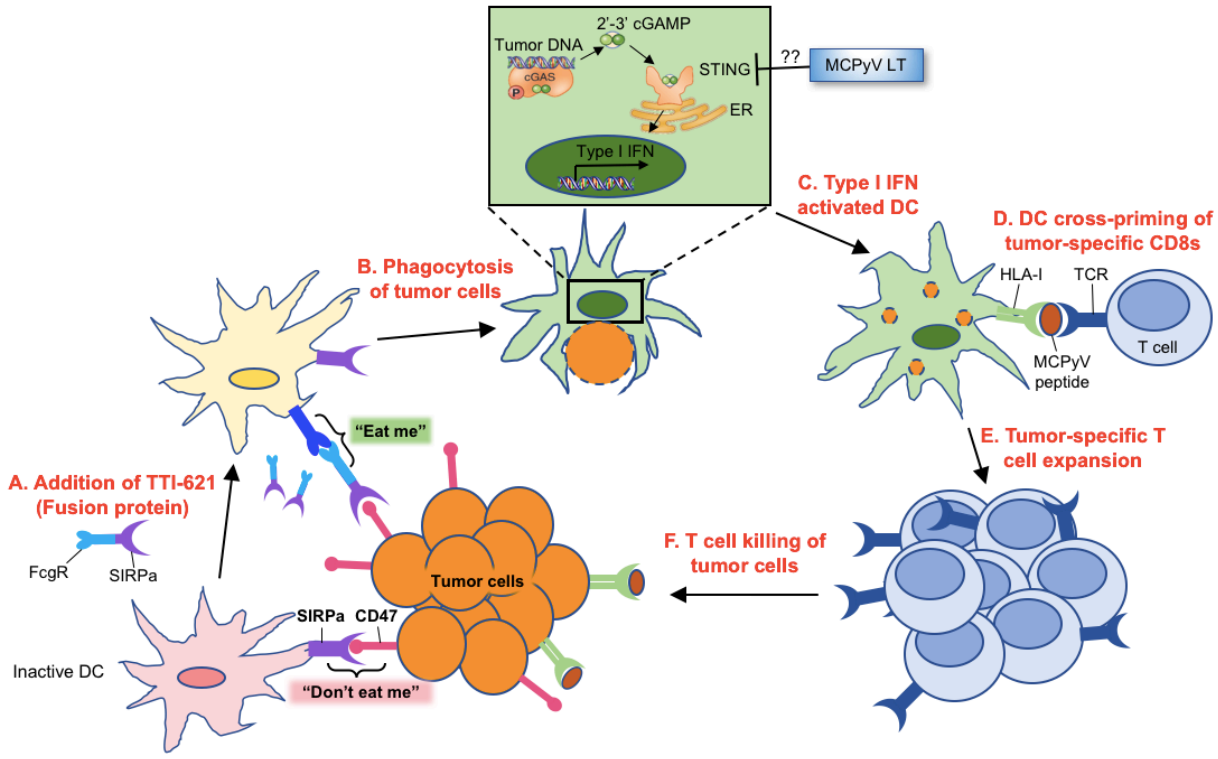


Figure 2: Model of CD47 blockade enhancing antigen-presentation by DCs to increase T cell priming. **A**: MCC tumor cells overexpress CD47 providing 'don't eat me signal' and preventing phagocytosis by macrophages and dendritic cells (DCs). Addition of SIRPa-FcgR fusion protein (TTI-621) blocks CD47 signaling and enables 'eat me' signaling. **B**: Phagocytosis of tumor cells is initiated by DCs and macrophages. Tumor cellular debris and DNA induce signaling via STING-cGAS induction. **C:** Type I IFN genes are expressed and activate DCs to elevate expression of HLA and co-stimulatory molecules. **D**: Cross-priming of CD8 T cells induces (**E**) tumor-specific T cell expansion. **F**: Expanded and activated tumor-specific CD8 T cells can mediate direct tumor cell killing. Adapted from Liu J Hematol Oncol 2017.

What about CD4 T cells in MCC? Another aspect of the immune response that is critical for initiating effective immunity against tumors is the activation and appropriate polarization of CD4 helper T cells[157,256]. CD4 T cells significantly improve the function of CD8 T cells, in particular they can enhance CD8 T cell survival, proliferation, tumor infiltration and function[299,435,436]. CD4 T cells can differentiate into several discrete subtypes each driven by distinct transcription factor expression resulting in the production of different but often overlapping cytokine profiles[4,157]. These CD4 subtypes and their role in

cancer immunology are described in detail in **Chapter 5**. Analysis of MCC tumoral mRNA transcripts indicates that elevated expression of genes associated with the majority of the known CD4 subtypes are correlated with improved outcome in MCC (**Figure 3**). These data suggests that CD4 T cells are important in controlling disease and that they likely mediate pleiotropic effects. Importantly, mRNA transcripts were derived from whole MCC tumors, therefore we have limited knowledge regarding the subtype of *MCPyV- specific* CD4 T cells. Numerous technical challenges have limited the study of cancer-

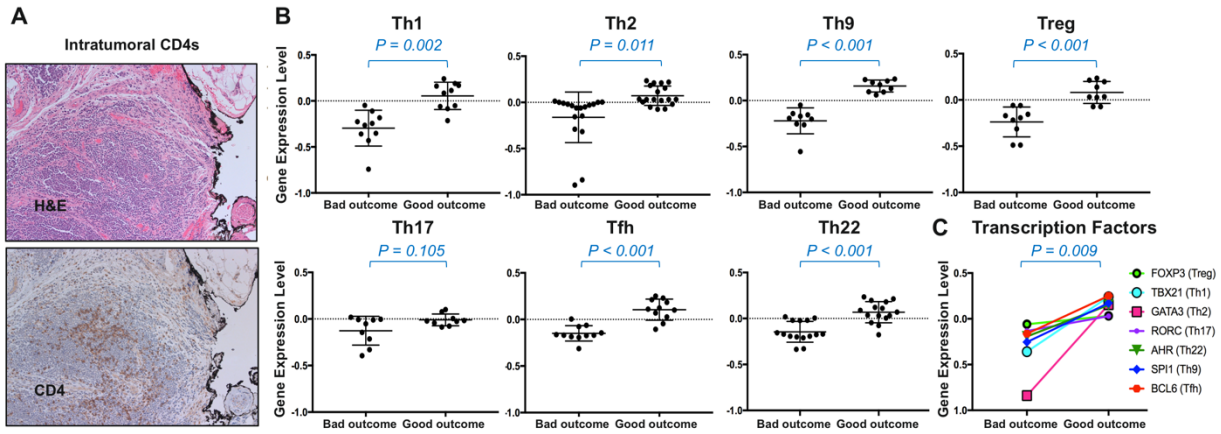


Figure 3: CD4 T cells can infiltrate MCC tumors and intratumoral CD4 mRNA expression profiles are correlated with outcome. A: Immunohistochemical staining of hematoxylin & eosin (upper panel) and CD4 (lower panel). **B:** mRNA gene expression profiles of CD4 subtypes as measured by Affymetrix RNA microarrays. Each dot represents a separate gene associated with the indicated process or CD4 T cell subset. Gene expression level is relative to given gene's expression across all MCC samples. Patients were binned as 'Bad outcome' (MCC presented with or progressed to distant metastasis) or 'Good outcome" (local disease presentation with no subsequent recurrence or nodal disease at presentation with no progression during follow-up of longer than 24 months). **C:** Shows the average mRNA expression of canonical transcription factors within tumors from patients with 'Bad' and 'Good' outcomes.

specific CD4 T cells. However, we have developed several approaches that have identified a total of 12 CD4 T cell epitopes (8 of which are published; **Chapter 6**) and generated reagents that now permit a detailed characterization of the MCPyV-specific CD4 response. These findings are the focus of **Chapters 6 & 7**. Additionally, several HLA class-II tetramers have been developed, allowing for the first time, isolation of MCPyV-specific CD4 T cells.

One identified CD4 epitope is of particular interest. This epitope, WEDLFCDESLSSPEPPSSSE (referred to as 'WED'), is located within MCPyV LT (LT-209-228) and has several attributes that make it a an ideal immune target. Importantly, it encompasses the LxCxE motif which binds retinoblastoma protein (Rb) thereby inducing cell cycle progression. This interaction is thought to be one of the main oncogenic

mechanisms in virus-positive MCCs[3,103] (detailed in **Chapters 3 & 7).** Consequently, expression and conservation of this epitope is required for oncogenesis and tumor persistence. Additionally, we show that 'WED' can be presented by at least three common HLA class-II allele types expressed by a total of 80% of the HLA class-II typed MCC patients within our cohort, indicating that this epitope is likely present in the majority of MCCs. These data suggest that inclusion of this epitope within an MCPyV-based vaccine would be capable of inducing CD4 T cell response in most MCC patients. However, ablation of oncogenic activity would be an essential prerequisite prior to administering this epitope in the context of the MCPyV LT-antigen. Oncogenic activity may be inhibited through two point mutations, both of which disrupt Rb binding. These include E216K, a mutation within the LxCxE motif[103], and S220A which prevents phosphorylation of a crucial serine residue just adjacent to the LxCxE motif[3]. We found that 'WED'-specific T cell clones generated against the wild-type, unphosphorylated LT sequence are capable of recognizing the S220A mutant, but not the E216K mutant (**Chapter 7**). These results suggest that oncogenic activity could be disrupted while retaining immunogenicity using the S220A mutant.

Crucially, additional experiments are required and on going prior to clinical testing of an MCC therapeutic cancer vaccine containing this epitope. The 'WED'-specific CD4 T cell clones described were generated against the WT-sequence and showed cross-reactivity to the S220A mutant, however, in the setting of a vaccine, T cells would be induced via S220A and must then be active against the WT-LT sequence. Therefore, this reverse cross-reactivity must be verified. Another critical aspect of vaccine development is to determine the appropriate method of delivery. Immunomic Therapeutics has developed DNA vaccine vectors encoding the lysosomal associated membrane protein 1 (LAMP1) which promotes lysosomal protein degradation and subsequent HLA class-II antigen presentation. Studies utilizing these vectors (LAMP1-vax) encoding various antigens have shown successful induction of CD4 T cell responses[437,438]. Therefore, in collaboration with Immunomic Therapeutics, we have generated several DNA vectors encoding WT-LT and sT as well as three mutants including LT-E216K, LT-S220A and sT-91-95AAAAA (reported to disrupt sT oncogenic function). Using a panel of MCPyV-specific CD8 and CD4 T cell clones, we will test the immunogenicity of each of these T-antigen sequences (WT and mutants) in vectors with and without the LAMP1 sequence to compare T cell proliferation and IFNγ secretion. If

MCPyV-specific T cells are capable of being stimulated by these vectors, we will then generate panels of autologous DCs and perform *in vitro* priming experiments to determine whether these vaccines can in fact induce *de novo* responses[386]. *In vitro* priming using these vectors would provide strong preclinical support for initiation of MCC therapeutic vaccination clinically. Should verifying detoxification of the MCPyV T-antigens prove too challenging for use of a DNA vector-based approach, we could use synthetic long peptides encoding known CD8 and CD4 epitopes (such as 'WED') that could provide an alternative strategy without fear of inducing oncogenesis[439].

Notably, if 'WED' were to be used therapeutically within a vaccine, it would also be critical to ensure that this sequence does not induce cross-reactivity against self-proteins, which could potentiate autoimmunity. BLAST analysis of the 'WED' epitope indicated that several human proteins share homology with WED and contain an LxCxE motif. In order to test for potential cross-reactivity, peptides encompassing the LxCxE motif within these self-peptides were synthesized (**Table 2**) and incubated overnight with 'WED'-specific clones. Reactivity was assessed via IFNγ ELISA and indicated that the none of the five tested clones reacted with these self-peptides (**Figure 4A**). Additionally, the LxCxE motif is conversed among all human polyomaviruses and several other DNA viruses, therefore, 13mer peptides spanning the LxCxE motif for all human polyomaviruses and select DNA viruses (selection based upon at least 30% homology to MCPyV in that region and a predicting binding affinity of < 1000 nM to HLA-

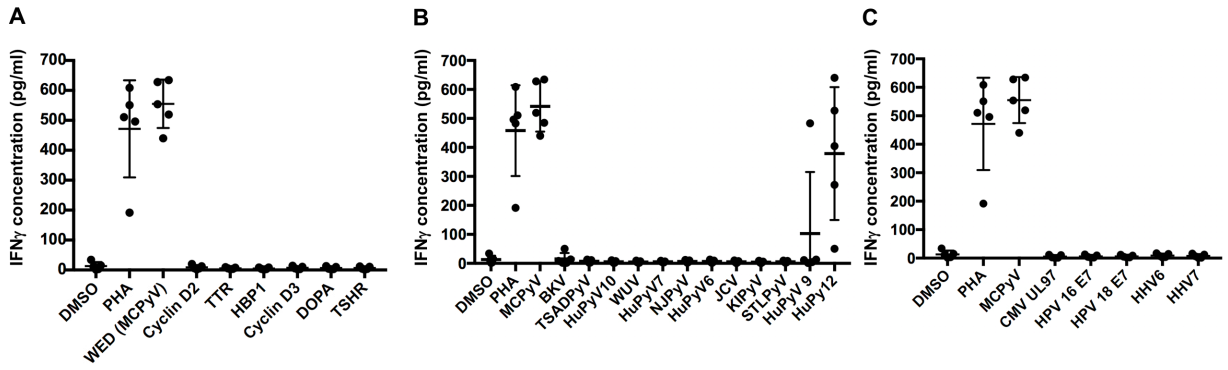


Figure 4: 'WED'-reactive clones cross-react with HPyV12 and HuPyV9 homologous sequences. **A**: MCPyV-WED clones (5 clones from 5 individuals) were screened for IFNγ secretion response against homologous sequences human proteins identified via BLAST against 'WED'. **B**: IFNγ ELISA was used to evaluated cross-reactivity against all other human polyomaviruses. **C**: MCPyV-WED clones (5 clones from 5 individuals) were screened for IFNγ response to other human DNA viruses containing a homologous region. Mean and SD are denoted in each panel.

TABLE 2: Peptides to test cross-reactivity with 'WED'-specific clones peptides

Source	Protein	Peptide sequence	Predicted IC50 binding affinity DRB1*0401 (nM)
Human Polyomaviruses			
MCPyV	Large T-antigen	EDLFCDESLSSPE	22.8
HuPyV12	Large T-antigen	SDLFADETLSSTS	47.5
HuPyV6	Large T-antigen	EDLYCDEHLSASE	146.2
HuPy7	Large T-antigen	EDLYCTEELSSSD	110.7
HuPyV10	Large T-antigen	EDLSCNESFAPSD	4795.5
WUV	Large T-antigen	EELRCNEEMPKSP	2940.2
BKV	Large T-antigen	DLFCHEDMFASDE	162.3
JCV	Large T-antigen	DLFCHEEMFASDD	177
MWPyV	Large T-antigen	EDLSCNESFAPSD	4795.5
TSADPyV	Large T-antigen	DLFCHESTIPSDE	10.9
NJ-PyV	Large T-antigen	ADLHCDESPISSS	366.5
STLPyV	Large T-antigen	DDLTCNESFNCSD	6870.7
KIPyV	Large T-antigen	EELRCNESMPSSP	613.1
HuPyV9	Large T-antigen	DDLFCSETISSSD	21.3
Human DNA viruses with LXCXE motifs and predicted binding to DRB1*0401 <1000 nM and at least 30% homology within core 9-mer sequence			
MCPyV	LT	EDLFCDESLSSPE	22.8
HPV 16	E7	TDLYCYEQLNDSS	191.3
CMV	UL97	GLRCRETSAMWSF	70
HHV7	U69	TDLLCHESLTVSP	134
HHV6	U69	EELLCHESLLDSP	393
Human self-proteins with LxCxE motifs, predicted binding to DRB1*0401 <1000 nM and at least 30% homology within core 9-mer sequence			
Human	Cyclin D2	MELLCHEVDPVRR	284.4
Human	Thyrotropin receptor (TTR)	ESLMCNESSMQSL	133.2
Human	HBP1	LELLQCNENLPSS	460
Human	DOPAchrome tautomerase precursor (DOPA)	HLLCLERDLQRLI	142.4
Human	thyroid stimulating hormone receptor (TSHR)	ESLMCNESSMQSL	133.2

DRB1*0401) were also generated (**Table 2**). Five 'WED'-specific clones from five different subjects were stimulated with these viral peptides and IFNγ secretion was measured as a marker of reactivity using an ELISA assay. Four of the five tested clones reacted strongly to the homologous region of HuPyV12 and one of five clones reacted to HuPyV9 (**Figure 4B**) while none of the tested clones responded to stimulation with homologous peptides from other DNA viruses (**Figure 4C**). Cross-reactivity to HuPyV12 is unlikely to mediate any negative consequences pertaining to a vaccination strategy against MCPyV. However, one hypothesis is that this cross-reactivity with HuPyV12 could potentially alter the response or function of MCPyV-specific CD4 T cells. More specifically, HuPyV12 has been detected via PCR within the gut and stool of humans[440] which could induce homing of MCPyV-specific T cells to the gut instead of

the skin. Future investigation into the homing, memory status and subtype analysis could shed light on whether this cross-reactivity results in altered anti-tumor function of these 'WED'-specific CD4 T cells.

Developing better reagents to identify MCPyV-specific CD4 T cells directly ex vivo

Aside from testing the potential of 'WED' as a therapeutic vaccine target, the generation of MCPyV-specific HLA class-II tetramers provides a unique opportunity to study the phenotype and function of MCPyV-specific CD4 T cells directly *ex vivo*. This has been broadly limited in the study of cancer immunology due to the incredibly low frequencies of antigen-specific CD4 T cell responses within the periphery. Using magnetic bead-based enrichment, MCPyV-specific CD4 T cells have been identified at frequency ranging from <1-5 per million CD4 T cells among healthy donors and 5-40 per million CD4 T cells among MCC patients (**Figure 5**). While this magnetic bead-based enrichment protocol is highly effective, there is a significant amount of cell loss and consequently, this method requires large volumes of blood. Often this volume requirement is limiting, therefore we developed a highly sensitive, novel platform

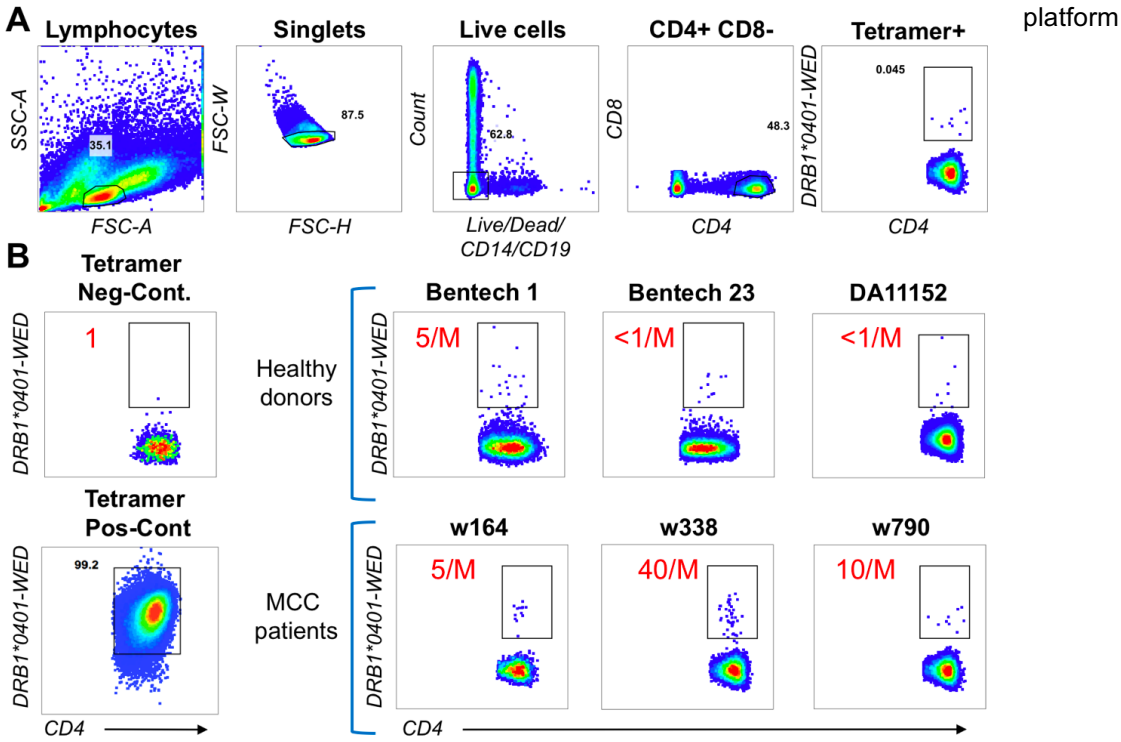


Figure 5: The frequency of 'WED'-specific CD4 T cells ranges from 0.5-40 per million CD4 T cells in the PBMC of healthy donors and MCC patients. A: The gating scheme for DRB1*0401-'WED' tetramer sorts. Sorted cells are Live CD4+Tet+ single lymphocytes and dumped cells are dead, CD8+, CD14+ CD19+ (these are included in the dump gate). B: PBMC from healthy donors and MCC patients were stained with DRB1*0401-'WED' tetramer and enriched using magnetic bead enrichment (EasySep). Red numbers denote the back calculated frequency among the CD4+ T cell population.

using a digital scanning microscope to identify and characterize rare antigen-specific T cells (described in **Chapter 8**).

While the generation of this novel platform and MCPyV-specific HLA class-II tetramers have enhanced our ability to find and isolate MCPyV-specific CD4 T cells, HLA class-II tetramers still have several important limitations especially in comparison to HLA class-I tetramers. The factors that contribute to the differences between HLA class-I and class-II tetramers are: 1) the lower affinity of HLA class-II/peptide complexes; 2) the lower affinity of TCRs for HLA class-II/peptide complexes; 3) the higher instability of soluble HLA class-II monomers in *in vitro* expression systems; 4) the potential for improper registry of peptides displayed by HLA class-II; 5) the lack of participation of CD4 coreceptors in HLA- binding[371]. Recent studies have suggested that the use of higher valency 'dextramers' can circumvent many of these challenges[371]. Structurally, dextramers are composed of dextran backbones, which are polymers of glucose molecules attached through 1–6 and 1–3 linkages[441]. A single dextran molecule carries multiple moieties of streptavidin to which biotinylated peptide-tethered HLA molecules can be assembled[441]. HLA dextramers therefore contain larger aggregates of HLA-peptide complexes than HLA tetramers allowing them to engage more TCRs [371]. In pilot studies comparing a 'WED'-specific tetramer and 'WED'-specific dextramer, we saw significantly higher mean fluorescent intensity with the dextramer than the tetramer and reduced non-specific staining in patients that are of an inappropriate HLA type (**Figure 6**). As a result, the antigen-specific T cell population had a larger degree of separation from the non-

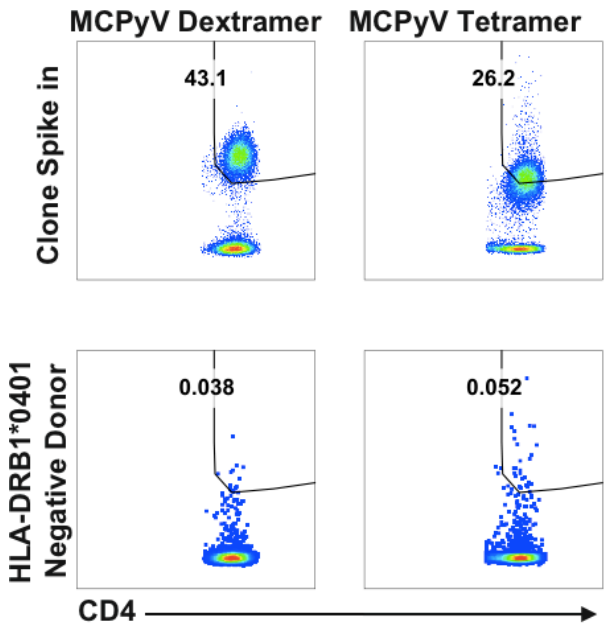


Figure 6: Greater population differentiation and reduced background with MCPyV-dextramer. Tumor infiltrating lymphocytes from a HLA-DRB1*0401 negative MCC patient (bottom row) or MCPyV-specific clone cells spiked into PBMC and stained with either MCPyV-dextramer or MCPyV-tetramer. Dextramer and tetramer staining were evaluated on singlet lymphocytes that were CD4+ and exclusion marker negative as previously described.

antigen-specific T cell population, thereby providing a more well defined dextramer+ population above background signal. There are also important differences in the staining protocols requires for these reagents. Dextramer staining can be performed at 4°C for only 30 minutes as compared to tetramer staining which requires 1 hour at 37°C. This significantly shortens staining times and maintains cells in a less activated/altered state. When using these reagents for sorting cells and performed down-stream functional analysis, it is critical to work quickly and minimize activation so as to retain the true cellular phenotype.

Improving strategies to phenotype MCPyV-specific CD4 T cells

Significant effort have been put into the generation of reagents and methods to identify MCPyV-specific CD4 T cells with the ultimate goal of determining their subtype, exhaustion status and memory phenotype. Insight into these functional aspects may identify novel therapeutic strategies or predictors of response to existing therapies. An initial phenotyping experiment was performed using 'WED'-specific tetramers to identify antigen-specific CD4 T cells and surface staining of a panel of chemokine receptors as surrogate markers of CD4 subtypes as has been described by others (**Figure 7**)[442]. This experiment was conducted on PBMC from two healthy donors and one MCC patient with blood available from two time points. MCPyV-specific CD4 T cells were compared to influenza-specific CD4 T cells isolated at the same time (influenza-specific HLA DRB1*0401 tetramer was obtained from Dr. Kwok). While these results were suggestive of MCPyV-specific CD4 Th2 skewing as compared to influenza-specific cells, the use of a single chemokine receptor to delineate discrete CD4 subtypes is suboptimal. Chemokine receptor expression is not binary but rather on a continuum and as a result, many cells express multiple chemokine receptors to varying degrees making definitive subtype determination challenging if not impossible. Consequently, the use of additional markers including canonical transcription factors and cytokines would greatly enhance our ability to accurately determine CD4 subtypes and would also enable evaluation of their exhaustion status and memory phenotype.

In order to achieve this, flow cytometry as the sole method of characterization is impractical as it would require the use of >20 markers making compensation a significant challenge. Methods such as CyTOF

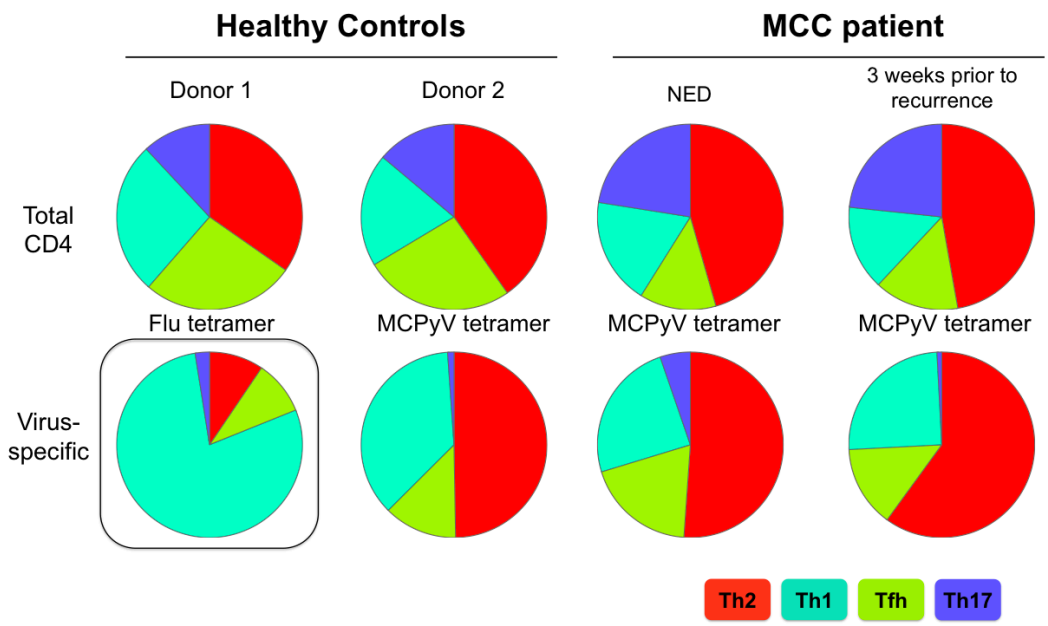


Figure 7: MCPyV-specific CD4 T cells are Th2 skewed relative to influenza specific CD4s. PBMC from two healthy donors and one MCC patient (two time points) were HLA-DRB1*0401-'WED' tetramer enriched and stained with a 12-color antibody panel. Surface chemokine receptors were used to determine subtype specificity including CXCR3 (Th1), CCR4 (Th2), CXCR5 (Tfh), CCR6 (Th17). Vandeven, unpublished.

can evaluate significantly more markers than conventional flow cytometry[322], however, CyTOF is not compatible with HLA tetramers. Recent advances in single cell RNA sequencing have enabled whole transcriptomic analyses at the single cell level. Therefore, we have established a collaboration with Dr. Alex Shalek (MIT) and Dr. Raphael Gottardo (FHCRC) to utilize SMART-seq2, a single cell RNA platform to more accurately phenotype MCPyV-specific CD4 T cells. In this study, we will use PBMC from three populations, 10 healthy donors (individuals who have never had MCC), 8 MCC patients with 'good outcomes' (as previously defined[91]), and 7 MCC patients with 'bad outcomes'. Antigen-specific cells will be sorted from PBMC using the MCPyV-dextramer and an influenza-tetramer in conjunction with several surface markers. Cells will be run on an Aria III cell sorter. Numerous exclusion markers will be included to enhance specificity. Based on preliminary data, we anticipate recovery of ~20-30 cells per donor, correlating to ~100-300 cells per group. Using this approach, the major questions to address are; 1) do MCC patients have 'WED'-specific CD4 T cells that are skewed' away from a Th1 response relative to 'WED'-specific T cells isolated from healthy donors? 2) Do MCC patients with poor outcome have 'WED'-specific T cells that are skewed away from a Th1 response as compared to MCC patients with good outcomes? 3) Are 'WED'-specific CD4 T cells broadly skewed away from an anti-viral Th1 response relative to influenza-specific T cells? 4) Are 'WED'-specific CD4 T cells more 'exhausted' than influenza-

specific CD4 T cells? While the number of cells isolated per person will be small, by having comparator groups composed of multiple individuals, we believe that meaningful differences in phenotype will be identifiable between these groups if they exist. Furthermore, in utilizing single cell RNA sequencing, we will be able to retain the heterogeneous data that single cells provide in the event that a particular individual has markedly desperate phenotypes from others within a given group. This exciting approach will be a major focus of MCPyV-specific CD4 studies moving forward.

An additional method of CD4 characterization using mIHC panels is also underway. CD4 subtype characterization will be performed on fixed tumor tissue and when possible, paired with matched peripheral CD4 T cell phenotyping to determine whether there are important differences between these two compartments. Notably, this panel will define CD4 subtypes based upon 1 or 2 markers each, a previously discussed limitation. However, in most patients we are unable to obtain fresh tumor tissue with enough lymphocytes to perform direct *ex vivo* staining. Therefore, the proposed mIHC panel would expand our general knowledge of CD4 function within MCC tumors. An additional caveat is that this method would assess the subtype of CD4 T cells broadly and would not identify MCPyV-specific CD4 T cells. In order to determine the subtype of MCPyV-specific CD4 T cells within fixed tissue, HLA class-II tetramers would have to be used *in situ*. Historically, *in situ* tetramer staining has been unsuccessful, largely due to the requirement for signal amplification and resulting high background[443,444]. Excitingly, HLA class-II dextramers can be used to also address this tetramer challenge and a recent publication successfully used to perform *in situ* autoreactive antigen-specific CD4 T cell staining in human brain sections[371]. Therefore, we are eager to attempt a similar method in MCC tumor sections using the 'WED'-dextramer. Using this powerful arsenal of tools, we are poised to perform an in-depth study of MCPyV-specific CD4 T cells with the hopes of improving our understanding of their role in this disease and how to employ them for therapeutic benefit.

MCC-specific neoantigens and neoantigen-specific T cells

While exciting progress has been made to permit the study of MCPyV-specific CD4 T cell responses, MCCs can also express UV-induced tumor-specific neoantigens[414]. Indeed, among the 20% of MCC that

are virus-negative, the number of non-synonymous single nucleotide variants (nSSNVs) detected per tumor is higher than most solid tumors including malignant melanoma or non-small cell lung cancer [60]. Evidence to suggest that immunity against these UV-neoantigens is protective in MCC is highlighted in a cohort of stage-matched patients who present with either a primary skin lesion or no detectable skin lesion (**Chapter 9**). Among advanced stage MCC patients, those presenting with nodal Merkel cell carcinoma without an identifiable (unknown) primary lesion (MCC-UP) had a significantly higher median number of nonsynonymous exome mutations (688 mutations/tumor) as compared to patients with detectable skin lesions (10 mutations/tumor, $p = 0.016$). This elevated mutation burden is associated with a 50% improved survival rate. Notably, there was a statistically significant difference in the number of SSNVs between patients with unknown and know primaries even among patients with virus-positive tumors. This result was highly surprising as virus-positive tumors had on average 16 SSNVs per tumor while virus-negative tumors had an average of >1050 per tumor. This suggests that even a small number of mutations may result in the expression of immunogenic neoantigens.

Indeed, preliminary data indicates that CD4 T cells from an MCC patient can respond to UV-induced neoantigens. Specifically, 5 neoantigen epitopes were recently identified in an MCC patient who responded to anti-PD-L1 immunotherapy (Dr. Candice Church; unpublished observations). Sequencing of this patient's tumor indicated mutated sites and then putative neoantigens were predicted based on the patients' HLA class-I allele types. Fascinatingly, all 5 of the identified epitopes were CD4 restricted despite using HLA class-I prediction algorithms to generate tested peptides. Other groups have reported similar findings, that identified neoantigen-specific responses are largely CD4 restricted[377]. Indeed, the use of neoantigen vaccine strategies in treating melanoma patients has indicated that CD4 T cell responses are critical in mediating efficacy [249,250]. These results suggest that further delineation into the CD4 T cell response against neoantigens in MCC is immunologically relevant and potentially therapeutically beneficial.

Novel CD4 T cell responses following successful immunotherapy

The presence of viral antigens and UV-induced neoantigens suggests that both etiologies of MCC are likely to be immunogenic, a notion which is strongly supported by the extraordinarily high response rates to immunotherapies including pembrolizumab (anti-PD-1), avelumab (anti-PD-L1) and nivolumab (anti-PD1)[1,445]. Additionally, novel immune-based therapies have shown promising results in some cases and CD4 T cells may play a crucial role in these responses. Indeed, evidence of CD4 T cell induction post-immune therapy has been observed in patients enrolled in two immune-based clinical trials. The first was observed in a patient treated with glucopyranosyl lipid adjuvant (GLA), a TLR4 agonist that has been shown to stimulate Th1 T helper responses (**Figure 8A)**[446,447]. This patient subsequently received radiation therapy and surgery and has remained free of disease for over 3 years (despite presenting with late stage disease). Analysis of tumor infiltrating lymphocytes (TIL) reactivity against MCPyV peptides, indicated that CD4 T cells specific to LT were present following GLA treatment, but were undetectable pre-treatment (**Figure 8B**). This suggests that immune stimulation by GLA led to novel priming, activation of existing T cells and/or recruitment of MCPyV-specific CD4 T cells to the tumor.

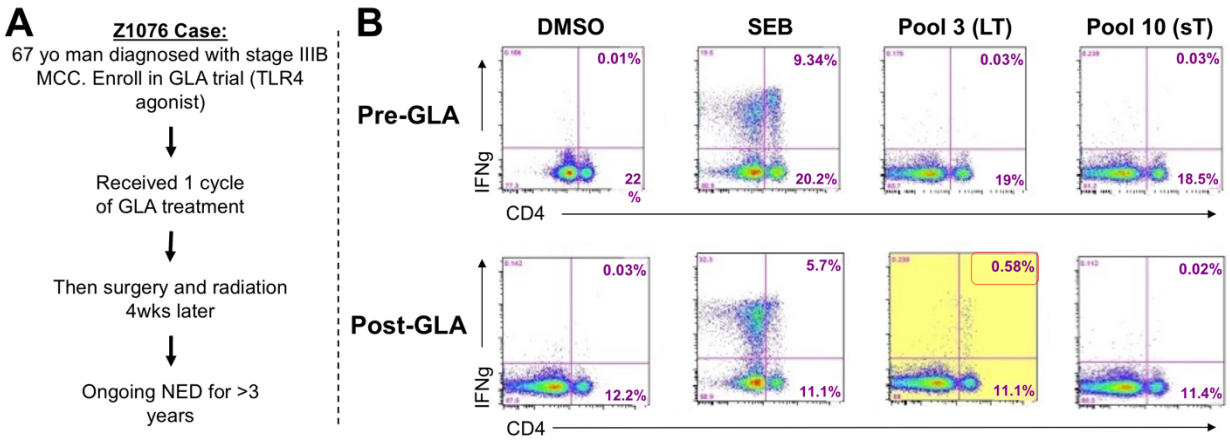


Figure 8: Evidence of epitope spreading following treatment with TLR4 agonist (GLA). A: Patient treatment summary. **B:** Tumor infiltrating lymphocytes (TIL) from z1076 were obtained pre and post-GLA treatment. TIL were cultured overnight with autologous PBMC, negative control (DMSO), positive control (SEB), and MCPyV peptide pools (Pool 3 and 10 depicted, Pools 1 & 2 were negative). IFNγ was measured on ICS.

In a second patient treated with autologous CD8 T cell infusions, HLA upregulation (via radiation) and anti-PD-L1 therapy (referred to as 'triple therapy'), a CD4 T cell response against peptides within the CT region of MCPyV was detected at days 28 and 160 post treatment initiation (**Figure 9**). This response was undetectable prior to treatment (Dr. Kelly Paulson; unpublished observations). This patient has done

clinically very well, with an ongoing complete response at >1.5 years. These two observations suggest that these treatments induced broadening of the antitumor immune response and in both cases, this response was associated with CD4 T cell activation and significant clinical benefit. Therefore, continued investigation into changes in the CD4 T cell responses pre- and post-immune therapy will be a crucial priority.

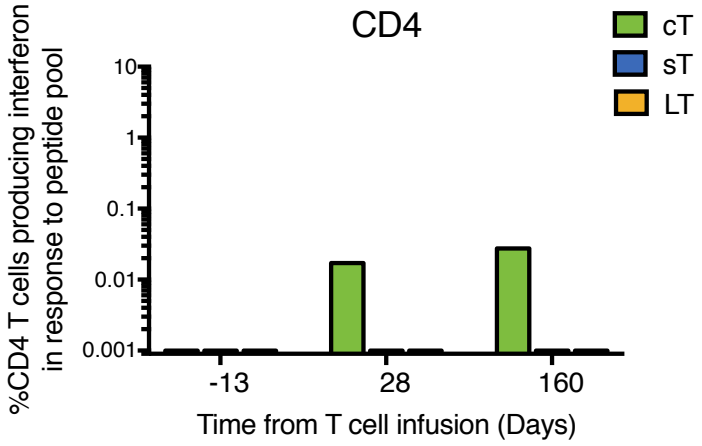


Figure 9: CD4 T cells respond to MCPyV common T peptides post immune therapy. PBMC from a patient who received 'triple therapy' was obtain 13 day prior to the first T cell infusion (-13 days) as well as 28 and 160 days after infusion). These cells were stimulated with peptide pools encompassing the MCPyV T-antigens and IFNγ was measured via an ICS assay.

Conclusions and the future of MCC immune-based treatments

While some mechanisms of response and resistance may be unique to MCC, the ability to deeply probe the immunobiology of this disease (via characterization of viral and neoantigen-specific T cell responses) provides a powerful opportunity to understand the dynamic host-tumor immune interactions during PD-1 blockade therapy. The data presented in this dissertation provides strong evidence for additional putative targets for immune therapies. These include targeting antiphagocytic molecules such as anti-CD47 and anti-CD200 and the development of an MCPyV therapeutic cancer vaccine. Additionally, the tools developed through this work has set the stage to deeply probe the MCPyV-specific and neoantigen-specific CD4 T cell responses which may further inform our understanding of the immune responses against these tumors and the importance of these cells in MCC outcomes and response to immune therapies.

I believe that within the next 10 years we will have radically changed our treatment approaches for MCC, tailored to maximize the immune response in each patient **(Figure 10**). To achieve this, I predict that new MCC patients with advanced disease (tumors requiring more than radiation and surgery alone) will have baseline biopsies sequenced using platforms such as 10x genomics to identify the tumor

immune status at baseline. Tumors densely infiltrated with immune cells that are Th1 skewed will likely be responsive to immune checkpoint blockade and sequencing data will indicate which checkpoints should be targeted. For poorly immunogenic tumors at baseline, the specific immune deficiencies observed based on sequencing data and the use of high throughput platforms such as multiplexed IHC, will indicate whether impairments exist in antigen-presentation, T cell infiltration, macrophage phenotype skewing etc. Agents that can target these specific deficiencies can then be combined in an intelligent, but 'off-the-shelf' manner to enable rapid, and rationale therapies. Once the tumor burden has been reduced via initial therapies, a therapeutic cancer vaccine could be administered, composed of immunodominant CD8 and CD4 epitopes presented within the tumor (either viral or neoantigen) to establish epitope spreading and provide a protective and robust immune response against the disease. If subsequent recurrences develop, these could be re- sequenced to identify escape mutations or newly induced mechanisms of immune evasion. These sophisticated and rationale approaches will enable patient-tailored treatment that I am highly optimistic will greatly improve the outcome for patients with this otherwise deadly skin cancer.

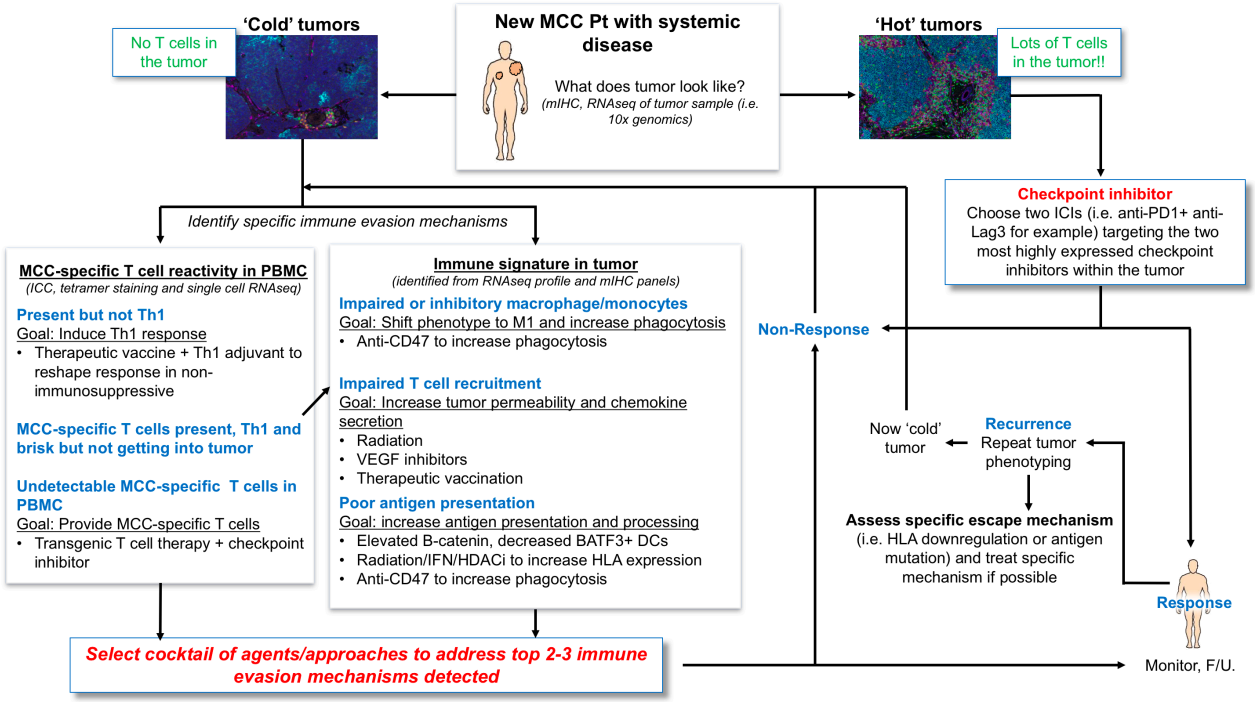


Figure 10: Predicted future immune assessment and immune-based treatment selection for advanced MCC patients.

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CURRICULUM VITAE

EDUCATION

University of Washington Medical School, Seattle, WA 06.2011 - present
- **Graduate Department:** Medical Scientist Training Program/Pathology
- **Laboratory:** Nghiem Lab
- **GPA**: 3.83

University of Michigan, Ann Arbor, MI 09.2006 – 12.2010
- **Major**: Cellular and Molecular Biology
- **GPA**: 3.85

AWARDS, GRANTS AND HONORS

Achievement Rewards for College Students (ARCS) Fellowship	2013 - 2018
University of Washington Graduate Medal Finalist	2016
Graduate School Fund for Excellence and Innovation (GSFEI) Travel Award	2015
Environmental Pathology and Toxicology T-32 Training Grant	2013 - 2016
University of Michigan Honors	2006 - 2010
Cullen Trust for Higher Education Fellowship Travel Award	2009
Thomas J. Bardos Science Education Award	2010 - 2011
Riecker Undergraduate Research Grant	2007

RESEARCH EXPERIENCE

Nghiem Laboratory, Dermatology Division; University of Washington 06.2012 - present
PhD Doctoral Candidate
Principal Investigator: Paul Nghiem MD PhD

Raible Laboratory, Department of Biological Structure; University of Washington 06.2011 – 08.2011
Rotation Student
Principal Investigator: David Raible PhD

Liu Laboratory, University of Michigan Cancer Center 2007-2010
Research Assistant
Principal Investigators: J. Rebecca Liu MD, Roland Kwok PhD, Anthony Opipari MD PhD, Valerie Castle MD

AKIL Laboratory, Boğazici Universitesi; Istanbul, Turkey 06.2009-08.2009
Research Assistant
Principal Investigator, Nesrin Özören PhD

PUBLICATIONS

Articles

[9] Vandeven N, Lewis CL, Makarov V, Riaz N, Paulson K, Hippe D, Bestick A, Doumani R, Marx T, Takagishi S, Chan TA, Choi J, Nghiem P. *Merkel cell carcinoma patients presenting without a primary lesion have elevated markers of immunity, higher tumor mutation burden and improved survival.* **Submitted**

[8] Paulson KG, Park SY, Vandeven NA, Lachance K, Thomas H, Chapuis AG, Harms KL, Thompson JA, Bhatia S, Stang A, Nghiem P. *Merkel Cell Carcinoma: Current United States Incidence and Projected Increases based on Changing Demographics*. **Accepted**, JAAD.

[7] Love JE, Thompson K, Kilgore MR, Murphy CE, Papanicolau-Sengos A, Westerhoff M, McCormick KA, Vandeven N, Shankaran V, Miller F, Blom A, Nghiem P, Kussick SJ. *CD200 Expression in Neuroendocrine Neoplasms.* **Am J Clin Path**. 2017, 148, 236-242.

[6] Vandeven N, Nghiem P. *Merkel cell carcinoma: An unusually immunogenic cancer proves ripe for immune therapy.* **J Oncol Pract**. 2016, 12(7), 649-650.

[5] Vandeven N, Nghiem P. *Rationale for immune-based therapies in Merkel polyomavirus-positive and -negative Merkel cell carcinomas*. **Immunotherapy**. 2016, DOI 10.2217/imt-2016-0009.

[4] Vandeven N, Nghiem P. *Pathogen-Driven Cancers And Emerging Immune Therapeutic Strategies.* **Can Immunol Res**. 2014, 2, 9-14.

[3] Afanasiev O, Nagase K, Simonson W, Vandeven N, Blom A, Koelle D, Clark R, Nghiem P. *Vascular E-Selectin Expression Correlates with CD8 Lymphocyte Infiltration and Improved Outcome in Merkel Cell Carcinoma.* **J. Inv. Derm**. 2013, (00)

[2] Vandeven N, Nghiem P. *Complete Spontaneous Regression of Merkel Cell Carcinoma Metastatic to the Liver: Did Lifestyle Modifications and Dietary Supplements Play a Role?* **Global Advances in Health and Medicine.** 2012 1(5).

[1] Vandeven N*, Hajra K*, Daudi S, Liu RJ. *The HIV Protease Inhibitor Saquinavir Induces Apoptosis and Autophagy in Ovarian Cancer Cell Lines.* **Gyn Onc,** 2009, 112(3), 623-30. *Co-first authors

Selected Conference Abstracts

[9] Paulson KG, Voillet V, Perdicchio M, Hunter D, Valente W, Wagener F, Koelle S, Church C, Vandeven N, Thomas H, Colunga A, Koelle D, Yee C, Kulikauskas R, Pierce R, Greenberg PD, Bhatia S, Bielas J, Gottardo R, Nghiem P, Chapuis A. Single Cell RNA Sequencing Reveals Mechanisms of Merkel Cell Carcinoma Escape from Intense Pressure of T Cell Immunotherapy. Society for Immunotherapy of Cancer Annual Meeting. National Harbor, MD. 2017.

[8] Paulson K, Perdicchio M, Kulikauskas R, Wagener F, Church C, Bui KT, Vandeven N, Thomas H, McAfee M, Yee C, Miller N, Chin KM, Su Z, Greenberg PD, Parvathaneni U, Bhatia S, Nghiem P, Chapuis A. Augmentation of Adoptive T cell Therapy for Merkel Cell Carcinoma with Avelumab. ASCO Annual Meeting. Chicago, Illinois. June 2017.

[7] Vandeven N, Xu Y, U'ren L, Hayes E, Yang J, Stillwell J, Kaldjian E, Warren E, Kwok W, Nghiem P. Novel platform for identifying rare antigen-specific CD4 T cells in Merkel cell carcinoma patients. Keystone Symposium: Cancer immunology and immunotherapy: Taking a place in mainstream oncology. Whistler, British Columbia. March 2017.

[6] Vandeven N, Viller NN, O'Connor AM, Chen H, Bossen B, Sievers EL, Uger RA, Nghiem PT, Johnson LDS. CD47 is over expressed on Merkel Cell Carcinoma and a target for SIRPaFc Therapy. Society for Immunotherapy of Cancer Annual Meeting. National Harbor, MD. 2016.

[5] Ibrani D, Iyer J, Vandeven N, Miller N, Afanasiev O, Koelle D, Nghiem P. Identifying Merkel polyomavirus-specific CD4+ and CD8+ T cells in Merkel cell carcinoma patients' tumor-infiltrating lymphocytes. Society for Investigative Dermotology Annual Meeting. Atlanta, GA. May 2015.

[4] Vandeven N, Iyer J, Ibrani D, Afanasiev O, Koelle D, Nghiem P. Identification of Merkel cell polyomavirus CD4 epitopes in Merkel cell carcinoma patients. Keystone Symposium: T cells: regulation and effector function. Snowbird, Utah, USA. 29 May - 3 April 2015.

[3] Afanasiev O, Yelistratova L, Miller N, Iyer J, Vandeven N, Paulson K, Ibrani D, Nghiem P. Phenotypic Differences In Tumor-Specific T Cells Among Patients Who Progress Or Control Merkel Cell Carcinoma. Keystone Symposia Conference Immune Evolution in Cancer. Whistler, British Columbia. March 2014.

[2] Vandeven N, Hajra K, Liu RJ, "HIV protease inhibitors induce apoptotic and non-apoptotic cell death in ovarian cancer cells". Biennial meeting for the International Gynecologic Cancer Society.Thailand. October 2008.

[1] Wahl H, Kirschkigar M, Daudi S, Vandeven N, Rhode J, Griffith K, Liu RJ, "P-mTOR and Glut-1: Metabolically Targeted Biomarkers in Endometrial *Carcinoma"* Society for Gynecologic Oncologists Annual Meeting on Women's Cancer. Tampa, Florida. March 9-12, 2008.

LEADERSHIP AND SERVICE

Phi Chi Medical Fraternity, Seattle WA — Present
Welfare Board Member, President of Seattle Chapter
Country Doctor Teen Clinic, Seattle, WA — 2014 - present
Front Desk Volunteer
One Step At A Time (OSAT), Seattle, WA — 2014 - present
Basic Mountaineering Skills Instructor
The Mountaineers, Seattle, WA — 2014 - 2016
Basic Mountaineering Skills Instructor, Trail Maintenance Crew
Himalayan Health Exchange, Lahaul, India — 07.2013-08.2013
Basic Health Care Provider
Ronald McDonald House, Seattle, WA — 2011 - 2013
Medical School Volunteer Coordinator for the Pediatric Interest Group
Musicians of the University of Washington, Seattle, WA — 2012 – 2013
Officer
University of Michigan Undergraduate Research Journal (UMURJ), Ann Arbor, MI — 2008 - 2009
Editor
Peer Utilizing Leadership Skills for Education (PULSE), Ann Arbor, MI — 2007 - 2009
Student Representative

TEACHING EXPERIENCE

Department of Medicine, *University of Washington*; Seattle, WA — 2012
Anatomy Course Teaching Assistant
Chemistry Department, *University of Michigan;* Ann Arbor, MI — 2009 – 2011
Organic Chemistry II Laboratory Instructor
Chemistry Department, *University of Michigan;* Ann Arbor, MI — 2009 - 2011
Organic Chemistry I Honors Structured Study Group Leader
Science Learning Center, *University of Michigan***;** Ann Arbor, MI — 2008
Organic Chemistry Study Group Leader