Merkel cell polyomavirus-specific T cell responses, immune evasion mechanisms & immune therapy in Merkel cell carcinoma

Olga Kochan Afanasiev

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Reading Committee:

Paul Nghiem, Chair
David Koelle
Marshall Horwitz

Program Authorized to Offer Degree:
Pathology
Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer with an increasing incidence and a 5-year disease-associated mortality of 46%. The Merkel cell polyomavirus (MCPyV), discovered in 2008, is involved in the pathogenesis of over 80% of MCC tumors. MCC typically requires persistent expression of immunogenic polyomavirus tumor-antigen (T-Ag) oncoproteins for growth and survival. We have found that these viral oncoproteins elicit T cell immune responses that specifically target virus-driven MCC tumors. Furthermore, in individual patients, these MCC-specific T cells in the blood increase with MCC disease progression and decrease with effective disease therapy. The presence of these tumor-targeting T cells in patients with MCC tumors suggested there may be several immune evasion mechanisms that prevent adequate immune control of this cancer. We first characterized the molecular pathways involved in inhibiting protective T cell immune responses. To assess the functional state of MCC-specific CD8 T cells, we determined the expression of physiologically relevant cell surface markers and cytokine production directly ex vivo. MCC-specific T cells fail to produce activation cytokines in response to the viral oncoproteins and they co-express PD-1 and Tim-3 inhibitory receptors, a phenotype strongly associated with T cell exhaustion and dysfunction. Importantly, these inhibitor pathways can be therapeutically targeted using existing or emerging agents to augment T cell function. Previous studies identified that high levels of CD8 lymphocytes within the tumor are strongly correlated with improved survival, although these cells are absent or sparse in ~80% of MCCs. We investigated specific mechanisms that
may prevent adequate T cell infiltration into MCC tumors. We found that intratumoral vascular E-selectin, critical for T cell recruitment and entry into skin, was downregulated in the majority (52%) of MCCs, which was associated with poor intratumoral CD8 lymphocyte infiltration and poorer survival. We have also identified that human leukocyte antigen class I (HLA-I) is downregulated in the majority (51%) of MCC tumors, which suppresses antigen presentation, thus promoting evasion of CD8 T cell responses. Excitingly, these findings have resulted in significant translational applications. Specifically, we have treated a patient with metastatic MCC by using an innovative therapeutic combination of polyclonal, tumor-specific cytotoxic T cells infused following a pre-conditioning regimen (interferon-beta or radiotherapy) that reverses local tumor immune evasion mechanisms. This combination resulted in the elimination of 2 of 3 metastatic tumors and yielded a distant metastasis free survival of 535 days, far beyond expected among such patients (95% CI: 154-260 days). Immunologic responses included persistence of MCPyV-specific T cells at several-fold above baseline and improved antigen-specific T cell responses that persisted beyond 100 days after treatment. Importantly, this therapy was well tolerated without significant side effects. In summary, these studies have characterized the cellular immune responses in MCC and have identified several immune evasion pathways that may be therapeutically targeted to augment the efficacy of immune therapy in MCC.
Dedication:

To my family
For your constant support and love

Acknowledgements:

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I am thankful to my thesis advisory committee for providing critical advice and direction, and especially to Professor David Koelle for his teaching and guidance in immunology techniques and considerable intellectual input during my PhD.

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Finally, I would like to thank all the patients who willingly participated in these studies and kindly provided their blood and tissue to make these studies possible.
# Table of Contents

1) Introduction to Merkel cell carcinoma (MCC) and the Merkel cell polyomavirus        1

2) Clinical overview of Merkel cell carcinoma  

3) Immunobiology of Merkel cell carcinoma  

4) T cells from Merkel cell carcinoma tumors and blood recognize viral oncoproteins  

5) Fluctuation and exhaustion of Merkel cell carcinoma-specific CD8 T cells  
   Afanasiev, Yelistratova, Miller, Kotaro, Paulson, Iyer, Ibrani, Koelle, Nghiem. Merkel cell carcinoma-specific T cells fluctuate with disease and express therapeutically targetable PD-1 and Tim-3 exhaustion markers. [submitted manuscript] .                                                                                      52

6) Downregulation of vascular E-selectin prevents CD8 lymphocyte entry into tumors  

7) Diminished antigen presentation: A reversible immune escape mechanism in MCC  

8) Immune stimulatory and adoptive T cell therapy induce MCC tumor regression  

9) Emerging prognostic and therapeutic approaches in Merkel cell carcinoma  

10) Conclusions................................................................................................................................. 146

11) Citations........................................................................................................................................ 154

12) Appendix  
   I. List of additional publications authored during period of dissertation research........ 156
Introduction to Merkel cell carcinoma

Background

Merkel cell carcinoma (MCC) is an increasingly common neuroendocrine skin cancer that is associated with UV-light exposure, advanced age, immune suppression (1) and a recently discovered polyomavirus (2). Despite its typically unimpressive clinical appearance as a red, painless nodule (1), MCC is very aggressive with a disease-associated mortality three times that of malignant melanoma (~46% versus 15%, respectively) (3). Chapter 2 further details the pathogenesis, clinical course and treatment for Merkel cell carcinoma.

MCC was first described by Toker in 1972, a relatively recently described disease, although the Merkel cell was described more than 100 years ago. In 1875, Friedrich S. Merkel first described human Merkel cells, which he correctly identified as likely having sensory touch function within the skin due to their association with nerves. Most recently, in 2009, a series of studies using elegant mouse models resolved several longstanding debates by conclusively establishing that 1) Merkel cells are essential for light touch responses, (4) 2) Merkel cells have an epidermal origin (4, 5) 3) Merkel cells do not divide, but are renewed by a reservoir of epidermal progenitor cells (5).

As the name of this cancer implies, Merkel cells were believed to be at the origin of MCCs due to their phenotypic similarities (6, 7). However, recent evidence suggests that the poorly differentiated MCCs likely develop from skin stem cells derived from the epidermal lineage (8).

Several lines of evidence suggest the importance of immune function in MCC. Patients with profound T-cell dysfunction (human immunodeficiency virus, chronic lymphocytic leukemia or medications after solid organ transplant) have 10- to 30-fold increased MCC risk (1) and their MCC sometimes spontaneously regresses following improvement in immune function (9-11). Indeed, systemic immune suppression is significantly associated with worsened survival (12).
Furthermore, several studies suggest that CD8 and CD3 lymphocyte infiltration into MCC tumors is strongly linked to survival (13, 14). However, this advantageous robust lymphocytic infiltration into MCC tumors is only present in approximately ~20% of patients (13). In addition, although T cell immunosuppression is associated with increased MCC risk, >90% of MCC patients have no known immune suppression. Together, these findings suggest a role for local immunosuppression in the tumor microenvironment.

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) (2). MCPyV is prevalent in the general population (15-17), integrates into most (>80%) Merkel cell carcinomas (MCCs) (2, 18, 19) and encodes oncoproteins required for MCC tumor growth (18). Chapter 3 further introduces recent discoveries in MCC, with a special focus on the pathogenic role of MCPyV and the immunobiology of the virus-associated MCC.

Relevance of studies

Despite the rising incidence and number of deaths from Merkel cell carcinoma and the increasing body of evidence acknowledging the importance of the immune system in preventing and controlling this cancer, the nature of the cellular immune response to the tumor remained largely uncharacterized.

Unfortunately, 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. Despite this growing health impact, very few clinical trials have focused on this unique virus-associated disease. Thus, there is an unmet need for biology-driven therapies in MCC.

The necessary and persistent expression of Merkel cell polyomavirus oncoproteins in MCC tumors provides a unique opportunity, not available for most cancers, to study anti-tumor immunity and immune evasion by assessing responses against a viral, tumor-specific antigen. It
is hoped that identifying and characterizing MCC-specific T cell responses and immune escape mechanisms may both expand the current understanding of MCC pathogenesis and guide the design of rational and synergistic therapies for MCC.

**Thematic Nature of Studies**

This dissertation is a compilation of six publications and manuscripts that focuses around the central theme of characterizing cellular immune responses and immune evasion mechanisms in Merkel cell carcinoma. These studies help to establish a new paradigm for immune therapy in MCC and point towards promising therapeutic directions in the future.

The first section helps to establish the immunogenicity of Merkel cell polyomavirus oncoproteins that are persistently expressed in MCC tumors. In Chapter 3, we first review the strong association of Merkel cell polyomavirus with Merkel cell carcinoma and the immunobiology of MCC. Primary data is then presented in Chapter 4 that establishes the presence of tumor-targeting MCPyV oncoprotein-specific CD8 and CD4 responses in the blood and tumors of MCC patients. We identified 24 epitopes derived from the persistently expressed regions of the virus that are recognized by T cells. The identified epitopes may be suitable candidates for peptide-specific vaccines and tumor-specific adoptive immune therapies. Indeed, as discussed below, T cell therapy targeting one of these identified epitopes has already been used in a patient with metastatic MCC.

In Chapters 5 through 7, we identify several immune evasion mechanisms that may help explain why MCC tumors often develop despite the presence of T cells specific for MCPyV oncoproteins. In Chapter 5, both cross-sectional and longitudinal studies indicate that MCPyV oncoprotein-specific T cells: 1) are detectable directly ex vivo in MCC patients (but not in control subjects) and 2) increase in frequency with larger tumor burden and decrease with smaller or absent tumor burden. Importantly, we also identify that two key inhibitory receptors involved in T cell dysfunction, PD-1 and Tim-3, are significantly upregulated and co-expressed on MCC-
specific T cells found in tumors and blood of MCC patients. These pathways can be targeted by existing or developing agents to overcome immune evasion. In Chapter 6, we investigated whether specific mechanisms of T-cell migration may be commonly disrupted in the majority (80%) of MCC tumors with poor CD8 lymphocyte infiltration. Indeed, we identified that a critical T cell recruiting marker on tumor vasculature, E-selectin, was downregulated in the majority (52%) of MCC tumors, and this defect was associated with poor intratumoral CD8 lymphocyte infiltration. Importantly, survival was significantly improved in MCC patients whose tumors had higher E-selectin expression. Chapter 7 establishes that most MCC tumors have prominent downregulation of human leukocyte antigen class I (HLA-I also known as major histocompatibility complex class I, MHC-I), a known mechanism for allowing tumor cells to escape host immunosurveillance by evading CD8\(^+\) T lymphocytes. We go on to also show that HLA-I downregulation can be reversed in MCC by intratumoral interferon injection or by a single dose of radiation. Ongoing expression of viral oncoproteins combined with reversibility of immune evasion mechanisms in MCC present exciting opportunities to develop rational therapy for this often-lethal cancer.

Chapters 8 and 9 move on to explore the therapeutic implications of the findings in these studies. In Chapter 8, we present data that support the use of a novel immunotherapy paradigm that combines the infusion of tumor-specific (and MCPyV-specific) adoptively transferred T cells after a pre-conditioning regimen that reverses local tumor immune evasion mechanisms. Chapter 9 further reviews current and developing translational therapies aimed at generating long-lasting and effective anti-tumor responses.

Together, these studies demonstrate the immunogenicity of the Merkel cell polyomavirus, identify specific T cell responses and characterize immune evasion mechanisms that prevent adequate control of MCC tumors. Furthermore, they provide the first promising translational applications of these findings with the ultimate goal to open new and effective therapeutic avenues for Merkel cell carcinoma.
Chapter 120 :: Merkel Cell Carcinoma
:: Andrew Tegeder, Olga Afanasieva, & Paul Nghiem

Merkel cell carcinoma (MCC) is an increasingly common neuroendocrine skin cancer that is associated with ultraviolet (UV)-light exposure, advanced age, immune suppression and a recently discovered polyomavirus. The reported incidence of MCC has more than tripled in the past 20 years to approximately 1,500 US cases/year and is expected to grow with the aging population. Although it is 40 times less common than malignant melanoma, it carries a markedly poorer prognosis, with disease-associated mortality at 5 years of 46% as compared with 9% for invasive melanoma. Management of MCC is challenging and optimal therapy is controversial, at least in part due to a lack of prospective or randomized data on which to base treatment decisions.

MCC is a relatively recently described entity, although the Merkel cell was identified more than 100 years ago. In 1875, human Merkel cells were first described by Friedrich S. Merkel (1845–1919). He named these cells Tastzellen (touch cells) assuming that they had a sensory touch function within the skin due to their association with nerves. In 2009, a series
Indeed, only about 10% of MCC cases are found in women and 65% for women.

As of 2008, there are approximately 1,500 MCC cases per year in the US. There are likely two factors that contribute to this increase in reported incidence that is more rapid than that of any other type of skin cancer. One factor is an increase in the incidence of melanoma which is the most common skin cancer. The second likely reason is a increased number of people over age 65 years with extensive sun exposure history and people living with prolonged immune suppression. Each of these factors is a known risk factor for MCC and several are discussed in Section "Etiology and Pathogenesis."

As a result of this increased incidence in MCC, there is an increased number of cases that present in patients under age 50 and it is extremely rare in childhood.

### Etiology and Pathogenesis

There are several known risk factors for MCC that will likely continue to lead to an increase in the incidence of this disease.

#### Epidemiology

Over the 20-year period between 1986 and 2006, the reported incidence of MCC quadrupled from 0.15 per 100,000 to 0.6 per 100,000. There are likely two factors that contribute to this increase in reported incidence that is more rapid than that of any other type of skin cancer. One factor is an increase in the incidence of melanoma which is the most common skin cancer. The second likely reason is an increase in the number of people over age 65 years with extensive sun exposure history and people living with prolonged immune suppression. Each of these factors is a known risk factor for MCC and several are discussed in Section "Etiology and Pathogenesis."

As a result of this increased incidence in MCC, there is an increased number of cases that present in patients under age 50 and it is extremely rare in childhood.

#### Age Greater Than 65 Years

The median age for diagnosis of MCC is 70 years, and there is a five- to tenfold increase in incidence after age 70 as compared to age less than 60 years, as shown in Fig. 120-1. Indeed, only about 10% of MCC cases present in patients under age 50 and it is extremely rare in childhood.

#### Sun Exposure

Sunlight, prolonged UV exposure, and photochemotherapy are all associated with an increased risk of MCC. As seen in Fig. 120-2, the vast majority (81%) of MCC tumors present on sun-exposed skin. However, it is clear that sun is not required for MCC to develop. MCC cases can occur on sun-protected skin, including buttocks and vulva as well as portions of the scalp that are covered by hair.

#### Immune Suppression

As compared to most cancers, MCC is strongly linked to immune suppression. Indeed, 7.8% of MCC patients are profoundly immune suppressed, a 16-fold overrepresentation compared with expected. Multiple forms of immune suppression are associated with an increase in MCC risk. These include HIV/AIDS, chronic lymphocytic leukemia, and the immune suppressive regimens associated with solid organ transplant. Additionally, there are 19 reported cases of complete spontaneous regression in the MCC literature, a far greater number than expected for its rarity, perhaps suggesting a sudden immune recognition and clearance of MCC.
In 2008, a new polyomavirus, the Merkel cell polyomavirus (abbreviated MCV or MCPyV), was discovered by extensive sequencing of MCC tumor RNA. MCPyV was reported to be present in 80% of MCC tumors compared to only 7% of skin controls.

Numerous groups across several continents (over 25 literature citations) have verified the association between MCPyV and MCC, and all groups observed that the virus is strongly associated with MCC. MCPyV is a small, circular, double-stranded DNA virus related to other known polyomaviruses (SV40, BK, JC, KI, WU). MCPyV is unique among the human polyomaviruses as it is the only one proven to integrate into a human cancer. Monoclonal integration of MCPyV into MCC tumors suggests that the virus is present prior to or very early in tumorigenesis.

A large fraction of the population has been infected with MCPyV. By the age of 5, prevalence of antibodies is about 35%, but increases to 50% by the age of 15. Interestingly, among MCC patients, the prevalence is significantly higher (88%) than age and sex-matched controls (53%). Importantly, despite how common exposure is to this virus in the general population, the incidence of MCC is very low. Therefore, viral infection alone is clearly insufficient for the development of this cancer.

**CLINICAL PRESENTATION OF MERKEL CELL CARCINOMA**

A systematic analysis of 195 MCC patients characterized clinical features that may serve as clues in the diagnosis of MCC. The most significant features can be summarized in an acronym: “AEIOU” (Table 120-1). This study found that 89% of 62 MCC cases exhibited at least three of the five features listed below. If a lesion exhibits at least three of these features, suspicion of MCC should increase and biopsy be considered. In particular, a lesion that is red or purple, rapidly growing, but nontender should be of concern. Strikingly, the clinician listed as clinical impression a benign diagnosis in over one half of cases. In particular, a cyst or acneiform lesion was the single most common of these (Box 120-1 and Figs. 120-3A and 120-3B) and nonmelanoma skin cancer was also relatively frequent as a presumptive diagnosis (Fig. 120-3C).

**PATHOLOGY**

As shown in Fig. 120-4A, the classic histologic features of MCC include sheets of small basophilic cells with scant cytoplasm, fine chromatin, and no nucleoli. There are numerous mitotic figures and occasional individual necrotic cells. Lymphovascular invasion is a very common feature and often can be found when it is specifically searched for even in a “negative” margin. This helps to explain the high local recurrence rate in MCC for narrow or even relatively wide margin excision when adjuvant radiation therapy is not given.

**TABLE 120-1**

<table>
<thead>
<tr>
<th>Clinical Features of Merkel Cell Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic (non-tender, firm, red, purple, or skin-colored papule or nodule; ulceration is rare; see Figs. 120-3A and 120-3B)</td>
</tr>
<tr>
<td>Expanding rapidly (significant growth noted within 1–3 months of diagnosis, but most lesions are &lt;2 cm at time of diagnosis)</td>
</tr>
<tr>
<td>Immune suppression (e.g., HIV/AIDS, chronic lymphocytic leukemia, solid organ transplant)</td>
</tr>
<tr>
<td>Older than 50 years</td>
</tr>
<tr>
<td>Ultraviolet-exposed site on a person with fair skin (most likely presentation, but can also occur in sun-protected areas; Fig. 120-2)</td>
</tr>
</tbody>
</table>
Chapter 120 :: Merkel Cell Carcinoma

HEMATOXYLIN AND EOSIN STAIN

Three histologic patterns have been described up to now and none is clearly associated with a better or worse prognosis. The most common type is the “intermediate type.” This has uniform small cells with minimal cytoplasm, pale nuclei, and a dispersed chromatin appearance. On hematoxylin and eosin staining, the differential diagnosis for this presentation is that of the small, blue-cell tumors including melanoma and lymphoma. The second most common pattern is the “small cell type.” This takes its name from small cell lung carcinoma, which is the principal differential diagnosis for this pattern. It shows irregular, hyperchromatic cells with scant cytoplasm and malignant cells that are arranged in linear patterns infiltrating stromal structures. The least common but perhaps most histologically distinctive type is the “trabecular” type. This is the pattern originally described by Toker in 1972. It has a lattice-like, or network appearance, and the differential diagnosis includes metastatic carcinoid tumor.

BOX 120-1 DIFFERENTIAL DIAGNOSIS OF MERKEL CELL CARCINOMA

**Most Likely**
- Cyst
- Basal cell carcinoma
- Squamous cell carcinoma (see Fig. 120-3C)
- Amelanotic melanoma
- Cutaneous lymphoma
- Adnexal tumor

**Consider**
- Metastasis
- Dermatofibromasarcoma protuberans
- Keratocanthoma
- Neuroblastoma

**Figure 120-3** Clinical appearance of Merkel cell carcinoma (MCC). A. MCC frequently has a “cyst-like” appearance. This is reflected in the differential diagnoses given by clinicians at the time of the biopsy, with cyst/acroform lesion being the most common clinical impression. B. MCC on the knee of a 70-year-old woman with chronic lymphocytic leukemia. For 6 months this lesion was thought to be a “cyst”; consequently, diagnosis and treatment were delayed. Pen marks indicate palpable satellite metastases that developed several months after the primary lesion, presumably tracking via lymphatics. C. MCC on the ear of an 87-year-old woman. The lesion grew rapidly and was nontender. After approximately 3 months, it was biopsied by a clinician who listed squamous cell carcinoma as the presumptive diagnosis.
IMMUNOHISTOCHEMICAL STAINS

The use of antibody-based stains has greatly facilitated the ease and specificity of MCC diagnosis (Table 120-2). The single most useful of these stains is cytokeratin-20.

CYTOKERATIN-20

Intermediate filament protein is expressed in MCC as well as in adenocarcinomas of the colon, stomach, and pancreas. However, within the skin the expression of cytokeratin-20 is limited to Merkel cells. A “perinuclear dot” pattern of cytokeratin is essentially pathognomonic for MCC (see Fig. 120-4B).

ANTIBODIES TO CAM5.2

CAM5.2, a cocktail of antibodies that detects multiple human cytokeratin epitopes, typically reacts with both MCC and small cell lung carcinoma. Although it is useful as an initial screening tool to detect tumors of squamous origin, its lack of selectivity relative to cytokeratin-20 means that it cannot be used to definitively diagnose MCC.

THYROID TRANSCRIPTION FACTOR-1

Thyroid transcription factor-1 is negative in MCC and positive in small cell lung cancer and thus useful for the differential diagnosis between these two tumors that can look identical by routine histology.

<table>
<thead>
<tr>
<th>TABLE 120-2</th>
<th>Immunohistochemistry Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytokeratin-20</td>
</tr>
<tr>
<td>Merkel cell carcinoma</td>
<td>+</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>-</td>
</tr>
<tr>
<td>Melanoma</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 120-3
Description of MCC Staging and Associated Predicted Survival Percentages

<table>
<thead>
<tr>
<th>Stage</th>
<th>Local, Tumor Diameter ≤2 cm</th>
<th>1-year Survival</th>
<th>3-year Survival</th>
<th>5-year Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Nodes microscopically negative and not clinically detectable</td>
<td>100%</td>
<td>86%</td>
<td>79%</td>
</tr>
<tr>
<td>IB</td>
<td>Nodes not clinically detectable (no pathologic evaluation of nodes done)</td>
<td>90%</td>
<td>70%</td>
<td>60%</td>
</tr>
<tr>
<td>Stage II: Local, Tumor Diameter &gt;2 cm</td>
<td>Nodes microscopically negative and not clinically detectable</td>
<td>90%</td>
<td>64%</td>
<td>58%</td>
</tr>
<tr>
<td>IIB</td>
<td>Nodes not clinically detectable (no pathologic evaluation of nodes done)</td>
<td>81%</td>
<td>58%</td>
<td>49%</td>
</tr>
<tr>
<td>IIC</td>
<td>Primary tumor is invading into bone/muscle/fascia/cartilage</td>
<td>72%</td>
<td>55%</td>
<td>47%</td>
</tr>
<tr>
<td>Stage III: Regional Nodal Disease</td>
<td>Micrometastasis</td>
<td>76%</td>
<td>50%</td>
<td>42%</td>
</tr>
<tr>
<td>IIIb</td>
<td>Macrometastasis (clinically detectable nodes)</td>
<td>70%</td>
<td>34%</td>
<td>26%</td>
</tr>
<tr>
<td>Stage IV: Distant Metastatic Disease</td>
<td></td>
<td>44%</td>
<td>20%</td>
<td>18%</td>
</tr>
</tbody>
</table>

STAGING AND PROGNOSIS

In 2010, the American Joint Committee on Cancer (AJCC) adopted a new staging system for MCC that replaced five existing—and conflicting—staging systems. According to the new AJCC guidelines, there are four stages for MCC based on clinical and pathological features at the time of presentation. This new staging system is summarized in Fig. 120-5 and Table 120-3. Survival after a diagnosis of MCC is highly dependent on the stage at presentation. Patients with local disease have estimated 5-year relative survival between 60% and 79%. Survival decreases markedly with nodal involvement and metastatic disease (Fig. 120-5). Unlike malignant melanoma, if MCC recurs it tends to do so rapidly with ~80% of recurrences occurring within 2 years of diagnosis.

Until 2009, MCC did not have its own specific International Classification of Diseases (ICD) code. Instead, MCC was often coded as “173.x: other malignant neoplasm of the skin.” Specific ICD codes are important because they allow for more streamlined therapy, faster insurance approvals and improved tracking of patients and costs for clinical research purposes. As of 2010, MCC now has its own set of ICD codes (209.3x) to better facilitate treatment and tracking of patients with MCC.

SENTINEL LYMPH NODE BIOPSY AND STAGING OF MERKEL CELL CARCINOMA

Over the past 20 years, sentinel lymph node biopsy (SLNB) has become quite common in staging malignant melanoma presenting with a depth of greater than 1 millimeter. More recently, several studies have indicated that SLNB is also a sensitive test for detecting MCC spread to the lymph nodes. Interestingly, MCC is far more likely to have occult lymph node involvement (approximately 30% for the average 1.7 cm MCC) than melanoma (~1% for melanomas with the average Breslow thickness of 0.63 mm). SLNB clearly has less morbidity than an elective lymph node dissection. Ideally, an SLNB should be performed at...
the time of the wide resection as opposed to after the wide resection when local lymphatics have been disturbed. The importance of performing an SLNB is that one-third of patients with local-only disease as determined by clinical palpation of lymph nodes, in fact, have microscopic disease in the lymph nodes that very much affects their subsequent survival (Fig. 120–5, Table 120–3).21 Specifically, one-third of patients would be understaged by clinical exam only, and would shift to stage IIIA disease with microscopic examination. Identification of microscopic nodal disease also importantly alters therapy for the node bed, as discussed below.

### RADIOLOGIC IMAGING STUDIES

Although many patients who present for management of MCC have not had an SLNB, the majority have had computed tomographic (CT) scans. We studied our own series of patients to determine the sensitivity and specificity of CT scans in patients presenting with MCC.21 We found that scans were not sensitive (missing 90% of positive cases) in terms of detecting nodal involvement of MCC as compared with SLNB. Among patients who had low-risk disease (negative sentinel lymph nodes or very small primary tumors) all of the “positive” scan results were, in fact, false positives.21 Therefore, we typically reserve CT or PET-CT scans for patients presenting with more advanced disease, such as positive nodal involvement or clinical evidence of metastatic disease.

### PROGNOSTIC FINDINGS ON HISTOLOGY

A recently published retrospective study evaluated several histologic features of MCC and performed survival analyses on these features. This large study had over 4 years of average follow-up time and analyzed the primary tumor lesions from 156 patients with MCC. In addition to tumor stage, two new histologic features were of particular interest: (1) lymphovascular invasion (LVI) and (2) tumor growth pattern. LVI was defined as tumor emboli within vascular spaces. Persons with MCC tumors with detectable LVI had a worse overall survival as compared to those with tumors without LVI (hazard ratio, 3.84; P = 0.007).25 Tumor growth pattern was described as nodular (well-circumscribed interface between tumor and surrounding tissue) or infiltrative (rows, trabeculae, or single cells that penetrate the dermis). Tumors that exhibited both features were considered infiltrative. An infiltrative tumor growth pattern was associated with poor outcomes as compared to MCC tumors with a nodular growth pattern (hazard ratio, 6.85; P = 0.001).23

### TREATMENT

Optimal therapy for MCC is controversial and there is no broad consensus on how to manage this disease. The best summary of consensus treatment for MCC is available through the National Comprehensive Cancer Network (http://www.nccn.org) and is updated annually (these guidelines and other useful information can also be found at www.merkelcell.org/usefulinfo/index.php). The best outcome for MCC is clearly obtained when multidisciplinary management is carried out by an experienced team. Each major treatment modality is summarized below.

#### SURGERY AT PRIMARY SITE

The initial management in most cases of MCC is surgical excision of the tumor. When carried out without subsequent adjuvant radiation, surgery may have a relatively high recurrence rate depending on the margins chosen and the risk profile for the tumor. Surgery alone with 0.5 cm margins resulted in a 100% recurrence rate in 38 patients.21 When wide local excision was carried out with 2.5 cm margins, the recurrence rate fell to 49%.23 For Mohs surgical excision, the local recurrence rate was 16% for surgery alone and 0% in patients who also had adjuvant radiation therapy, although this difference was not statistically significant.26 There are no data to suggest that extremely wide excision margins improve overall survival. Depending on the location of the tumor, significant morbidity can result when 2–3 cm margins are taken. Numerous studies show that if surgery is the sole treatment, recurrence rates are significantly higher than if radiation therapy is added to the regimen. Excision with narrow but clear margins (carried out at the time of SLNB) followed by adjuvant radiation therapy is a reasonable approach to management in many cases. Overly aggressive surgery, including amputation, or very wide margins in cosmetically sensitive areas, decreases quality of life, increases morbidity, delays time to initiation of adjuvant radiation, and does not appear to improve survival or local control rates.

#### SURGERY AT THE DRAINING NODE BED

Completion lymphadenectomy is typically carried out if there is gross involvement of the draining node bed detected clinically. However, the role of completion lymphadenectomy in MCC is controversial. For example, a 2010 study suggests that patients with nodal MCC disease who underwent complete lymphadenectomy had comparable outcomes with patients who only underwent radiation therapy to the nodal bed.27 Two options have similar and excellent control rates for node-positive disease. Although either can be chosen, depending on the clinical situation, it is clear that for microscopic nodal disease, only one of these two modalities should be performed. This is because regional control rates were 100% for each modality and the combination of radiation and surgery to the lymph node bed greatly increases risk of chronic lymphedema.
MCC is an unusually radiosensitive tumor. Recently, there have been reports of successful treatment of MCC with radiation as monotherapy. One study of 43 patients with MCC for whom surgical excision was not possible demonstrated an excellent infield control rate (75%) after treatment with radiation therapy alone. In a separate cohort, there were no recurrences among nine patients treated with radiation monotherapy. One of our cases treated with radiation monotherapy is shown in Fig. 120-6. In most of these cases, the lesion was felt to be inoperable and radiation was given for palliation, but typically resulted in long-lasting local control.

The much more common use of radiation is as an adjuvant to surgery. Adjuvant radiation clearly is critical if surgical margins are positive or if microscopic margins are relatively narrow (<0.5 cm). A retrospective review of the literature indicates a statistically significant improved local and nodal rate of control in this cancer if radiation is added. A cancer registry-based study also indicates improved survival in patients with adjuvant radiation therapy. In this study, patients who received adjuvant radiation therapy had a median survival of 63 months compared to median survival of 45 months in patients who did not receive adjuvant radiation. The typical doses of radiation for MCC are 50–56 Gy for a primary site with negative excision margins. Detailed dosing regimens can be found in the National Comprehensive Cancer Network’s MCC Guidelines. Radiation doses are typically given in 2-Gy fractions, five times/week over 4–6 weeks. Acute side effects from radiation therapy include erythema at the site and mild-to-moderate fatigue that peaks toward the end of radiation and usually resolves within 1–2 months of completing a 5-week course. Chronic radiation skin changes include temporary or permanent alopecia within the irradiated field, epidermal atrophy, loss of adnexal structures leading to skin or mucosal dryness, and risk of subsequent secondary skin cancers in the irradiated region in patients with a life expectancy of greater than 20 years after the radiation treatment. Perhaps the most significant potential side effect is lymphedema. This is more commonly an issue in lower extremities, when radiation therapy is given to the inguinal lymph nodes, especially after surgery has also been carried out in that region. Early referral to a physical therapist trained in lymphedema management is indicated to minimize the severity and incidence of this potential complication in higher risk cases.

Figure 120-6  A. Merkel cell carcinoma on the eyelid arising 3 months before biopsy. The lesion was initially presumed to be a chalazion or cyst. B. Using a thin lead shield to protect the globe, the eyelid, the surrounding tissues, and draining lymph node bed were treated with radiation monotherapy. C. The patient remains recurrence-free at 4.5 years after diagnosis, and thus has a greater than 97% chance of cure at this point.
CHEMOTHERAPY

The most commonly used chemotherapeutic regimen for MCC is the combination of etoposide and either cisplatin (perhaps more clinically effective) or carboplatin (less nephrotoxic). Chemotherapy is often useful in palliation for symptomatic disease that is otherwise inoperable. MCC patients receiving chemotherapy for the first time usually have a significant response with shrinkage of the tumor. Unfortunately, in almost all cases, the tumor grows back and is resistant to chemotherapy even if entirely different agents are used on a subsequent round of chemotherapy. After careful analysis of the literature, we currently do not recommend adjuvant chemotherapy for patients whose MCC has been treated with surgery, radiation therapy, or both. There are six reasons why we currently do not recommend adjuvant chemotherapy:

1. **Mortality:** There is a 4%–7% acute death rate due to adjuvant chemotherapy in MCC partly due to the fact that these patients are often elderly.

2. **Morbidity:** Neutropenia has been reported to occur in 60% of patients with fever, and sepsis in 40%.

3. **Decreased quality of life:** This can be quite severe in this older population, including fatigue, hair loss, nausea, and vomiting.

4. **Resistance to chemotherapy:** MCC that recurs after chemotherapy is less responsive to later palliative chemotherapy.

5. **Immunity:** Chemotherapy suppresses immune function, and this is known in general to be very important in preventing and controlling MCC.

6. **Apparent poorer outcomes:** Among patients with nodal disease, there was a 60% survival if chemotherapy was not given among 53 patients. In contrast, survival was only 40% among node positive MCC patients who did receive adjuvant chemotherapy.

In general, optimal treatment for MCC should involve obtaining pathologically clear margins by surgery, typically with 1- to 2-cm margins as possible, depending on the site. More narrow margins or even positive margins can often be effectively treated by local radiation, typically extending 3–5 cm beyond the tumor bed. We also recommend treating the draining lymph node bed, likely with radiation therapy, for patients with high-risk disease including a positive SLNB, immune suppression, or a tumor greater than 2 cm in diameter. Although still controversial, we currently do not recommend adjuvant radiation therapy for MCC patients with all of the following five good prognostic features: (1) primary tumor diameter ≤ 1 cm; (2) microscopic margins that are confidently negative following surgery; (3) no lymphovascular invasion noted in the tumor; (4) no profound immune suppression (HIV, chronic lymphocytic leukemia, etc.); and (5) SLNB that was negative with proper immunohistochemistry studies.

**CLINICAL COURSE AND COMPLICATIONS**

In more than 90% of cases, MCC is an unanticipated diagnosis at the time the pathology results become known. Most commonly, the lesion in question was thought to be a cyst or acneiform lesion. Although most patients and physicians are not familiar with this disease or its specific management, MCC can be lethal and there is a need to initiate therapy rapidly. In one Australian study, a high fraction of patients (45%) developed progressive disease while waiting for adjuvant radiation therapy to begin (median wait time 41 days). Because of the rarity of this cancer, patients and physicians increasingly resort to the Internet for medical information. One Web site, http://www.merkelcell.org, is devoted to aiding patients and physicians by presenting current data and referral center availability.

Among patients who experience a recurrence of their MCC, ~80% of these happen within 2 years of diagnosis. The most common site of recurrence is the draining nodal basin or adjacent skin. For those who have recurrences, the locations and frequencies are skin (28%), lymph nodes (27%), liver (13%), lung (10%), bone (10%), brain (6%), bone marrow (2%), pleura (2%), and other sites (4%). Once MCC has spread to viscera, it is typically incurable.

Fortunately, for more than 50% of patients, a good outcome can be anticipated. Those who have had no recurrences for 3–5 years, unlike for melanoma, enjoy a greatly reduced risk of recurrence. The complications for those who do not experience a metastasis depend very much on the therapy they received. We believe that treatment with surgical excision and radiation therapy as outlined in this chapter has relatively minimal complication rates and the best possible chance of cure based on current literature. However, for those who receive very aggressive surgical excision, amputation or chemotherapy for low-risk disease, complication rates tend to be higher without improved outcomes.

**KEY REFERENCES**

Full reference list available at www.DIGM8.com

- **DVD contains references and additional content**


Chapter 121  ::  Mammary and Extramammary Paget's Disease


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Section 121.1  ::  Mammary and Extramammary Paget's Disease

**MAMMARY AND EXTRAMAMMARY PAGET’S DISEASE AT A GLANCE**

Rare intraepithelial adenocarcinoma occurring in apocrine gland-bearing skin in patients over 50.

Erythematous, scaly, eczematous plaque frequently misdiagnosed as inflammatory or infectious dermatitis.

Most commonly affected sites: unilateral nipple/areola complex [mammary Paget’s disease (MPD)], vulva, perianal skin, scrotum, and penis [extramammary PD (EMPD)].

Most cases are associated with underlying breast carcinoma (MPD); EMPD not usually associated with underlying neoplasm but may be present in minority (15%) of cases.

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Section 121.2  ::  Clinical Findings

**HISTORY**

Both MPD and EMPD present with a long-standing history of pruritic, erythematous, scaly, or velvety patches on the breast or in apocrine-rich areas such as the groin, perineum, or axilla. Given the rather non-descript appearance, there is frequently a significant delay in diagnosis as initial treatment often involves topical steroids and/or antifungal agents for presumed inflammatory or infectious dermatitis. After continued recalcitrance to therapy, a diagnostic biopsy is performed and the correct diagnosis is made.

**CUTANEOUS LESIONS**

MPD frequently presents as a unilateral, erythematous, scaly plaque involving the nipple and/or the...
Chapter 120
Merkel Cell Carcinoma
Andrew Tegeder, Olga Afanasiev, & Paul Nghiem

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Immunobiology of Merkel Cell Carcinoma: Implications for Immunotherapy of a Polyomavirus-Associated Cancer

Shailender Bhatia • Olga Afanasiev • Paul Nghiem

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Abstract Merkel cell carcinoma (MCC) is an aggressive skin malignancy with a high mortality rate and an increasing incidence. The recent discovery of Merkel cell polyomavirus has revolutionized our understanding of MCC pathogenesis. Viral oncoproteins appear to play a critical role in tumor progression and are expressed in the majority of MCC tumors. Virus-specific humoral and cellular immune responses are detectable in MCC patients and are linked to the natural history of the disease. Despite persistent expression of immunogenic viral proteins, however, MCC tumors are able to evade the immune system. Understanding of the mechanisms of immune evasion employed by MCC tumors is rapidly increasing and offers opportunities for development of rational immune therapies to improve patient outcomes. Here we review recent discoveries in MCC with a special focus on the pathogenic role of Merkel cell polyomavirus and the immunobiology of this virus-associated disease.

Keywords Merkel cell carcinoma • Immunotherapy • Merkel cell polyomavirus • MCV • MCPyV • Cancer virus • Viral cancer • Immune evasion • Immune escape • MHC • Tumor immunology • Tumor infiltrating lymphocytes • TILs • Viral oncoproteins • T-antigen • Immune suppression

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer with a disease-associated mortality three times that of malignant melanoma (46% vs 15%, respectively) [1]. MCC is an uncommon cancer with an estimated 1,600 cases/year in the US [2, 3]. The reported incidence has more than tripled over the past 20 years [3, 4], and the health impact of MCC is growing rapidly with the proportional increase in the aging population [2, 3]. This increasing incidence is in part due to improved detection following availability of a specific immunohistochemical marker, cytokeratin-20 [5], but is also likely due to the higher prevalence of known risk factors for MCC: T-cell immune suppression and Caucasians over 50 years of age with extensive prior sun exposure [6]. MCC now kills more patients than cutaneous T-cell lymphoma and a similar number as chronic myelogenous leukemia, both well-known and frequently studied cancers [2, 7, 8].

MCC is an aggressive cancer with prognosis dependent on the stage at presentation. Stages I and II represent low-risk and high-risk primary disease, respectively, while stages III and IV represent the presence of nodal and distant metastases, respectively. The reported 5-year relative survival for patients with local, nodal, and metastatic disease is 64%, 39% and
18%, respectively [1•]. Although surgery and/or radiation therapy (RT) may be curative for patients with locoregional MCC without distant metastases, relapses are common and often incurable. There is no established adjuvant therapy after definitive management. For patients with distant metastatic disease, systemic chemotherapy is considered. The objective response rate (ORR) with platinum-based chemotherapy regimens is around 60% [9]; however, responses are usually short-lived and the impact on survival is unclear. Also, the chemotherapy regimens are associated with significant toxicity and may not be suitable for many MCC patients who usually tend to be older with multiple comorbidities. There are no established second-line treatments for patients who have progressed on initial systemic chemotherapy regimens. There is therefore a strong and unmet need for novel, biology-driven therapies in this disease.

Fortunately, rapid strides are being made in our understanding of the biology of MCC that have opened up new avenues for investigation of rational therapies in this aggressive disease. We review the recent discoveries in MCC, with a special focus on the emerging importance of immune mechanisms in the pathogenesis of this disease.

Link with Immune Suppression Leads to Discovery of Merkel Cell Polyomavirus

Epidemiologic data suggest a strong link between MCC and the immune system. Individuals with T-cell dysfunction (solid organ transplant recipients [10, 11], HIV-infected patients [12], or chronic lymphocytic leukemia patients [6]) are at fivefold to 50-fold increased risk of developing MCC. MCC tumors sometimes regress following improvement in immune function [13, 14], underscoring the importance of immune surveillance in the development of MCC. Additionally, there are several reported cases of complete spontaneous regression in the MCC literature (a far greater number than expected for its rarity) that suggest a sudden recognition by the immune system leading to the clearance of MCC [15–20]. These epidemiologic data raised the possibility of an infectious etiology for MCC. Indeed, the recent discovery of the Merkel cell polyomavirus (MCV or MCPyV) has provided the missing link between MCC and its association with immune suppression [21•].

The Merkel cell polyomavirus was discovered in 2008 [21••]. Yuan Chang, Patrick Moore, and their colleagues created cDNA libraries from MCC tumor mRNA and used the Digital Transcriptome Subtraction method to identify a novel transcript with high homology to the African green monkey lymphotropic polyomavirus (AGM LPyV). The circular genome of MCPyV (~5,200 base pairs) has an early gene expression region containing the oncoprotein tumor (T) antigen locus with large T (LT) and small T (ST) open reading frames. A late gene region contains the viral structural proteins that encode capsid proteins. MCPyV was found to have the highest homology with the murine polyomavirus subgroup (includes AGM LPyV) and lesser homology to the known human polyomaviruses (BK or JC viruses) or to simian virus 40 (SV40). PCR-Southern hybridization revealed MCPyV sequences to be present in 8 of 10 (80%) MCC tumors, but uncommon in non-MCC tissues (8%) and normal skin or non-MCC skin tumor tissues (16%), suggesting strong association between MCPyV infection and MCC. The monoclonal pattern of integration of the viral genome into the tumor genome was suggestive of MCPyV infection and genomic integration prior to or very early in tumorigenesis. Since the original description of the virus in 2008, several groups around the world have independently verified the association between MCPyV and MCC [22–26•, 27, 28].

Epidemiology of MCPyV Infection

Similar to the other known human polyomaviruses (BK, JC, KI, and WU viruses) [29], exposure to MCPyV as measured by serum antibodies to viral capsid proteins appears to be widely prevalent among healthy subjects [30–32]. In one study, the prevalence of MCPyV seropositivity was 0% in infants, 43% among children aged 2–5 years old, and increased to 80% among adults older than 50 years [30]. A similar trend of increasing seroprevalence with age was seen in another study, suggesting that primary exposure to MCPyV occurs during childhood [29]. Consistent with the serologic data, MCPyV DNA was detected in cutaneous swabs from clinically healthy subjects with a prevalence of 40%–100% in three independent studies [33–35]; it appears that the virus is being shed chronically from clinically normal skin in the form of assembled virions [33]. Besides the skin, viral DNA has been detected in lower frequencies among respiratory secretions, on oral and anogenital mucosa, and in the digestive tract [36–41]. The exact mode of transmission remains to be elucidated and could involve cutaneous, fecal-oral, mucosal, or respiratory routes. Importantly, although widely prevalent, active MCPyV infection appears to be asymptomatic and with the exception of MCC, this virus has not yet been convincingly associated with any other human disease.

Role of MCPyV in Pathogenesis of MCC

Cancer-associated viruses may contribute to carcinogenesis directly via expression of viral oncogenes that promote cell transformation or indirectly via chronic infection and...
inflammation, which may predispose host cells to acquire carcinogenic mutations [42••]. Polyomaviruses are a genus of non-enveloped viruses with a circular double-stranded DNA genome of approximately 5,000 base pairs. The ability of certain polyomaviruses to transform mammalian cells is well known. The best studied example is the SV40 polyomavirus that was originally discovered in the primary monkey kidney cells used to prepare polio vaccines. Alarmingly, SV40 was found to induce multiple tumors in newborn hamsters [43]. Fortunately, despite their prevalence, the known polyomaviruses other than MCPyV have not been associated with formation of any human tumors. Typically, human polyomavirus infection is asymptomatic except in immunosuppressed individuals who can develop nephropathy (BK virus) or progressive multifocal leukoencephalopathy (JC virus). In humans, MCPyV is the first polyomavirus with demonstrated integration into genomic DNA. Several significant observations suggest that MCPyV contributes to the pathogenesis of MCC (Fig. 1): 1) it is present in a substantial portion of MCC tumors [21••]; 2) monoclonality of MCPyV integration in MCC tumor cells suggests viral integration is an early event in tumorigenesis [21••]; 3) T-antigen transcripts and oncoproteins are expressed in most MCC tumors [58]; 4) the MCPyV LT-antigen expressed in MCC tumors is truncated due to mutations that preserve critical cell-cycle progression functions, but eliminate cell-lethal virus-replication activities [44••]; and 5) persistent expression of these MCPyV proteins is required for continued growth of MCC cell lines in vitro [26•, 98]. These findings strongly suggest that MCPyV plays a key role in MCC carcinogenesis rather than merely being a passenger virus that secondarily infects tumor cells. The MCPyV LT-antigen appears to retain the major conserved features of other polyomavirus LT-antigens, including the DnaJ motif (binds to heat-shock proteins) and the LxCxE motif (inactivates retinoblastoma family proteins), and with IHC anti-LT antibody, CM2B4) that plays a key role in MCC pathogenesis [26•, 42••, 46]. Importantly, in parallel, local, systemic, or tumor-induced loss of immune surveillance may allow for an unsupervised increase in both wild-type virus burden and T-Ag–dependent MCC disease. Oftentimes, disease progression can be monitored via immune biomarkers such as anti–T-Ag antibody titers with disease [60•], and disease outcome can be predicted by levels of CD8 T-cell infiltration [63•].

![Diagram](image_url)
the origin-binding and helicase/ATPase domains (promote viral replication) [44••]. These various domains allow the polyomaviruses to use host cell machinery for viral genome replication, but can also target tumor suppressor proteins resulting in cellular transformation [45]. The LT-antigen transcripts are commonly expressed in MCC tumors [44••]. However, tumor-specific truncating mutations retain LT-antigen DnaJ and LxCxE motifs that promote cellular growth, but eliminate origin-binding and helicase domains that are essential for production of progeny virions [44••]. This acquired inability of tumor-derived LT-antigen to initiate constitutive viral genome replication protects virus-infected tumor cells from apoptosis triggered by DNA-damage response mechanisms.

The mechanisms by which MCPyV may contribute to MCC carcinogenesis continue to be elucidated. MCPyV T-antigen appears to be essential for cell survival among tumors infected with the virus. In MCPyV-infected MCC cell lines and xenograft models, the expression of T-antigen appears to be essential for sustained proliferation; knockdown of this viral protein leads to growth arrest and/or cell death while restoration of T-antigen expression rescues cell growth [26•, 46, 98]. Furthermore, interaction with the retinoblastoma (Rb) tumor suppressor protein appears to be critical to the observed growth-promoting effects of LT-antigen [46]. Immunohistochemistry (IHC) data from human MCC tumors shows strong positive association between tumor Rb expression and MCPyV LT-antigen expression, with LT-antigen showing strong positive association between tumor Rb expression [45]. This acquired inability of tumor-derived LT-antigen to initiate constitutive viral genome replication protects virus-infected tumor cells from apoptosis triggered by DNA-damage response mechanisms.

The mechanisms by which polyomaviruses contribute to transformation is interference with the p53 tumor suppressor pathway. The usual functions of p53 are not conducive to viral replication as p53 transactivates genes that lead to cell cycle arrest, which could deprive the virus of essential replication factors. Additionally, active p53 could lead to cellular apoptosis in response to the presence of viral or cellular oncoproteins. In order to complete their normal infectious cycles, the polyomaviruses have developed the ability to block p53 function through several mechanisms. The bipartite domain of the SV40 LT-antigen can bind directly to the specific DNA-binding domain of p53, hence interfering with p53-dependent gene transcription [49, 50] (this binding has also been shown to increase the half-life and steady-state levels of p53 in cells [51]). As the MCPyV LT-antigen seems to be prematurely truncated in the MCC tumor cells lacking the helicase domain and the supposed p53-binding sites [44••], the significance of the p53 pathway in pathogenesis of MCPyV-associated MCC is unclear. However, even if the truncated T-antigen does not bind to p53, MCPyV may play a role in suppressing p53 function in MCC tumors via other mechanisms. For example, there is evidence that the binding of T-antigen to p53 in SV40 may not be sufficient to block p53 function and that other indirect mechanisms (involving small T-antigen and/or the J-binding and Rb-binding domains of the LT-antigen) are also important in functional suppression of p53 [52, 53]. Consistent with MCPyV somehow disabling p53 function in MCC tumors, inactivating mutations in TP53 gene and/or overexpression of p53 have been seen only in a small subset of MCC tumors [54, 55]. Moreover, recent studies have indicated an inverse relationship between p53 expression and MCPyV viral abundance in MCC tumors as well as p53 overexpression potentially being associated with poor outcome [56, 57].

In addition to the processes described above, there are likely additional mechanisms by which MCPyV contributes to the development/maintenance of MCC tumors. For example, the small T-antigen (ST) that shares the N-terminus with LT-antigen has recently been found to play an important role in 1) activating the AKT-mTOR signaling pathway, 2) inducing loss of contact inhibition, and 3) promoting anchorage- and serum-independent growth [98]. While some of these MCPyV-associated pathways may also be relevant to MCPyV-negative MCC tumors (albeit via non-viral mechanisms), the virus-associated MCC subgroup is likely to have important biological distinctions from the virus-negative subgroup. Understanding the molecular mechanisms that contribute to disease progression in various MCC subgroups will be crucial to the development of mechanism-based targeted therapies for this disease.

**Immunology of Merkel Cell Cancer**

The discovery of MCPyV and its role in MCC pathogenesis raises several interesting questions about interactions between the host-immune system and MCC tumor cells. The sero-epidemiologic data (discussed above) suggests that exposure to MCPyV is widely prevalent and that viral capsid proteins are recognized by the human immune system in infected individuals [30, 31]. Also, as discussed above, MCC tumor cells commonly express the MCPyV LT-antigen [44••, 58] and the LT-antigen is essential for continued growth of cells infected with the virus [26•, 46]. Despite this persistent expression of viral proteins, however, MCC tumor cells are
somehow able to evade the immune system. While this can be explained by the presence of generalized T-cell dysfunction in a small subset of MCC patients with comorbidities such as HIV infection, immunosuppressive medications, or concurrent hematologic malignancies, the vast majority (> 90%) of MCC patients have no clinically apparent immune dysfunction [6]. Our understanding of host-virus immune interactions in MCC pathogenesis is increasing rapidly with new insights into the humoral and cellular immunity in MCC patients (Fig. 1).

Humoral Immune Response

Although the prevalence of antibodies to viral capsid proteins (VP) in the general population is high, all studies have found that IgG antibodies to MCPyV VP1 and VP2 are even more prevalent in MCC patients [27, 30, 31, 59]. Interestingly, the titer of antibodies to viral capsid proteins is typically higher in MCC patients than in control populations [30–32]. This finding is not attributable to increased viral capsid antigen production by tumor cells because MCC tumor cells do not express viral capsid proteins [31, 32]. One possible explanation for higher antibody titers in MCC patients could be exposure to a greater virus burden in MCC patients. Supporting this hypothesis, the MCPyV DNA levels in cutaneous swabs from MCC patients were found to be significantly higher than levels in control population [34], and another study reported a positive correlation between serum MCPyV antibody titers and MCPyV DNA levels in skin biopsies [59]. The apparently higher virus burden in MCC patients could possibly be a risk factor that predisposes to subsequent development of MCC in these patients; alternatively, the development of MCC could somehow have resulted in a MCPyV-specific immunodeficiency that leads to the higher virus levels on the skin of MCC patients (further discussed below). Interestingly, higher anti-MCPyV capsid antibody titers have also been associated with better progression-free survival in MCC patients [32]; whether this indicates the presence of a more robust host immune system remains unclear.

The limited serologic data from patients with MCPyV-negative MCC tumors suggests that the majority of these patients have been exposed to MCPyV [27], and in many patients, antibody titers can be very high, similar to patients with MCPyV-positive MCC [30]. This raises the fascinating possibility of MCPyV infection possibly playing a role in tumor initiation with subsequent selection for less immunogenic, MCPyV-negative MCC tumor subclones in these patients. Indeed, the heterogeneity of MCPyV DNA or T-antigen expression levels in MCC tumors supports immune selection within the tumors and is consistent with the “hit and run” hypothesis for tumorigenesis in MCPyV-negative MCC tumors.

As compared to antibodies to viral capsid proteins, antibodies to MCPyV T-Ag oncoproteins are more specifically associated with MCC; these antibodies are rarely detected in the general population (< 1%) but appear to be present in a substantial proportion (~40%) of patients with active MCC [60•]. Importantly, the titer of antibodies to T-antigen oncoproteins correlates strongly with the presence of MCPyV DNA and the expression of T-antigens in MCC tumor cells [60•]. Moreover, the antibody titer to T-Ag oncoproteins can potentially serve as a biomarker of MCC disease burden; the antibody titer drops rapidly after successful treatment of MCC tumors and a rising titer in a previously treated patient has been shown to herald disease progression prior to development of symptoms [60•]. This apparent correlation between the humoral response to T-antigens and MCC disease burden is not completely unexpected because T-antigen expression is selectively linked to MCC tumors. Specifically, in contrast to viral capsid proteins that are readily visible to the host humoral immune system, T-antigens are not present in viral particles, are only expressed after viral entry into host cells, are located in the nucleus [61], and are thus less likely to trigger an antibody response except in the setting of dying or diseased tissue (such as a tumor that persistently expresses T-antigens).

Cellular Immune Response

The presence of MCPyV T-antigen–specific antibodies that appear to correlate with tumor burden in MCC patients [60•] suggests ongoing expression of viral proteins in tumor cells and their recognition by the adaptive arm of the immune system. Histologic analyses have revealed the presence of variable numbers of tumor-infiltrating lymphocytes (TILs) in the MCC tumors with possible prognostic significance [62]. Our group has recently documented that intratumoral (but not peritumoral) infiltration of CD8+ lymphocytes is an independent predictor of improved survival among MCC patients. In this study, unbiased gene expression analyses revealed overexpression of immune response genes in tumors with favorable prognoses. These immune response genes included genes that encode components of cytotoxic granules (granzymes), chemokines (CCL19), lymphocyte-activation molecules, and CD8 receptor molecules [63•]. Importantly, in an independent cohort of 156 cases, patients with robust CD8+ intratumoral infiltration had 100% MCC-specific survival as compared to 60% survival among patients with sparse or no CD8+ intratumoral infiltration [63•]. This evidence highlights the important role of cellular immune responses in the natural history of MCC and further explains the increased incidence of MCC in patients with cellular immune suppression. Furthermore, we have identified MCPyV-specific epitopes that are immunogenic to CD8 and CD4 T cells isolated from blood and MCC tumors [97]. These epitopes and corresponding tumor-specific T cell responses represent candidate targets for therapeutic manipulation in MCC patients.
Immune Evasion Mechanisms in MCC

Despite the expression of immunogenic virus-encoded oncoproteins in the majority of tumors [44••, 60•], MCCs that became clinically evident were significantly able to evade host immune responses. According to the cancer immunoediting hypothesis [64••], development of tumors generally requires cancer cells to navigate successfully through three distinct (and usually sequential) phases of the interaction between the cancer and the host immune system: 1) elimination phase, an immunosurveillance phase in which the innate and adaptive immune systems work together to detect the presence of nascently transformed cells and destroy them before a tumor becomes clinically apparent; 2) equilibrium phase, a tumor dormancy phase in which the adaptive immune system restrains the outgrowth of tumors and sculpts the immunogenicity of the tumor cells; and 3) escape phase, a tumor progression phase in which the tumor cells are able to circumvent the host immune response manifesting as clinically progressing tumors. The lack of a good animal model for MCC pathogenesis and the inherent challenges of conducting longitudinal studies in at-risk individuals for a rare cancer render it difficult to study the precise events during the elimination and equilibrium phases of MCC tumorigenesis. However, the potential mechanisms of immune escape by MCC tumors are becoming increasingly apparent (Fig. 1).

The progression from equilibrium to the escape phase may occur due to changes in tumor cell population that may acquire new immune evasive characteristics or due to changes in the host immune system that may get suppressed either generally or more selectively toward the tumor cells. Both of these broad mechanistic categories appear relevant to MCC.

Tumor Cell Changes

Under the pressures of immune selection, MCC tumor cells may acquire new features to become either “less visible” to the immune system or “more resistant” to the effects of the cytotoxic immune cells. The former may occur via loss of tumor antigen expression. Cell surface major histocompatibility complex class I (MHC-I) serves to present intracellular peptides to CD8+ T lymphocytes; specifically, viral oncoproteins expressed in MCC tumor cells would be presented to T cells via MHC-I. Indeed, multiple viruses (eg, adenovirus and HSV) and virus-associated cancers (eg, Kaposi’s sarcoma, cervical cancer) are known to directly or indirectly down-regulate the expression of MHC-I as a key mechanism of immune escape [65–70]. Besides MHC-I loss, dysregulation of other components of cellular antigen-presenting machinery such as the transporter associated with antigen processing (TAP) [71] or down-regulation of appropriate tissue-specific T-cell homing signals may also preclude the presentation of persistently expressed tumor antigens to T cells and need to be investigated further in MCC. Indeed, our laboratory findings suggest that 46% of MCC tumors exhibit a “stalled phenotype” of lymphocytic infiltration where CD8+ cells accumulated near the tumor-stroma border but were unable to infiltrate into the tumors [63•]. Such “peritumoral” T cells were not associated with significantly improved survival. These features together likely lead to poor visibility of the MCC tumor cells to the immune system and may explain the sparse infiltrates of T cells in most MCC tumors that are associated with poor outcomes [63•]. Another important adaptation at the tumor cell level that can result in immune escape is increased resistance of the tumor cell to immune control mechanisms. Innate immune signaling networks and tumor suppressor pathways share some key proteins such as p53 [72] and cyclin-dependent kinase inhibitor p21 [73]. Due to this functional overlap, the targeting of tumor-suppressor pathways by MCC oncoproteins may also serve as an immune evasion mechanism for MCC. In addition, tumor cells may secrete proteins that interfere with the functioning of the immune cells (discussed below).

Immune System Changes

Immunosuppression resulting in T-cell dysfunction may predispose to the immune escape of transformed cancer cells; however, clinically evident systemic immunosuppression due to comorbidities such as post-transplant status, concurrent hematological malignancy, HIV infection, etc. is present only in fewer than 10% of MCC patients. What may be of even greater relevance to the pathogenesis of MCC, a disease of the elderly population, could be the altered phenotype and functional incapacity of an aging immune system that allows the development and progression of the disease (Fig. 1). This phenomenon of immunosenescence, an erosion of the immune response with aging, is associated with phenotypic and functional changes in both innate and adaptive arms of the immune system, including a contracted repertoire of naïve and cytotoxic T-cells and impaired function of effector T cells [74]. Ultraviolet radiation (UVR), another risk factor for MCC, may not only promote critical LT-antigen mutations and ST-antigen upregulation [75], but may also play a key role in cutaneous immune system inhibition and tolerance [76]. Specifically, UVR has been implicated in recruitment of regulatory T cells and in inhibition of antigen presentation via direct damage to antigen presentation cells (APCs) or via functional inhibition of APCs by cytokines (interleukin 10, tumor necrosis factor-α) released by kerati-
nocytes and mast cells [77, 78]. In addition to systemic immune dysfunction contributing to immune escape, it is likely that MCC tumor cells establish a local immunosuppressive microenvironment in order to thrive. In this scenario, immunologically sculpted tumor cell subclones may overproduce immunosuppressive cytokines, such as TGF-β [79], Fas-L [80], IL-10 [81], or inhibitors of T-cell responses such as galectin-1 [82] and indoleamine 2,3-dioxygenase (IDO) [83]. Tumors could also suppress proinflammatory danger signals through pathways involving activated STAT3, leading to impaired dendritic cell maturation [84], or could downregulate the NKG2D receptor on immune effector cells by secretion of soluble forms of the MIC NKG2D ligands thereby attenuating lymphocyte-mediated cytotoxicity [85]. Tumor cells may also facilitate the generation, activation, or function of immunosuppressive cells [86], such as CD4+ CD25+ regulatory T cells (T-regs) [87] or myeloid-derived suppressor cells [88]. T-cell exhaustion, originally described in the context of chronic viral infection in mice [89, 90], is being found to be increasingly relevant to human cancers. In response to chronic antigen exposure, antigen-specific CD8+ T-cells often develop an exhausted phenotype with poor effector function, sustained expression of inhibitory receptors, and a transcriptional state distinct from that of functional effector or memory T cells. The final stage of exhaustion may involve physical deletion of antigen-specific T cells [89, 91]. In the context of viral infection, more severe CD8+ T-cell exhaustion has been correlated with higher viral load. Moreover, in the setting of the same viral load, epitopes that were present in larger amounts led to more extreme exhaustion and/or deletion than epitopes present in smaller amounts [91]. This phenomenon may possibly be relevant in MCC as well and could explain the observed higher MCPyV viral load on the skin of MCC patients as compared to the general population (discussed above) if MCPyV-specific T cells are exhausted by chronic antigen exposure in the tumors and hence fail to suppress MCPyV colonization [34]. The interaction of programmed death (PD)-1 expressed on T-cells with its ligand B7H1 or PDL-1 is an important mechanism of T-cell exhaustion [92] that could be harnessed for therapeutic purposes.

Moving Toward Biology-Driven Immunotherapy

The discovery of the MCPyV and the increasing recognition of the importance of the immune system in MCC pathogenesis suggest several new targets for therapeutic exploration; rational immunotherapeutic approaches can possibly advance outcomes for this aggressive disease. The critical role of viral oncoproteins in tumorigenesis of MCPyV-positive MCC tumors and the resultant cellular expression of viral peptides could not only be exploited to develop virus-targeting therapies interfering with the function of the oncoproteins, but also be harnessed to stimulate immune responses against virus-infected tumor cells. As an example, the T-antigen–specific antibody response is confined to a 78 amino acid N-terminus domain shared by the small and large T-antigens [60*], which could provide a suitable vaccine or adoptive T-cell therapy target. Similarly, other non-viral tumor-associated antigens such as survivin [93] or the oncoprotein HIP1 that interacts with c-KIT [94] may also be suitable immunotherapy targets. Immunostimulatory cytokines, such as interferons, interleukin (IL)-2, IL-12, IL-15, or IL-21, could be delivered systemically or intratumorally to counteract immune evasion mechanisms employed by MCC tumors. A phase 2 trial using intratumoral delivery of IL-12 plasmid DNA followed by in vivo electroporation of MCC tumors will be opening to accrual soon. Other therapeutic agents that look appealing to investigate for MCC treatment include CTLA-4 receptor-blocking agents such as Ipilimumab (recently approved by the FDA for metastatic melanoma), drugs targeting the PD-1/PDL-1 pathway to reverse immune exhaustion of infiltrating lymphocytes, or drugs targeting the co-stimulatory 4-1BB pathways that could promote T-cell infiltration, proliferation, and cytokine production [95, 96].

Given the heterogeneity of MCC tumors and individual variations in host immune systems, it is unlikely that one single approach will be effective in all patients. Rather, a combination of various strategies and personalization to the unique biologic characteristics of MCC tumors in individual patients will be required. Nevertheless, it is an exciting time for investigation of novel targeted and/or immune therapies in this fascinating malignancy.

Conclusions

The discovery of Merkel cell polyomavirus has revolutionized our understanding of MCC pathogenesis. The immune system appears to be playing a major role in MCC biology with increasing evidence of virus-specific cellular and humoral immune responses that influence the prognosis of MCC patients. MCC tumors are able to evade the immune system by establishing a local immunosuppressive microenvironment. Understanding the mechanisms of immune evasion by MCC tumors will offer opportunities for development of biologically driven therapies to improve patient outcomes from this often lethal virus-associated cancer.

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Disclosure No potential conflicts of interest relevant to this article were reported.
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•• Of major importance

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Merkel Cell Polyomavirus-Specific CD8+ and CD4+ T-cell Responses Identified in Merkel Cell Carcinomas and Blood

Jayasri G. Iyer1, Olga K. Afanasiev1,2, Christopher McClurkan3, Kelly Paulson1,2, Kotaro Nagase1,4, Lichen Jing1, Joshua O. Marshak4, Lichun Dong4, Joseph Carter6,9, Ivy Lai10, Erik Farrar12, David Byrd5,12, Denise Galloway6,8,9, Cassian Yee4,10,12, David M. Koelle3,4,7,11,13, and Paul Nghiem1,2,10,12

Abstract

Purpose: Merkel cell polyomavirus (MCPyV) is prevalent in the general population, integrates into most Merkel cell carcinomas (MCC), and encodes oncoproteins required for MCC tumor growth. We sought to characterize T-cell responses directed against viral proteins that drive this cancer as a step toward immunotherapy.

Experimental Design: Intracellular cytokine cytometry, IFN-γ enzyme-linked immunospot (ELISPOT) assay, and a novel HLA-A*2402–restricted MCPyV tetramer were used to identify and characterize T-cell responses against MCPyV oncoproteins in tumors and blood of MCC patients and control subjects.

Results: We isolated virus-reactive CD8 or CD4 T cells from MCPyV-positive MCC tumors (2 of 6) but not from virus-negative tumors (0 of 4). MCPyV-specific T-cell responses were also detected in the blood of MCC patients (14 of 27) and control subjects (5 of 13). These T cells recognized a broad range of peptides derived from capsid proteins (2 epitopes) and oncoproteins (24 epitopes). HLA-A*2402–restricted MCPyV oncoprotein processing and presentation by mammalian cells led to CD8-mediated cytotoxicity. Virus-specific CD8 T cells were markedly enriched among tumor infiltrating lymphocytes as compared with blood, implying intact T-cell trafficking into the tumor. Although tetramer-positive CD8 T cells were detected in the blood of 2 of 5 HLA-matched MCC patients, these cells failed to produce IFN-γ when challenged ex vivo with peptide.

Conclusions: Our findings suggest that MCC tumors often develop despite the presence of T cells specific for MCPyV T-Ag oncoproteins. The identified epitopes may be candidates for peptide-specific vaccines and tumor- or virus-specific adoptive immunotherapies to overcome immune evasion mechanisms in MCC patients. Clin Cancer Res; 17(21); 6671–80. ©2011 AACR.

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer. Its reported incidence has quadrupled in the past 20 years to approximately 1,600 cases per year in the United States (1). In 2008, the Merkel cell polyomavirus (MCPyV) was discovered and found to be integrated into the host genome in approximately 80% of MCC tumors (2). This association of MCPyV with MCC has been confirmed by multiple groups worldwide (3). MCPyV infection, defined by serology or detection of viral DNA, is prevalent in both healthy persons and MCC patients (4). However, in MCC patients, MCPyV acquires oncogenic potential via rare integration and T-antigen (T-Ag) truncation mutations (5, 6). These large T-Ag (LT) truncation mutants bind and inactivate the retinoblastoma tumor suppressor but no longer induce lytic viral replication that would be lethal to a cancer cell (7). The small T-Ag (ST) shares the N-terminus with LT and plays an important role in activating the AKT–mTOR signaling pathway (8, 9). Importantly, MCPyV/T-Ag oncoproteins are persistently expressed in MCCs and are required for growth (6, 9).

The cellular immune system appears to be critical in preventing and controlling MCC. Patients who are chronically immunosuppressed by HIV infection, chronic lymphocytic leukemia, or medications after solid organ transplant have a 3- to 30-fold increased risk of MCC and represent approximately 10% of MCC patients (10). There

Authors’ Affiliations: Departments of 1Medicine/Dermatology, 2Pathology, 3Laboratory Medicine, 4Medicine, 5Surgery, 6Microbiology, and 7Global Health, University of Washington; Divisions of 8Human Biology, 9Public Health Sciences, 10Clinical Research, and 11Vaccine and Infectious Diseases, 12Seattle Cancer Care Alliance, Fred Hutchinson Cancer Research Center; 13Benaroya Research Institute, Seattle, Washington; and 14Department of Medicine/Dermatology, Saga University, Nabe-shima, Japan

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J.G. Iyer and O.K. Afanasiev contributed equally to this work.

D.M. Koelle and P. Nghiem are co-senior authors.

Corresponding Author: Paul Nghiem, Department of Dermatology, University of Washington, 815 Mercer Street, Seattle, WA 98109. Phone: 206-221-2632; Fax: 206-221-4364; E-mail: pnghiem@uw.edu
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are numerous cases of spontaneous MCC regression, suggesting immune-mediated cancer control (11, 12). Furthermore, intratumoral infiltration of CD8 lymphocytes is an independent predictor of improved survival in MCC (13). The high prevalence of antibodies to MCPyV T-Ag (14) and VP1 capsid proteins (15–17) in MCC patients is suggestive of virus-specific helper CD4 T-cell responses. Despite the virus dependence of most MCC tumors and the high seroprevalence of MCPyV in the general population (15–17), there have been no reports of MCPyV-specific T-cell reactivity in MCC patients or control subjects. We hypothesized that MCPyV proteins are immunogenic and are recognized by T lymphocytes in MCC tumors and peripheral blood of patients and control subjects. Here, we show that MCPyV-specific CD8 and CD4 T cells can localize to MCC tumors and report 26 novel MCPyV T-cell epitopes, some of which may serve as targets for immunotherapy in the future.

**Materials and Methods**

**Human subjects and clinical samples**

This study was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board and conducted in accordance with Helsinki principles. Subjects (Supplementary Table S1) gave informed consent. Peripheral blood mononuclear cells (PBMC) obtained from heparinized blood samples were cryopreserved. Tumor samples obtained from medically necessary biopsies or surgeries were transported in T-cell medium (TCM; ref. 18).

**Synthetic viral peptides**

A total of 428 peptides (13-mers, overlapping by 9 amino acids) were synthesized (Sigma-Aldrich; Supplementary Table S2) from MCPyV reference sequence GenBank #EU375803 and strain variant #EU375804. Peptide pools (~25 peptides per pool) were organized by viral domains (Fig. 1). Additional truncated MCPyV peptides and polyomavirus-derived homologous peptides (predicted by CLUSTAL 2.0.12 sequence alignment software) were synthesized (Supplementary Table S3).

**Tumor-infiltrating lymphocytes culture**

Minced MCC tissue was plated in 48-well plates in TCM plus 1.6 μg/mL phytohaemagglutinin (PHA; Remel), 32 units/mL natural human interleukin-2 (nIL-2; Hemagen Diagnostics), and allogeneic-irradiated PBMCs. nIL-2 in

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**Figure 1.** MCPyV proteome and synthetic peptide pools. MCPyV proteins (gray boxes) are annotated with nucleotide (nuc) and amino acid (a.a.) numbers. Peptides spanned the entire MCPyV proteome and were grouped into pools (boxes with a.a. range indicated) on the basis of protein domains. Double outlined boxes represent persistently expressed LT, ST, and CT (shared T-Ag N-terminus, light-gray slashed lines) domains. Jagged line at nucleotide 429 represents the genomic splice site. c(#) indicates the corresponding complementary DNA nucleotide position number.
fresh TCM was added every second day for 14 to 20 days (18). When conducted, a second tumor-infiltrating lymphocyte (TIL) expansion used anti-CD3 monoclonal antibody (mAb) OKT3 (Ortho), recombinant IL-2 (Chiron Corporation), and feeder cells (19). Cultures expanded with anti-CD3 mAb are comprised entirely of CD3-Qdot655 (Invitrogen)-positive cells (Supplementary Fig. S1).

T-cell cloning

Subject w347 primary tumor TILs were cloned by limiting dilution and subsequently expanded (20). Subject w347 metastatic tumor was collagenase (Sigma)-digested and plated as single-cell suspensions in TCM plus PHA and IL-2 for limiting dilution cloning. Subject w447 bulk TILs were tetramer-sorted and expanded (19).

ELISPOT

**Cultured assays.** PBMCs plated in TCM at 3 × 10^5 cells per well in 96-well flat bottom plates were stimulated on day 1 with MCPyV peptide pools (1 μg/mL of each peptide), individual peptides (1 μg/mL), cytomegalovirus (CMV), Epstein–Barr virus (EBV), influenza peptides (CEF, 2 μg/mL; Cellular Technology Ltd.) as positive control or dimethyl sulfoxide (DMSO; <0.2%) and/or TCM as negative control and cultured for 10 days. Fresh TCM with 20 U/mL IL-2 (Chiron Corporation) and 20 ng/mL IL-7 (R&D Systems) were added on days 3, 6, and 9. On day 11, cells were plated in 96-well multiscreen IP plate (Millipore) precoated with anti-CD3 mAb are comprised entirely of CD3-Qdot655 (Invitrogen)–positive cells (Supplementary Fig. S1).

Plasmids, transfection, and immunoblot

Cos7 cells were transfected with plasmid-encoding MCPyV LT and/or HLA-A*2402 cDNA using FuGENE6 transfection reagent (Roche) following manufacturer-recommended protocol (Supplementary Methods). Protein product was confirmed by immunoblotting for MCPyV LT-specific mAb CM2B4 (Santa Cruz; ref. 24). Expression of HLA-A*2402 was confirmed by flow cytometry with A23/A24 mAb (One Lambda) followed by phycoerithrin (PE)-labeled IgG secondary antibody (Invitrogen) at 48 hours. Controls included HLA-A*2402–positive and negative LCLs. Transfected Cos7 or control cells were used as APCs in intracellular cytokine (ICC) cytometry.

Cytotoxicity assay

Target cell lysis was determined by a 4-hour ^51^Cr release assay in quadruplicates (25). Negative controls included LTL-transfected Cos7 plus effectors and Cos7 transfected with LT and HLA-A*2402 without effectors. Positive control included detergent-lysed target cells.

Tetramer staining

PE- and APC-conjugated complexes of HLA-A*2402 and peptide LT-92-101 (EWWRSGGSF; CPC Scientific) or irrelevant peptide (NY-ESO-158-166; ref. 26), were synthesized at the Immune Monitoring Laboratory (FHCRC, Seattle). Cells were stained with tetramer for 60 minutes at 37°C, followed by anti-CD8-APC mAb (BD Biosciences) for 30 minutes at 4°C, then washed with fluorescence-activated cell-sorting (FACS) buffer, fixed, and analyzed.

MCPyV DNA sequencing and detection

DNA extraction, amplification, and sequencing are detailed in Supplementary Methods (3, 27). Sequence data were submitted to GenBank for subjects w447 (accession number JF912157) and w347 (accession number JF912158).

HLA typing

HLA-typing genotypes from blood-derived DNA were determined by sequence-based typing of exons 2 and 3 (ref. 28; FHCRC, Seattle) or with commercially available (One Lambda; Qiagen) sequence-specific primer PCR kits (Puget Sound Blood Center, Seattle; Hematologisk Laboratorium, Denmark).

Immunohistochemistry

MCPyV T-Ag staining was done using CM2B4 antibody (Santa Cruz; ref. 14, 24). Anti-CD8 (Novocastra) and anti-CD4 (Cell Marque) antibodies were used at 1:200 and 1:25 dilution, respectively.
Statistical analyses
Analyses were conducted using 2-tailed Fisher exact test in Stata v11.0 (StataCorp). A value of \( P < 0.05 \) was considered significant.

Results
To study the T-cell response to MCPyV proteins, we isolated lymphocytes from MCC tumor tissues and blood donated by MCC patients and control subjects (Supplementary Table S1). Fresh tumor specimens were obtained from 10 MCC patients requiring diagnostic or therapeutic surgery. TILs were successfully cultured from each tumor in sufficient numbers to test T-cell reactivity to regions of T-Ag that are persistently expressed in MCC tumors (peptide pools 1–3 and 10; Fig. 1 and Supplementary Table S2).

MCPyV T-Ag oncoproteins were present in 24 of 33 tumors (72%) as evaluated by immunohistochemistry (IHC). T-cell responses to MCPyV T-Ag as assessed by IFN-\( \gamma \) secretion were found in 2 of 6 T-Ag protein-expressing tumors. T-Ag–specific T cells were not detected among the 4 tumors that did not express viral oncoproteins.

Identification of the MCPyV large T-Ag epitope recognized by CD8\(^+\) TILs
Subject w447 presented at age 67 with a 1.5-cm primary MCC lesion on his left hip. The patient underwent wide local excision of the tumor, a portion of which was used for obtaining TILs. IHC revealed CD8\(^+\) cells within an MCPyV T-Ag protein–positive tumor that had low HLA class I expression (Fig. 2A). After 14 days of expansion in the presence of PHA and nIL-2, TILs were screened by ICC.
of the immunogenic peptide (MCPyV LT 92-101) to HLA-peptide binding algorithms (30) predicted strong binding. To address this, we cotransfected full-length MCPyV LT and HLA-A2402 into the primate cell line Cos7 (Fig. 2E–F). Transfection analysis identified a 10-amino acid section, LT-92-101 (EWWRSGGFSF), that generated strong CD8 IFN-γ responses that persisted in peptide titrations to as low as 1 ng/mL (Fig. 2B and C). Notably, engagement of TCR signals may downregulate CD8 expression (29), explaining slightly low CD8 signal among IFN-γ–producing cells. Sequencing of DNA amplified from this patient’s tumor confirmed the predicted MCPyV large T-Ag amino acid sequence (amino acids 92–101, data not shown).

We next determined whether TILs from this subject cross-reacted to peptide homologues of MCPyV LT-92-101 derived from other human polyomaviruses. Cross-reactivity with other polyomaviruses could indicate T-cell priming by other pathogens prior to tumor infiltration. Conversely, if the TIL response was MCPyV specific, this would suggest that MCPyV was the relevant immunogen. To address this, we tested 13-mer peptides homologous to MCPyV LT-92-101 (Supplementary Table S3) and did not detect cross-reactivity among TILs to these peptides (Fig. 2D).

**HLA restriction and endogenous processing of MCPyV large T-Ag**

We sought to determine the restricting HLA allele from among this patient’s genotype (A*03G1, A*24G1, B*1501, B*3502, Cw*0304, and Cw*04G1). Two published HLA-peptide binding algorithms (30) predicted strong binding of the immunogenic peptide (MCPyV LT 92-101) to HLA-A*2402. This in silico prediction was confirmed using cells matched to subject w447 only at HLA-A*2402. Although allelically partially matched PBMCs induced IFN-γ secretion in CD8 TIL from subject w447, peptide-loaded PBMCs that were not matched at any HLA locus induced minimal IFN-γ secretion (Supplementary Fig. S2).

To study MCPyV oncoprotein processing into short peptides, we cotransfected full-length MCPyV LT and HLA-A*2402 into the primate cell line Cos7 (Fig. 2E–F). Transfected cells were exposed to subject w447 TILs, and T-cell activation was measured by IFN-γ production. Mock-transfected cells, or cells transfected with A*2402 alone, did not activate subject w447 TIL. However, cotransfection of LT and A*2402 led to readily detectable activation of CD8 TIL by IFN-γ ICC (Fig. 2G). Furthermore, we observed specific lysis of Cos7 cells that expressed MCPyV LT and HLA-A*2402 by a TIL-derived CD8 T-cell clone (Supplementary Fig. S3). These MCPyV-specific CD8 cells also have excellent cytolytic potential against peptide-pulsed autologous cells (91% and 100% lysis of PBMCs pulsed with peptide LT-92-101 at 1 μg/mL and 5 μg/mL, respectively; Supplementary Fig. S3). We conclude that (i) MCPyV LT can be processed into appropriate peptides, (ii) these peptides can access the HLA class-I antigen presentation pathway for proper load-
to MCPyV CT-57-69, we investigated the cytokine secretion profile of the expanded CD4 TIL population. Interestingly, we observed the secretion of both Th1 and Th2 type cytokines, namely, IFN-γ, IL-5, and IL-10 (Fig. 4C), although the latter cytokine was only modestly increased compared with background. Moreover, 2 of 58 tested T-cell clones isolated by limiting dilution from the primary TILs also secreted IFN-γ and 1 of 1 clones tested also secreted IL-10 in response to peptide (Fig. 4D).

Two months after primary tumor excision, subject w347 developed a skin recurrence of MCC (Fig. 4E, left). After clonal microculture of the digested tumor tissue, 1 of 53 clones tested produced a robust IFN-γ response to the same peptide (CT-57-69) as in the primary tumor (Fig. 4E, right).

T-cell responses to MCPyV in the peripheral blood

PBMC reactivity was screened against the MCC-associated T-Ag oncoprotein domains (pools 1, 2, 3, and 10) in MCC patients and control subjects. We observed a trend toward a higher frequency of T-cell responses in patients than in control subjects for pool 1 (37% of patient PBMC samples vs. 8% of control subject samples), pool 2 (31% vs. 17%), and pool 10 (37% vs. 17%; Fig. 5A). The magnitude of pool-level responses for each subject in Fig. 5A is included as Supplementary Fig. S6. We detected PBMC responses against T-Ag in 12 of 19 patients with MCPyV-positive tumors. In contrast, we found T-Ag–specific T-cell reactivity in blood from 1 of 6 MCC patients with MCPyV-negative tumors ($P = 0.07$).

Twenty-four additional immunogenic MCPyV epitopes were identified. Breakdown of the reactive pools to single reactive peptides is shown in Fig. 5B for 2 MCC patients and 2 control subjects. A T-cell response to a single peptide (SI-125-137) was common to both subjects C7 and w147. Responder T-cell phenotype to a single peptide was determined for 5 immuno-reactive T-Ag–derived peptides in control subject C4 by a cultured ICC assay. In each case, the reactive cells were CD4 positive (data not shown). To explore whether these CD4 T-cell responses were likely to be MCPyV specific, we compared the sequence of the reactive MCPyV 13-mers with the homologous regions from other known polyomaviruses (Supplementary Table S3). Among these 5 peptides, 3 were from an MCPyV unique protein domain and 1 was sequence divergent when compared with homologous peptides, suggesting MCPyV specificity in most cases.

T-cell responses to the entire MCPyV proteome (18 peptide pools) were also assessed in 4 control subjects. PBMCs from each subject showed strong reactivity to at least 1 portion of the MCPyV proteome (Fig. 6A). Of note, PBMCs from all 4 control subjects reacted to peptides in pool 13 (derived from the capsid protein VP1). Single peptide-level responses were deconvoluted for 2 subjects (C7 and C8). Subject C7 showed robust T-cell responses to multiple peptides within pool 13 (Fig. 6B, top). The peptide with the strongest response (VP1-225-237) was shown to be CD4 positive (Fig. 6B, top, inset). This particular peptide shares amino acid identity at 5 of 13 positions, with all of the homologous peptides derived from 5 other polyomaviruses (Supplementary Table S3). All 6 of these related peptides elicited T-cell reactivity from this subject’s PBMCs (Fig. 6B, top, right). In subject C8, VP1-245-257 was the only reactive peptide within pool 13 and it stimulated CD4 cells (Fig. 6B,
bottom, inset). This immunoreactivity was MCPyV specific, as homologous peptides from other polyomaviruses were nonreactive (Fig. 6B, bottom, right). Aggregate results for all 26 peptides described in this study are shown in Supplementary Table S4.

**Discussion**

The strong association of MCC with both a polyomavirus and T-cell dysfunction suggests that dissection of the specificity, trafficking, and effector functions of MCPyV-specific T cells is medically significant. We show that circulating and tumor-infiltrating T cells can recognize specific peptides from MCPyV T-Ag oncoproteins and capsid structural proteins and that both CD8 and CD4 MCPyV-specific T cells are locally enriched in at least some MCC tumors. Twenty-six novel MCPyV T-cell epitopes have been identified. We confirmed that the T-Ag oncoprotein could be processed and presented to stimulate cell lysis by virus-specific CD8 cells isolated from a patient’s tumor. We developed an MCPyV tetramer that should serve as the first of several tools for isolating and determining the function of MCPyV-specific CD8 T cells directly ex vivo. These findings advance our understanding of the association of immunosuppression with MCC and help enable rational therapies for MCC that augment virus-specific T-cell activity.

Although not previously described, several lines of evidence suggest that MCPyV-specific T cells should be present...
in patients and control subjects. Serologic data indicate that MCPyV infection is commonly acquired during childhood (16, 32, 33). The presence of MCPyV-specific antibodies in 53% to 88% of healthy persons (15–17) implies the presence of MCPyV-specific CD4 T-cell help (indeed, all MCPyV-responsive T cells identified in our PBMC-based assays were CD4^+). Similar to these findings in MCPyV, T-cell responses have been described (34–37) in other

Figure 5. Prevalence of PBMC reactivity to MCPyV oncoproteins and determination of immunoreactive epitopes. A, PBMC T-cell reactivity (as described in ELISPOT interpretation criteria in Supplementary Methods) to specified MCPyV peptide pools among MCC patients (black) and control subjects (gray). Although there is a trend, data did not reach statistical significance. B, identification of immunoreactive epitopes derived from previously reactive T-Ag peptide pools by cultured IFN-γ 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.

Figure 6. MCPyV proteome-wide screen of T-cell responses and characterization of select epitopes. A, PBMCs from 4 control subjects showed reactivity to both MCPyV T-Ag and structural proteins as measured by cultured IFN-γ ELISPOT assay. B, characterization of T-cell responses to pool 13 peptides derived from VP1 capsid protein in control subject C7 (top) and C8 (bottom). Left, stimulation with CEF-positive control, pool 13, or single 13-mer peptides from pool 13. Inset, ICC/flow cytometric analysis identifies IFN-γ^+ CD4^+ T cells upon peptide stimulation. Right, PBMCs challenged with relevant peptide or homologous peptides from indicated polyomaviruses.
cancer-associated viruses, such as human papilloma virus and hepatitis C virus HCV, but are typically of low abundance (38, 39). Cultured ELISPOT or ICC assays are usually required for their detection (35, 40), but occasionally, their abundance is sufficient to be detected ex vivo by tetrasters (41).

Our data document significant enrichment of MCPyV-specific T cells in an MCC tumor compared with blood. In subject w447 (with a small, slow-growing tumor), we detected a marked enrichment of antigen-specific CD8+ T cells in TILs compared with blood using tetramer staining. Because primary tumor tissue was sparse, TILs were expanded using a nonspecific mitogen (PHA + IL-2) cocktail that uniformly expands the peripheral T-cell repertoire (42). We therefore believe the relative frequency of antigen-specific TILs remains stable after expansion. Regardless, this finding indicates that local immune evasion mechanisms in this tumor did not block the trafficking and enrichment of virus-specific T cells. However, the tumor may persist despite these antigen-specific CD8+ T cells due in part to low HLA-I expression. Conversely, chronic exposure to antigen may mediate an exhausted phenotype of antigen-specific CD8+ T cells with poor effector function and sustained expression of inhibitory receptors, such as PD-1 and CTLA-4 (43). Interestingly, in subject w323 (with a large, rapidly growing tumor), we detected MCPyV T-Ag-specific T cells only in the blood but not in TILs derived from her T-Ag-expressing tumor. In the latter case, there are several possible explanations about antigen-specific T cells: (i) they were present in the blood but failed to home to the tumor; (ii) they homed to the tumor but were undetectable because they failed to produce IFN-γ; or (iii) they were present in the tumor but failed to expand in culture.

It is possible that local MCPyV-specific T cells actually promoted tumor growth in some cases. Chronic antigen exposure, a common feature of MCC tumors, has been reported to induce IL-10 and IFN-γ-secreting CD4 T cells that have a regulatory function (44). In subject w347, we observed MCPyV-specific IFN-γ and IL-10 cytokine secretion by CD4 T cells isolated from the primary tumor. Notably, we also detected FoxP3+ cells within the tumor microenvironment, which may be similar to the antigen-specific, IL-10–secreting, FoxP3+ T-regulatory cell subset found to be immunosuppressive in metastatic melanoma (45). Further investigation is required to determine the role of T-regulatory cells in MCC.

There are several limitations to this study. PBMC availability prevented proteome-wide screens for some subjects; therefore, we focused on the oncogenic portions of T-Ag persistently expressed in tumors. Cultured ELISPOT assay may sometimes generate false-positive reactivity via in vitro priming (46). To eliminate such artificial results, we used stringent ELISPOT interpretation criteria (Supplementary Methods). Finally, it was not possible to determine the extent of prior exposure to MCPyV for all studied subjects because no "gold standard" serologic or viral shedding test exists. The single most relevant indicator of MCPyV status is viral oncoprotein expression in the tumor, which was assessed for 33 of 38 patients.

In conclusion, this study is the first report of T-cell immune responses to the MCPyV. The necessary and persistent expression of T-Ags to drive MCC proliferation makes these oncoproteins especially promising targets for measuring and manipulating tumor-specific immune responses. As has been accomplished in other cancers (20, 47, 48), the identified MCPyV T-cell epitopes may be used to design peptide-specific vaccines, to generate virus-specific T cells for adoptive immunotherapy, or to track tumor-associated antigen-specific T-cell responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Iyer et al.


Supplementary data associated with: “Merkel cell polyomavirus-specific CD8+ and CD4+ T-cell responses identified in Merkel cell carcinomas and blood”

Supplementary Methods:

ELISPOT Interpretation Criteria. Whole data sets from a patient or a control subject were excluded from analysis if no SFU were detected from any stimulus including the positive control. A peptide pool was considered “reactive” if it contained ≥10 net SFU. Single constituent peptides from reactive pools were tested by cultured ELISPOT assay. A single peptide was considered “reactive” if both the parental pool and constituent peptide(s) showed ≥10 net SFU in the same experiment, or two separate experiments confirmed reactivity with the same peptide. Assays were excluded at the pool or single peptide level if the negative controls (DMSO or TCM) had ≥10 spots, or if more than 50% of peptides from a single pool had ≥10 net SFU due to concern for non-specific reactivity.

Plasmids. Full-length MCPyV LT was PCR-amplified from MCC tumor w156 (Genbank HM355825.1), using primers GTCGACACCATTGAATTAGTC and GCGGCCGCTTATTGAGAAAA. The amplicon was digested with Not-I and Sal-I and cloned into similarly digested pCMV-sport6 (Invitrogen). Identity was confirmed by sequencing.

MCPyV DNA sequencing and detection. DNA was extracted using DNeasy kit (Qiagen) or QiAamp DNA FFPE Tissue kit (Qiagen) and quality assessed with a Nanodrop spectrophotometer (Thermo Scientific). Primers amplified nucleotides 151-589 (151F: TTGCTCCTCTGCTGTTTCTG, 589R: TACAATGCTGGCGAGACAAC) or 729-1287 (729F: CTGCTTACTGCATCTGCACC, 1287R: GGGAGGAAAGTGATTCATCG) of the MCPyV genome (Genbank EU375803). 40 cycles of PCR was performed using 5 Prime Mastermix (5prime, Inc) using manufacturer-recommended protocol. Products visualized on 1% agarose gels were of the expected size, with no product seen for human lymphocyte DNA (Promega) negative control. Amplification primers were used for fluorescent dideoxy bi-directional sequencing (Functional Biosciences). Sequence data were submitted to GenBank for subjects w447 (accession number JF912157) and w347 (accession number JF912158). For MCPyV DNA evaluation, DNA from MCC was extracted and polyomavirus DNA was detected by quantitative PCR (1, 2).

Intracellular cytokine cytometry (ICC) tetramer pre-staining. For intracellular assessment of cytokines in tetramer-specific T cells, T-cells were first pre-stained with fluorescently-labeled tetramer for 30 min at 4°C (3). This “pre-staining” protocol, recently described by Baumgaertner et al, allows for efficient visualization of antigen-specific T-cells by tetramer even after stimulation with peptide (conventional ICC techniques preclude efficient tetramer-staining post stimulation due to TCR downregulation). Conventional ICC methods described in the Methods section were employed thereafter, with an additional step of tetramer-staining for 60 minutes at 37°C, prior to anti-CD8-APC and anti-IFN-γ-PE mAbs staining.
Supplementary Figures:

Figure S1. Expanded lymphocyte cultures are comprised entirely of CD3-positive cells. Ex-vivo PBMC (shaded gray) were gated by FS/SS and set the threshold discriminating CD3-positive versus CD3-negative cells. Expanded TIL (black outline) were gated on FS/SS and CD8 (left) or CD4 (right). The bracket indicates the percent of CD3-positive cells among TIL (black outline).
Figure S2. Validation of peptide LT.92-101 HLA-A*2402 restriction. TIL from HLA-A*2402-positive subject w447 were incubated with allogeneic DMSO- or peptide-pulsed antigen presenting cells (APCs): PBMC matched at HLA-A*2402-only (top panel) or HLA-mismatched PBMC (bottom panel). Numbers shown in plots are the percentage of CD8-gated TIL.

APCs:
- Allogeneic PBMC matched at HLA-A*2402-only
- HLA-mismatched allogeneic PBMC
Figure S3. MCPyV-specific CD8 TIL recognize and kill large T-antigen expressing cells and autologous cells pulsed with peptide. (A) w447 TIL clone was used as the effector cells against the specified target cells at the indicated Effector:Target cell ratios. Fractional cytotoxicity of Cos7 cells is presented as the percentage of all Cos7 cells, and does not take into account the decreased number of cells expected to be successfully double-transfected (well below 100%). (B) At a 20:1 Effector:Target cell ratio, w447 TIL clone was used as the effector cells against autologous PBMC pulsed with peptide LT.92-101 at the indicated concentration.
**Figure S4. Negative controls for A24/MCPyV.LT.92-101 tetramer.** MCPyV-specific tetramer staining of lymphocytes from three HLA-A24-positive subjects and an HLA-A24-negative subject. Unlike in Figure 3A, no CD8+Tetramer+ clusters are observed. Numbers shown in plots are the percentage of CD8-gated lymphocytes.
Figure S5. Functional status of tetramer-positive CD8 T cells in tumor and blood of MCC subject w447. Interferon-γ intracellular cytokine assay was performed using the “pre-staining” protocol described in Supplementary Methods. The gray shaded histogram represents IFN-γ production in response to DMSO. The black outlined histogram represents IFN-γ production in response to peptide LT.92-101. Data represent a minimum of 700 gated CD8+Tetramer+ cells. Brackets indicate the percent of IFN-γ-producing tetramer-positive cells after incubation with peptide.
Figure S6. Magnitude of PBMC reactivity against MCPyV pools for each subject. Net SFU/well is presented in log-scale on Y-axis. MCC patients’ (black) and control subjects (gray) study ID is listed on the X-axis. Dotted line represents threshold response set by our criteria detailed in Supplementary Methods. The data indicated below each graph (percent of MCC patients or controls with PBMC reactivity to a given pool) has been compiled as Figure 5A. The table with indicated reactivity to ≥ 1 or ≥ 2 pools among subjects did not reach statistical significance.
## Supplementary Tables:

**Table S1. Characteristics of MCC patients and control subjects.**

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*As determined by large T-Ag tumor IHC or tumor DNA PCR*
Table S2. List of all synthetic peptides used as antigens in T cell assays. Peptides are derived from MCPyV sequence Genbank EU375803 unless otherwise specified. *Peptide nomenclature: abbreviation of viral protein name: common T-antigen, CT; large T-antigen, LT; small T-antigen, ST followed by fist-last amino acid position. a.s.c. = alternate splice site; alt. strain = alternate strain (EU375804)

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**xxx** PDIPEFHEHEPTS failed quality control

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### Supplementary data, Iyer, Afanasiev, et al

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| VP2/3-230-241 | 12 | pool 17 | VP2/VP3 |
| VP2/3-194-206 | 13 | pool 17 | VP2/VP3 |
| VP2/3-198-210 | 13 | pool 17 | VP2/VP3 |
| VP2/3-202-214 | 13 | pool 17 | VP2/VP3 |
| VP2/3-206-218 | 13 | pool 17 | VP2/VP3 |
| VP2/3-210-222 | 13 | pool 17 | VP2/VP3 |
| VP2/3-214-226 | 13 | pool 17 | VP2/VP3 |
| VP2/3-218-230 | 13 | pool 17 | VP2/VP3 |
| VP2/3-222-234 | 13 | pool 17 | VP2/VP3 |

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| VP2-21-33 | 13 | pool 18 | VP2 |
| VP2-25-37 | 13 | pool 18 | VP2 |
| VP2-29-41 | 13 | pool 18 | VP2 |
| VP2-33-45 | 13 | pool 18 | VP2 |
| VP2-37-49 | 13 | pool 18 | VP2 |
| VP2-41-53 | 13 | pool 18 | VP2 |
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| LT-327-339 | 13 | pool 9 | LT, a.s.c. |
| LT-331-342 | 13 | pool 9 | LT, a.s.c. |
| LT-336-348 | 13 | pool 9 | LT, a.s.c. |
| LT-340-352 | 13 | pool 9 | LT, a.s.c. |
| LT-105-117 | 13 | pool 9 | LT, alt strain |
| LT-109-121 | 13 | pool 9 | LT, alt strain |
| LT-113-125 | 13 | pool 9 | LT, alt strain |
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| LT-205-217 | 13 | pool 9 | LT, alt strain |
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| VP1-177-189 | 13 | pool 15 | VP1, alt strain |
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| VP1-357-369 | 13 | pool 15 | VP1, alt strain |
| VP1-361-373 | 13 | pool 15 | VP1, alt strain |

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### Failed quality control

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| xxx | 13 | pool 17 | VP2/VP3 |
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| xxx | 13 | pool 17 | VP2/VP3 |
| xxx | 13 | pool 17 | VP2/VP3 |
| xxx | 13 | pool 17 | VP2/VP3 |

### Failing quality control

| VP2/3-146-158 | 13 | pool 17 | VP2/VP3 |
| VP2/3-226-238 | 13 | pool 17 | VP2/VP3 |
| VP2/3-230-241 | 13 | pool 17 | VP2/VP3 |
| VP2/3-194-206 | 13 | pool 17 | VP2/VP3 |
| VP2/3-198-210 | 13 | pool 17 | VP2/VP3 |
| VP2/3-202-214 | 13 | pool 17 | VP2/VP3 |
| VP2/3-206-218 | 13 | pool 17 | VP2/VP3 |
| VP2/3-210-222 | 13 | pool 17 | VP2/VP3 |
| VP2/3-214-226 | 13 | pool 17 | VP2/VP3 |
| VP2/3-218-230 | 13 | pool 17 | VP2/VP3 |
| VP2/3-222-234 | 13 | pool 17 | VP2/VP3 |
**Table S3. Accession numbers and homologous peptides from other polyomaviruses that correspond to immunogenic MCPyV peptides.** Homologous sequence alignment to MCPyV peptides using CLUSTAL (v2.0.12). Hyphens ("-".) indicate there was no optimal alignment or amino acid match from the indicated polyomaviruses to the corresponding MCPyV peptide.

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Table S4. Novel MCPyV T-cell epitopes.

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*Epitope nomenclature: abbreviation of viral protein name (common T-antigen, CT; large T-antigen, LT; small T-antigen, ST) followed by first-last amino acid position of the peptide. *Subject ID beginning with “w” indicates MCC patient, “C” indicates control subject. *Minimal reactive epitope is HLA-A*2402 restricted. Bolded epitopes were recognized by more than one subject. NA = not available.
Supplementary references:


Merkel polyomavirus-specific T cells fluctuate with Merkel cell carcinoma burden and express therapeutically targetable PD-1 and Tim-3 exhaustion markers

Olga K. Afanasiev1,2, Lola Yelistratova1, Natalie Miller1,2, Kotaro Nagase8, Kelly Paulson1,3, Jayasri Iyer1, Dafina Ibrani1, David M. Koelle3-7, Paul Nghiem1,2,6

Departments of 1Medicine/Dermatology, 2Pathology, 3Medicine, 4Laboratory Medicine, 5Global Health, University of Washington, Seattle Washington; 6Fred Hutchinson Cancer Research Center, Seattle, Washington; 7Benaroya Research Institute, Seattle, Washington; 8Department of Medicine/Dermatology, Saga University, Nabeshima, Japan

Running title: Fluctuating and exhausted CD8 T cells in MCC

Corresponding author:
Paul Nghiem
850 Republican Street
Seattle, WA 98109
Phone: 206-221-2632
Fax: 206-221-4364
Email: pnghiem@uw.edu

The authors disclose no potential conflicts of interest.
**Translational relevance:**

Merkel cell carcinoma (MCC) is an aggressive skin cancer (46% 5-year disease-associated mortality) without available disease-specific therapies. Prior studies show a requirement for persistent expression of Merkel cell polyomavirus (MCPyV) oncoproteins and the frequent presence of virus-specific T cells in MCC patients. It is thus likely that immune evasion mechanisms are important in the pathogenesis of this immunogenic cancer. In this report, we identify several immune inhibitory pathways that are active in MCPyV-specific T cells in MCC patients. These findings have implications for the use of existing and emerging agents that may augment immune responses in this virus-associated cancer.
Abstract

Purpose: The persistent expression of Merkel cell polyomavirus (MCPyV) oncoproteins in Merkel cell carcinoma (MCC) provides a unique opportunity to characterize immune evasion mechanisms in human cancer. We isolated MCPyV-specific T cells and determined their frequency and functional status.

Experimental Design: Multi-parameter flow cytometry panels and HLA/peptide tetramers were used to identify and characterize T cells from tumors (n=7) and blood (n=18) of MCC patients and control subjects (n=10). PD-1 ligand (PD-L1) and CD8 expression within tumors were determined using mRNA profiling (n=35) and immunohistochemistry (n=13).

Results: MCPyV-specific CD8 T cells were detected directly ex vivo from the blood of 7 of 11 (64%) patients with MCPyV-positive tumors. In contrast, 0 of 10 control subjects had detectable levels of these cells in their blood (p<0.01). MCPyV-specific T cells in serial blood specimens increased with MCC disease progression and decreased with effective therapy. MCPyV-specific CD8 T cells and MCC-infiltrating lymphocytes expressed higher levels of therapeutically targetable PD-1 and Tim-3 inhibitory receptors compared to T cells specific to other human viruses (p<0.01). PD-L1 was present in 9 of 13 (69%) MCCs and its expression was correlated with CD8 lymphocyte infiltration.

Conclusions: MCC-targeting T cells expand with tumor burden and show evidence of inhibition by PD-1 and Tim-3 exhaustion mechanisms. Reversal of these inhibitory pathways is therefore a promising therapeutic approach for this virus-driven cancer.
**Introduction**

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer with a disease-associated mortality three times that of malignant melanoma (~46% versus 15%, respectively) (1). MCC is increasingly common with an estimated 1,600 cases/year in the US (2), and the reported incidence has more than tripled over the past 20 years (3). This increasing incidence is partly due to improved detection using a specific immunohistochemical marker, cytokeratin-20 (4), but may also be due to the higher prevalence of known risk factors for MCC: chronic T-cell immune suppression and the number of Caucasians over 50 years of age with extensive prior sun exposure (5). Furthermore, the recent discovery of the Merkel cell polyomavirus (MCPyV) and its causal association with at least 80% of MCCs (6-8) has provided insight into MCC pathogenesis and underscores the importance of characterizing MCPyV-specific immune responses.

The necessary and persistent (7) expression of MCPyV T-antigen (T-Ag) oncoproteins in MCC tumors provides an opportunity to study anti-tumor immunity by assessing responses against a viral, tumor-specific antigen. Although the role of T cells is variable among different human cancers, multiple lines of evidence suggest that cellular immune function is unusually important for survival in MCC. We have previously demonstrated that intratumoral CD8 lymphocyte infiltration (9) and lack of systemic immune suppression (10) are each significantly associated with improved survival. Furthermore, recent evidence suggests that MCC patients have T cells that are specific for persistently expressed viral oncoproteins (11). In this study, we made use of an extensive collection of clinically annotated longitudinally collected blood specimens to track the frequency and function of MCPyV-specific CD8 T cells. It is hoped that characterizing the molecular pathways involved in the inhibition of MCPyV-specific T cell responses may guide the design of rational therapies to overcome tumor immune escape.

To assess the functional state of MCC-targeting CD8 T cells, it was critical to determine the expression of physiologically relevant cell surface markers directly *ex vivo* from tumors or blood. Key pathways examined included those associated with T cell inhibition (programmed death 1, PD-1; T cell immunoglobulin and mucin-domain, Tim-3; cytotoxic T-lymphocyte antigen 4, CTLA-4), co-stimulation and activation (CD28, CD69, CD137). Many of these molecules are the targets of therapeutic agents that are FDA approved (ipilimumab for CTLA-4) or are in clinical (PD-1, CD137 or 4-1BB) (12, 13) or pre-clinical (Tim-3) (14, 15) trials. We show that while MCPyV-specific T cell frequency increases and decreases in parallel with disease burden, these cells display an exhausted phenotypic profile throughout the disease course. Importantly, this study identifies key inhibitory and activation pathways that may be suitable therapeutic targets for reversing T cell dysfunction and promoting anti-tumor responses.
Materials and Methods

Human subjects and samples. This study was approved by the Fred Hutchinson Research Center IRB and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Blood was obtained from HLA-A*2402+ , HLA-A*2301+ or HLA-A*0201+ subjects based on HLA restriction of available tetramers. Tumors were obtained from medically necessary procedures. Tumor MCPyV status was assessed by RT-PCR for MCPyV T-Ag, immunohistochemistry (CM2B4 antibody, Santa Cruz) and/or T-Ag serology (9). Extent of disease was determined by clinical evaluation and staging by AJCC 7th edition guidelines.

T-cell analysis and flow cytometry. Virus-specific T cell frequencies in blood were assessed directly ex vivo using tetramers indicated below. Tumor infiltrating lymphocytes (TIL) were obtained from fresh MCC tumors that were minced and digested with 0.1mg/ml DNase-I, 0.4mg/ml collagenase-IV, 0.1mg/ml hyaluronidase (all from Worthington Biochemical) in serum-free RPMI for 3 hr at 37°C then passed through a 70µm nylon cell strainer. Isolated lymphocytes were incubated for 30 min at 37°C with APC-conjugated tetramers specific for MCPyV (11), CMV or EBV (HLA-A24/MCPyV.LT-92-101, A2/CMV.pp65.495-503 or A2/EBV.BMLF1.280-288, respectively). Fc receptor block (Miltenyi Biotec) was added for 10 min at 4°C, and cells were stained for 30 min at 4°C with: CD3-Qdot605 (Invitrogen), CD8-V500 (BD), PD-1-BrilliantViolet421 (BioLegend), Tim-3-PE (R&D Systems), CTLA-4-FITC (Cedarlane), CD28-ECD (Beckman Coulter), CD69-PeCy5.5 (Invitrogen), CD137-PeCy7 (BioLegend) or isotype control antibodies. Cells were washed and fixed. At least 2 million events were collected on FACSAriaII machine (BD) and analyzed using FlowJo (Tree Star, Inc). Mean fluorescence intensity for PD-1 was determined for PD-1-positive populations.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was stained with anti-PD-L1 (clone 5H1) at BioPillar Laboratories (Monmouth Junction) using previously described methods (16) and scored in a four-tiered system according to staining intensity, as previously described (17): strong (Grade 3), moderate (Grade 2), weak (Grade 1) or no (Grade 0) expression, in comparison to external controls (tonsil). As previously described, Grades 3 and 2 were grouped as high expresser cases and Grade 1 or 0 were defined as low expresser cases (18). Tumor infiltrating CD8 lymphocytes were scored as previously described (9). MCPyV T-antigen staining was done using CM2B4 (Santa Cruz) (19) and/or Ab3 (8) antibodies.

mRNA profiling. mRNA profiling and analysis was performed as previously described (9) and relevant expression data was extracted from the publically available GEO database (accession number GSE22396).

IFN-γ functional assays.
CD8 cells were negatively selected (MACS kit, Miltenyi Biotec), plated at 1-2x10^5 cells/well with 1.5x10^4 autologous PBMC used as APC, and stimulated in duplicate wells with peptides specific for MCPyV (MCPyV.LT-92-101, 10µg/mL), EBV (BMLF1.280-288, 10µg/mL), or media (negative control). Blocking anti-Tim-3 (10µg/ml, Biolegend) and anti-PD-1 (10µg/ml, R&D Systems), or isotype control mAbs (10µg/ml) were added. For assays performed directly ex vivo, cells were plated directly onto 96-well multiscreenIP plates (Millipore) pre-coated with anti-IFN-γ capture antibody (1-D1K; Mabtech). For cultured assays, cells were stimulated on day 0 as above in 96-well round bottom plates with fresh TCM and 20U/ml IL-2 (Chiron Corporation), 20ng/ml IL-7 (R&D Systems) and 10ng/ml IL-15 (R&D Systems) added on days 2, 4, and 6. On day 7, cells were transferred to IFN-γ pre-coated ELISPOT plates and mitogens corresponding to the prior stimulation cycle were added. ELISPOT plates were developed after 20 hours, scanned with an enzyme-linked immunospot reader (AID), counted using EliSpot Reader software (AID) and verified for quality control. Representative experiments are shown, with each experiment performed at least twice. Data are presented as net spot forming units (SFU), which is the average SFU of duplicate wells minus the average SFU in the negative control well. Experiments in patients whose cells failed to proliferate with culture or exhibited high background signal were not interpretable and were not included.

The intracellular IFN-γ assay was performed as previously described (11) and is detailed in Supplemental Methods.

**Statistical analysis.** For quantitative comparisons, Fishers’ exact test, Wilcoxon’s rank sum test or Student’s t-test was performed with Stata11 (StataCorp); p<0.05 was considered as significant.
Results

CD8 T cells specific for MCPyV T-Ag are detectable in MCC patients, but not in control subjects

In order to investigate the prevalence of MCPyV-specific T cells found in the blood of MCC patients and control subjects, we used an HLA-peptide tetramer (HLA-A24:MCPyV.LT.92-101) in a direct *ex vivo* screen of HLA-compatible PBMC from first available blood draw. Viral oncoprotein-specific CD8 T cells were not detectable in blood from any of ten HLA-A24 control subjects in this assay (detection sensitivity of approximately 0.01% of CD8 T cells). In contrast, 64% of HLA-compatible MCC patients (7 of 11; p<0.01) had MCPyV-specific T cells in their blood (range: 0.03-0.24% of CD8 T cells) (*Figure 1A, Supplemental Figure S1*). Patients with detectable virus-specific T cells had a significantly greater disease burden (average 3.7 cm in the longest dimension; range 1.8 cm to 5.5 cm; n=6), compared to those without detectable T cells (average 0.7 cm; range 0.3 cm to 1.0 cm; n=3; p<0.05). Furthermore, among the seven patients with detectable virus-specific T cells, blood was drawn near the time of known disease (at an average of 32 days since last detectable disease) and prior to completion of treatment. In contrast, in the four patients with no virus-specific T cells, blood was drawn at an average of 281 days after diagnosis or after last recurrence. As expected, in an HLA-compatible patient whose MCC tumor did not have detectable MCPyV oncoprotein expression (by CM2B4 (19) or Ab3 (8) antibody immunohistochemistry, data not shown), no tetramer-positive T cells were found in peripheral blood obtained at a time point at which the patient had a sizable tumor burden. Collectively, our results demonstrate that circulating MCPyV-specific T cells are more likely to be found among MCC patients with larger MCPyV-associated tumors.

MCPyV oncoprotein-specific T cells fluctuate with tumor burden and anti-viral antibodies

The greater likelihood of detecting MCPyV-specific T cells among MCC patients with large tumors and in blood drawn near the time of disease prompted us to investigate how the frequency of virus-specific T cells changed over time in individual patients. As a baseline comparison of T cell responses to other prevalent human viruses, we tracked the frequency of CD8 T cells specific for CMV or EBV in MCC patients (without known clinically active CMV or EBV infection). There were no appreciable differences in the frequency of T cells specific for CMV or EBV over time (*Figure 1*). In contrast, MCPyV-specific T cell frequencies varied dramatically over time, correlating directly with tumor burden (*Figure 1*). Interestingly, frequencies of T cells specific for the viral T-Ag oncoprotein also correlated directly with T-Ag antibody titers that have previously been reported to reflect tumor burden (20). Thus, both cross-sectional (*Figure 1A*) and longitudinal (*Figure 1B-F*) studies indicate that MCPyV-specific CD8 T cell levels increase with larger tumor burden and fall, sometimes to undetectable levels, with smaller or absent tumor burden.

MCPyV-specific and MCC-infiltrating CD8 T cells co-express high levels of immune checkpoint receptors PD-1 and Tim-3
To determine the functional status of MCC-targeting T cells, we used a multi-parameter flow cytometry phenotyping panel to characterize tumor infiltrating lymphocytes (TIL) and circulating MCPyV-specific T cells in MCC patients. Since culture can alter protein expression patterns, specimens were phenotyped directly \textit{ex vivo} for markers associated with co-stimulation (CD28, CD137), activation (CD69, CD137) and T cell inhibition (PD-1, Tim-3, CTLA-4) (Figure 2; Supplemental Figure S2). T cells specific for CMV or EBV were used as controls. Activation and co-stimulation markers, CD28, CD69 and CD137 (4-1BB), suggestive of appropriate antigen recognition, were expressed on significantly more MCPyV-specific T cells from blood and MCC-infiltrating lymphocytes compared to other viruses (Figure 2B).

PD-1 was expressed on a significantly higher percentage of MCC TIL (mean = 71±8%; n=7) and circulating MCPyV-specific T cells (96±4%, n=5) compared to T cells specific for CMV and EBV (Figure 2B). Tim-3 was also significantly more likely (>3-fold) to be expressed on TIL from MCCs (34±17%, n=7) and MCPyV-specific T cells from PBMC (46±21%, n=5) as compared to control virus-specific T cells (Figure 2B). Surface expression of another inhibitory molecule, CTLA-4, was generally low among TIL and CD8 T cells specific for MCPyV, CMV, and EBV (Figure 2B).

Since simultaneous upregulation of multiple inhibitory receptors has been shown to be associated with T cell dysfunction in other cancers (21), we evaluated the fraction of T cells that co-expressed key inhibitory receptors among TIL and PBMC specific for EBV, CMV or MCPyV in MCC patients (Figure 3A). The combination of PD-1 and Tim-3 co-expression was present among MCC TIL and MCPyV-specific PBMC at significantly higher levels (at least 8-fold higher) than on T cells specific for EBV or CMV (p<0.05; Figure 3B). Over 90% of those Tim-3$^+$ cells co-expressed PD-1 (Supplemental Figure S2). Furthermore, Tim-3 expression was most often observed among TIL with high-positive PD-1 levels as compared to cells with intermediate-positive PD-1 levels (Figure 3C). MCPyV-specific PD-1$^+$ cells had a significantly higher MFI (>4-fold) compared to the PD-1$^+$ T cell subset specific for CMV or EBV (Figure 4A). We did not observe any differences in the density of Tim-3 or CTLA-4 expression (data not shown). Longitudinal studies revealed high PD-1 expression by MCPyV-specific CD8 T cells throughout the disease course, while there was minimal fluctuation in T cells specific for CMV or EBV (Figure 4B).

To test function, we assayed the IFN-γ response of MCC-infiltrating lymphocytes and MCPyV-specific PBMC. Since none of the available TIL were from HLA-A24-positive patients, we used phorbol myristate acetate (PMA) and ionomycin to stimulate the cells. Of the four tested TIL samples, two failed to produce IFN-γ when stimulated with PMA/ionomycin directly \textit{ex vivo}. This dysfunction was reversed after a period of cell division initiated by phytohemagglutinin followed by a 6-day culture with IL-2 and IL-15 (Supplementary Figure S3). Virus-specific PBMC responses could be evaluated in only one patient, w678, because others either lacked sufficient PBMC for this study or had baseline experimental characteristics (e.g. inability to expand in vitro) that were not interpretable as outlined in Methods. In this patient, the baseline number of MCPyV-specific CD8 T cells that secreted IFN-γ was markedly lower than would be expected based on the
number of virus-specific cells that were plated (Figure 5; 1x10^5 CD8 T cells were plated, 0.87% of which were MCPyV tetramer-positive cells (data not shown)). In contrast, while a similar number of EBV-specific CD8 T cells were plated (0.74% of 1x10^5 CD8 T cells), these cells produced a more robust IFN-γ response to the cognate antigen (Figure 5).

Because PD-1 and Tim-3 are targets of agents in clinical development and are potentially relevant to the MCC immune response as described above, we tested if blocking these inhibitory receptors could improve the function of MCPyV-specific T cells. Upon short (20 hour) ex vivo stimulation with cognate peptide, antibodies that functionally block PD-1, Tim-3 or the combination did not significantly augment MCPyV-specific IFN-γ response (Figure 5A). In contrast, when CD8 T cells were pre-incubated with the relevant peptide and blocking antibodies in a 7-day stimulation assay, we observed an augmented T cell IFN-γ response to MCPyV peptide compared to similarly cultured cells to which blocking antibodies were not added (Figure 5B).

In summary, we show that MCPyV-specific CD8 T cells from blood and MCC-infiltrating T cells predominantly co-express PD-1 and Tim-3 inhibitory receptors that may prevent adequate control of MCC tumors in vivo. In addition, we show that MCPyV-specific CD8 T cells from peripheral blood secrete minimal IFN-γ in response to cognate peptide, and that this response can be augmented with antibodies targeting the relevant inhibitory receptors.

**PD-L1 is expressed within MCC tumors and correlates with CD8 lymphocyte infiltration**

Given the high level of PD-1 expression on MCC-infiltrating lymphocytes and MCPyV-specific CD8 T cells from blood, we investigated if PD-1 ligand, PD-L1, was present within MCC tumors and if it was associated with CD8 lymphocyte infiltration. We evaluated PD-L1 and CD8 mRNA expression in 35 MCC tumors and protein expression in 13 formalin-fixed paraffin embedded tumors. Expression of PD-L1 mRNA was correlated with CD8α mRNA (R^2 = 0.6; Figure 6A). A non-overlapping set (relative to the mRNA data) of archival tumor specimens was analyzed for PD-L1 and CD8 protein expression. Biopsy specimens from 9 of 13 patients (69%) had positive PD-L1 expression at levels that were weak (n=2), moderate (n=4) or high (n=3) as assessed using a previously established scoring guide (17). Further analysis was carried out by grouping specimens as low expressers (no or weak PD-L1 levels) and high expressers (moderate or strong PD-L1 levels) as previously described (18). The intratumoral CD8 lymphocyte infiltrate was scored on a 0 to 5 scale (0=absent to 5=strong) as previously described (9). Consistent with the mRNA data, tumors with high PD-L1 expression were significantly more likely to have more intratumoral CD8 lymphocytes than those with low PD-L1 expression (p<0.05; Figure 6B). Representative histopathological photographs are provided in Figure 6C. This pattern of PD-L1 staining suggests that tumor infiltrating PD-1^+ T cells have a high chance of encountering their relevant inhibitory ligand in the MCC microenvironment.
Discussion

The purpose of this study was to investigate the mechanisms that prevent Merkel cell polyomavirus-specific T cells from controlling Merkel cell carcinoma. Here, we show that MCPyV-specific T cells: 1) dynamically correlate in frequency with clinical disease burden and with antibodies against the viral oncoprotein (T-antigen), 2) co-express therapeutically reversible markers of exhaustion, PD-1 and Tim-3 at far higher levels than T cells specific for other common human viruses, 3) are likely to encounter the relevant inhibitory receptor ligand, PD-L1, within the MCC tumor microenvironment. These findings may help us optimize targeted approaches to overcome tumor immune evasion mechanisms in MCC.

While the concept that circulating antigen-specific CD8 T cells may fluctuate in number with viral (22) or tumor (18) load has precedent in the literature, to our knowledge, longitudinal tracking of tumor-specific T cells together with disease burden has not been previously reported. To track the frequency and function of Merkel polyomavirus-specific T cell responses, we relied on an extensive collection of clinically annotated serial blood specimens from individual MCC patients with variable disease burdens. In MCC patients, we speculate that increased tumor burden (and the associated viral oncoprotein load) leads to the expansion of the oncoprotein-specific CD8 T cell pool in the blood. An increase in MCPyV-specific CD8 T cells may thus provide a clinical biomarker of increasing disease. These data suggest that in order to obtain sufficient T cells for adoptive T cell immunotherapy, it may be important to use PBMC acquired at times of higher tumor burden. Additionally, because T cell number increases with disease burden, there is a need for careful interpretation of immunotherapy efficacy data aimed at increasing tumor-specific T cell frequency.

The presence and expansion of MCPyV-specific T cells with increasing tumor burden is highly suggestive that tumor immune escape mechanisms are active in MCC. T cell dysfunction mediated by surface expression of inhibitory molecules may, at least in part, explain why MCC tumors grow despite the presence of an immune response. We observed that among the majority of MCPyV-specific T cells in blood and MCC-infiltrating lymphocytes PD-1 and Tim-3 are simultaneously co-expressed, a combination that is often associated with chronic antigen exposure and reversible T cell dysfunction (14, 21, 23-27). Our observations that most of the MCPyV-specific T cells in the blood are functionally exhausted, while at the same time these cells increase in number in parallel with tumor burden, suggests that more than one population of MCPyV-specific cells are present. Memory T-cells are generally segregated into effector-memory cells that traffic to sites of antigen and respond to peptide by secreting cytokines or executing a cytotoxic program, and central memory T-cells that traffic to lymph nodes and are specialized for proliferation rather than effector functions (Kaech, 2002). Our data suggest that the defect in MCC may preferentially involve the effector-memory population rather than the central-memory population, and this can be clarified in future work using markers for these cell subsets.
The present report suggests that the therapeutically targetable PD-1/PD-L1 pathway is particularly relevant in MCC. In contrast to prior studies that show upregulation of PD-1 with acute infection (22) or with increasing tumor stage (18), PD-1 expression on MCPyV-specific T cells was maintained at high levels throughout the MCC disease course. Furthermore, we observe a particularly high PD-1 receptor density level compared to control viruses, and speculate this may be associated with decreased function. The relevant ligand, PD-L1, is often expressed within the tumor microenvironment (17, 28-30), and in melanoma, PD-L1 expressing tumors cells are often localized immediately next to tumor infiltrating lymphocytes (31). In MCC tumors, using both histologic and mRNA-based analyses in independent cohorts, we observed that PD-L1 expression within the tumor microenvironment is positively correlated with the number of infiltrating CD8 lymphocytes. The heterogeneous expression of PD-L1 suggests that it is not necessarily confined to the tumor cells. Indeed, a recent study reports that PD-L1 and PD-L2 expression within MCC tumors is mostly restricted to a subset of dendritic cells and macrophages (but not the cancer cells themselves) (32). The presence of both PD-1 and PD-L1 within the tumor microenvironment suggests that the PD-1/PD-L1 inhibitory axis is a likely immune evasion strategy in MCC tumors. Importantly, the blockade of the PD-1/PD-L1 pathway has been recently shown to effectively induce durable tumor regression and stabilization of disease in a subset of several diverse types of cancer (12, 33). We noted that IFN-γ responses directly ex vivo required the synergistic activity of anti-PD1 and anti-Tim-3, while in contrast, after in vitro expansion, either checkpoint inhibitor could increase responses and synergism was not observed. The reason for this difference is not clear, but we believe that the direct ex vivo data more closely correspond to the in vivo situation and that combination therapy is therefore rational.

There are several limitations to this study. Given the rarity and aggressive clinical course of MCC, a limited number of HLA-A24-positive (prevalence ~20%) patients’ samples were available for longitudinal studies and for antibody blockade experiments. Additional experiments will be required to determine the prevalence of this functional phenomenon in additional persons with MCC and MCPyV-specific CD8 T cells. We also focused on a single, but well-established (11), MCPyV-specific epitope, which may provide a limited representation of the total antigen-specific immune response to MCC. Future studies may investigate if circulating virus-specific CD8 T cells with different epitope specificities display differences in frequency and inhibitory receptor expression.

In summary, this study demonstrates that the frequency of MCPyV-specific CD8 T cells dynamically fluctuates with tumor burden and with viral oncoprotein-specific antibody titer. These cells are also characterized by high expression of multiple inhibitory and activation markers and by augmentation of MCPyV-specific T cell function upon inhibitory receptor blockade. Therefore, our data support the investigation of agents currently in clinical or pre-clinical trials, such as blockers of the PD-1/PD-L1 (12, 33) and of the Tim-3
axis (14, 15), or agonists of co-stimulatory molecules such as CD137 (13) in patients with advanced Merkel cell carcinoma.
Acknowledgements

We thank James DeCaprio for the gift of the Ab3 antibody to MCPyV large T-antigen.

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Figure Legends

Figure 1. CD8 T cells specific for MCPyV were detected in the majority of MCC patients and tracked with tumor burden and with anti-T-Ag antibodies.

A. MCPyV-specific T cell frequencies among patients or control subjects. Dashed line indicates the threshold of tetramer detection. Solid line indicates the median among patients with detectable MCPyV-specific T cells. All analyses were from the first available blood draw of subjects who were HLA-A*24 or HLA-A*23 positive. Representative flow cytometry plots are shown cases that were tetramer-positive or tetramer-negative. **p<0.01, Fisher’s exact test.

B-F. MCPyV-specific T cells (gray bars, percent of CD3+CD8+ cells) and anti-T-Ag antibody titers (black line) measured in serial blood draws from four MCC patients at indicated times in their disease course. Days since diagnosis of primary tumor are indicated. Clinical extent of disease at time of blood draw is as indicated: (-) = none through (+++) = heavy burden.

G. CMV-specific (black dashed line) or EBV-specific (gray dashed line) T cells were measured in serial blood draws from MCC patients (circle, w678; X, w334; triangle, w672; square, w131) at the indicated times.

Figure 2. MCPyV-specific T cells and MCC TIL express multiple inhibitory receptors and activation markers.

A. Evaluation of three inhibitory receptors: PD-1, Tim-3, CTLA-4 and three activation markers: CD28, CD69, CD137 as assessed by flow cytometry. Representative histograms are gated on CD3+CD8+ TIL or CD3+CD8+Tetramer+ PBMC from blood.

B. Box plots summarize the data of all MCC patients analyzed: tumor infiltrating lymphocytes (TIL) from MCC tumors (n=7); tetramer-positive PBMC specific for MCPyV (n=5), CMV (n=7), EBV (n=5). Bottom and top of box indicate 25th and 75th percentile, whiskers extend out to lowest or highest observation within 1.5 of the interquartile range. Single dots indicate outlying values. The horizontal line within the box indicates the median. Statistical comparisons were made between MCC TIL and MCPyV-specific PBMC and between all virus-specific T cells in the blood. *p<0.05, **p<0.01, Wilcoxon rank sum test.

Figure 3. Co-expression of PD-1 and Tim-3 inhibitory receptors is elevated among MCPyV-specific T cells and MCC-infiltrating lymphocytes.

A. Co-expression of inhibitory receptors from four representative samples analyzed with SPICE software (34). Pie chart indicates number of co-expressed markers. Outer arcs correspond to the extent of indicated surface marker expression on CD3+CD8+ TIL or CD3+CD8+Tetramer+ PBMC as assessed by flow cytometry.
B. Comparison of the fraction of cells that co-express PD-1 and Tim-3 in MCC CD8+ TIL (n=7), PBMC specific for MCPyV (n=5), CMV (n=7), EBV (n=5) and all CD3+CD8+ T cells (n=11). Data measured by flow cytometry. The mean and SEM are shown. **p<0.01, Wilcoxon’s rank sum test.

C. CD3+CD8+ TIL (n=7) assessed for PD-1 and Tim-3 co-expression by flow cytometry. Three distinct populations of PD-1 expression are often detected. Relative expression is indicated on the first plot as (-) = negative, (+) = positive, (++) = high-positive.

Figure 4. PD-1 is highly expressed on MCPyV-specific T cells and its expression is maintained throughout the MCC disease course.

A. Median fluorescence intensity (MFI) of CD3+CD8+PD-1+ T cells specific for MCPyV (n=5), CMV (n=7) and EBV (n=5) measured in the first available blood draw from 12 MCC patients. Most MCC patients only had detectable tetramer-positive T cells for one of these viruses. Line indicates median. Tet* = tetramer-positive. *p<0.05, **p<0.01, Wilcoxon’s rank sum test.

B. Percent PD-1 expression among CD3+CD8+ T cells specific for MCPyV (solid lines, left panel), CMV (dashed lines, right panel) or EBV (dotted lines, right panel) measured in serial blood draws from MCC patients (diamond, w447; circle, w678; X, w334; triangle, w672; square, w131) at indicated times following diagnosis. Sizes of the black diamonds or circles on the solid black line represent relative disease burden among MCC patients with MCPyV-specific T cells (smallest data points represent no detectable disease burden). Not all patients had T cells that were reactive to each tetramer, but all tetramer-positive T cells results are shown.

Figure 5. Culture with inhibitory receptor blocking agents augments MCPyV-specific T cell function.

A. PBMC from case w678 were analyzed directly ex vivo for ELISPOT-based IFN-γ cytokine production by CD8 T cells exposed to EBV or MCPyV peptide in the presence of indicated blockers or IgG isotype control antibody. A representative experiment is shown and similar findings were obtained in a separate experiment. Net SFU (spot forming units) is the average SFU of indicated duplicate wells minus the average SFU in the negative control (media only) wells. Error bars represent mean +/- SEM.

B. PBMC from case w678 were cultured for 7 days as described in Methods and were assessed for IFN-γ production in an ELISPOT assay. A representative experiment is shown and analogous findings were obtained in a separate experiment. Error bars represent mean +/- SEM. *p<0.01, Student’s t-test.

Figure 6. PD-L1 expression in MCC tumors correlates with CD8 lymphocyte infiltration

A. Among 35 MCC tumors, CD8α and PD-L1 mRNA expression were closely correlated.

B. Correlation in an independent set of MCC tumors (compared with Figure 6A) between protein expression of PD-L1 and intratumoral CD8 infiltration in 13 MCC tumors. *p<0.05, Wilcoxon’s rank sum test.
C. Immunohistochemical analysis of CD8 infiltration (left) and PD-L1 expression (right) in two representative MCC tumors as assessed on serial sections of the same tumors. Scale bar: 100μm.
Fluctuating and exhausted CD8 T cells in MCC

Figure 1

A

B

C

D

E

F

G

Control-virus T cells:
- CMV
- EBV

tet+ tet+
### Figure 2

#### A

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<th>PD-1</th>
<th>Tim-3</th>
<th>CTLA-4</th>
<th>CD28</th>
<th>CD69</th>
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#### B

- **PD-1**: *******, ****
- **Tim-3**: *******, ***, ***
- **CTLA-4**: *******, ***, ***
- **CD28**: *******, ***, ***
- **CD69**: *******, ***, ***
- **CD137**: *******, ***, ***
Figure 3

A

Tumor
(CD3+CD8+ cells)

MCC TIL
(w415)

MCPyV
(w678)

CMV
(w334)

EBV
(w678)

Blood
(CD3+CD8+ Tetramer+ cells)

Inhibitory receptors (% expression)

PD-1

Tim-3

CTLA-4

# co-expressed markers

3

2

1

0

B

PD1+Tim3+CTLA4
(% positive cells)

Tumor

Blood

TIL

CD8+

MCPyV
tet

CMV
tet

EBV
tet

All

CD8+

C

Case w847

Case w845

Case w853

Case w768

Case w778

Case w415

Case w781

PD-1

Tim-3
Figure 5

Panel A: 
- **Drug**: IscAb control, αPD-1, αTim-3, αPD-1 + αTim-3
- **Peptide**: EBV peptide, MCPyV peptide
- **Net SFU**

Panel B: 
- **Drug**: IscAb control, αPD-1, αTim-3, αPD-1 + αTim-3
- **Peptide**: EBV peptide, MCPyV peptide
- **Net SFU**

*Directly ex vivo*  
*Post 7-day culture*
**Figure 6**

A. Graph showing the correlation between CD8 mRNA expression and PD-L1 mRNA expression. The correlation coefficient $R^2 = 0.6$.

B. Bar chart showing the intratumoral CD8 score at different levels of PD-L1 protein expression.

C. Immunohistochemical images for two cases, w209 and w197, showing CD8 and PD-L1 expression.
References:


Vascular E-Selectin Expression Correlates with CD8 Lymphocyte Infiltration and Improved Outcome in Merkel Cell Carcinoma

Olga K. Afanasiev1,2, Kotaro Nagase3, William Simonson2,4, Natalie Vandeven2, Astrid Blom1, David M. Koelle4,5,6,7, Rachael Clark8 and Paul Nghiem1,2,6

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 (Lemos et al., 2010). 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) (Feng et al., 2008), as well as the increased MCC incidence among immune suppressed individuals with HIV, chronic lymphocytic leukemia, or solid organ transplantation (Penn, 1999; Engels et al., 2002; Heath et al., 2008). Indeed, MCPyV oncoproteins that are persistently expressed in MCC tumors have recently been shown to be targets for CD8 and CD4 T cells (Iyer et al., 2011). Furthermore, several studies suggest that CD8 and CD3 lymphocyte infiltration into MCC tumors is strongly linked to survival (Paulson et al., 2011; Sihto et al., 2012). However, this advantageous robust lymphocytic infiltration into MCC tumors is only present in ~20% of patients (Paulson et al., 2011). 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 down-regulation 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 (Kupper and Fuhlbrigge, 2004) and cancer (Clark et al., 2008). Indeed, human squamous cell carcinomas have been shown to evade the immune response by downregulating E-selectin on tumor

Abbreviations: CLA, cutaneous lymphocyte antigen; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NO, nitric oxide; RNS, reactive nitrogen species

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vasculature (Clark et al., 2008). 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 (Gehad et al., 2012). 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 (Kasic et al., 2011), show markedly elevated levels of nitrotyrosine, which are associated with a lack of functional tumor-infiltrating lymphocytes (Bronte et al., 2005; Nagaraj et al., 2007). 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.

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 (Kupper and Fuhlbrigge, 2004). 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 or peritumoral areas into low, moderate, or high bins (<1%, 1–5%, and >5% of vessels being E-selectin-positive,

![Figure 1. Vascular adhesion molecules and CD8 infiltration on representative serial tumor sections.](image)

(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 μm. (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 μm.
E-selectin and stage, gender, age, or lesion type. Figure 2b). There were no significant associations between tumor vasculature (a higher fraction of E-selectin-positive vessels 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 (Paulson et al., 2011). 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 into 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). Of the 20 tumors, 80% had CLA/CD8 coexpression 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

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) (Paulson et al., 2011). 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 (Paulson et al., 2011). 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 into 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). Of the 20 tumors, 80% had CLA/CD8 coexpression 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

![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.](www.jidonline.org)
primed in other nonskin 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 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 (Gehad et al., 2012). Thus, we stained 236 MCC tumors from 181 patients using an antinitrotyrosine antibody to evaluate protein nitration (Molon et al., 2011), which is a consequence of local NO-mediated production of reactive nitrogen species (RNS) (Eiserich et al., 1995; Sawa et al., 2000; Radi, 2004; Szabó et al., 2007; Nathan and Ding, 2010). 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 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 bars indicate mean. **P < 0.01, Student’s t-test.

DISCUSSION

The cellular immune system is particularly important in controlling MCC given that immune dysfunction is associated with increased incidence (Penn, 1999; Engels et al., 2002;
Heath et al., 2008) and diminished survival for MCC (Paulson et al., 2012). 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. E-selectin is typically expressed in a subset of vessels in normal, noninflamed skin (Chong et al., 2004). 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 facilitates attachment and transmigration of tumor cells through the endothelium, effectively promoting cancer progression, metastasis, and poorer survival (Mann and Tanaka, 2011). 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 (Weishaupt et al., 2007; Clark et al., 2008; Gehad et al., 2012). 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

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 = 50 μm. (b) CLA expression in blood as evaluated by flow cytometry. (b, top left) Summary data of CLA expression among CD3+ CD8+ cells from control subjects (subs; n = 10) and MCC patients (pts; n = 8). (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.
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 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 (Gehad et al., 2012). 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/CD3\(_{\zeta}\) nitration (Nagaraj et al., 2010), (2) block of TCR/HLA interactions and tumor recognition by TCR/CD8 nitration (Nagaraj et al., 2007), and (3) prevention of T-cell migration via nitration of chemokines that renders them dysfunctional (Molon et al., 2011).

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 (Clark et al., 2008) and protein nitration (Molon et al., 2011). Specifically, E-selectin induction has been observed in vitro with tumor necrosis factor-\(\alpha\) and IL-1 cytokines (Wyble...
et al., 1997), angioptatins (Luo et al., 1998), and topical imiquimod (Clark et al., 2008). Recent studies also showed that inhibitors of NO synthase activity were effective in both E-selectin upregulation (Gehad et al., 2012) and reversal of nitrotyrosine-associated T-cell dysfunction (Bronet et al., 2005; De Santo et al., 2005). 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 (Molon et al., 2011).

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; Czéh et al., 2010) 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-κB regulation of adhesion molecules and chemokines (Liou, 2002). 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 (Schaefer et al., 2004) and platelet (P)-selectin mediated cutaneous T-cell migration (Kulidjian et al., 2002).

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.

**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.

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**Immunohistochemistry and immunofluorescence**

Serial tumor sections were stained with hematoxylin and eosin, and with antibodies against E-selectin (clone 16G4, 1:50 dilution; Novoceastra), CD31 (clone JC70A, 1:100 dilution; Dako), CD8 (clone 4B11, 1:200 dilution; Novoceastra), CLA (clone HEC4-452, 1:100 dilution; BioLegend), and nitrotyrosine (rabbit polyclonal, 1:250 dilution; Millipore). 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) (Sawa et al., 2000; Molon et al., 2011). 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 = <1%, moderate = 1–5%, and high >5%, 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 (Fleiss, 1981).

Fifty-six MCC tumors from 55 patients were assessed for CD8 lymphocytes using a previously described scoring system (Paulson et al., 2011). 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 mm² were quantified for each 0–5 bin (with an average of 0, 90, 306, 508, 675, 732 + CD8 cells per mm², 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 ≤ 2), brisk (intratumoral CD8 score ≥ 3), or stalled (intratumoral CD8 score ≤ 1 and peritumoral CD8 score ≥ 3).

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). The same sections were stained with anti-CD8 (1:50, clone C8/44B; Dako) followed by goat-anti-mouse AlexaFluor-647 (1:50; Invitrogen). 4,6-diamidino-2-phenylindole was used for nuclear staining. CLA/CD8 coexpression 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 coexpressing CLA was assessed in the whole tissue specimen and was categorized as none/low (<5%), moderate (5–50%), or high (>50%). Sections were captured using ScanScope model FL (Aperio), 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 semiquantitative 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 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 (Fleiss, 1981).

Statistical analysis
Wilcoxon rank-sum test was used to assess significance among categorically ordered groups. Cuzick’s nonparametric test for trend (Cuzick, 1985) 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 < 0.05 was considered significant. All analyses were performed with Stata software (StataCorp).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

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Downregulation of MHC-I expression is prevalent but reversible in Merkel cell carcinoma

Kelly G. Paulson, Andrew Tegeder, Cristoph Willmes, Jayasri Iyer, Olga K. Afanasiev, David Schrama, Shinichi Koba, Renee Thibodeau, Kotaro Nagase, William T Simonson, Aaron Seo, Janell Schelter, James S. Hardwick, David M. Koelle, Margaret Madeleine, Mary L. Disis, Michele Cleary, Shailender Bhatia, Hideki Nakajima, Shigetoshi Sano, Jürgen C. Becker, Paul Nghiem

1. Departments of Medicine (Dermatology/Medical Oncology) and Pathology, University of Washington, Seattle, Washington
2. Department of Dermatology, University Hospital Würzburg, Germany
3. Merck and Co. Inc, North Wales, PA
4. Divisions of Epidemiology, Vaccine and Infectious Disease, and Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington
5. Department of Dermatology, Kochi Medical School, Kochi University, Japan
6. Department of Dermatology, Medical University of Graz, Austria

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Figures 4
Tables 1
References 31

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Corresponding author: Paul Nghiem, MD, PhD
University of Washington
850 Republican St, Brotman Rm 240, Seattle, WA 98109
Phone: 206-221-2632
Fax: 206-221-4364
Email: pnghiem@uw.edu


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Abstract

Purpose: Merkel cell carcinoma (MCC) is an aggressive, polyomavirus-associated skin cancer. Robust cellular immune responses are associated with excellent outcomes, but are typically absent. We determined the prevalence and reversibility of class I MHC (MHC-I) downregulation, a potentially relevant immune evasion mechanism.

Patients and methods: Cell surface MHC-I expression was assessed on 114 human MCC tumors using immunohistochemistry and in MCC cell lines using flow cytometry. After IRB approval and patient consent, tissues were obtained and analyzed from a case series of 9 MCC patients who had received intralesional interferon as part of definitive (n=1) or neoadjuvant therapy (n=8).

Results: 84% of MCCs (n=114) demonstrated reduced MHC-I expression as compared to surrounding tissues and 51% had poor or undetectable expression. Expression of MHC-I was lower in polyomavirus-positive MCCs as compared to virus-negative MCCs (P<0.01). The MHC-I downregulation mechanism was multifactorial and did not depend solely on HLA gene expression. Treatment of MCC cell lines with ionizing radiation, etoposide, or interferon resulted in MHC-I upregulation, with interferons most strongly upregulating MHC. Intralesional interferon-beta treatment in a retrospective case review was associated with lesional regression in 7 of 8 cases and with MHC-I upregulation and CD8+ lymphocyte infiltration in 3 of 3 evaluable cases.

Conclusion: MCC tumors may be amenable to immunotherapy, but downregulation of MHC-I is frequently present in these tumors, particularly those that are polyomavirus-positive. This downregulation is reversible with any of several clinically available treatments that may thus promote the effectiveness of immune stimulating therapies for MCC.

Statement of clinical relevance

Merkel cell carcinoma is a frequently lethal skin cancer that is typically driven by expression of immunogenic polyomavirus oncoproteins. Intratumoral T cell responses have been associated by multiple groups with improved clinical outcomes, however most MCCs lack these responses. We report that MCCs frequently downregulate MHC-I expression, one means of T cell evasion. Indeed, this downregulation was more pronounced in virus-expressing tumors. Although presenting an obstacle to immunotherapy, MHC-I downregulation was reversible with multiple available therapies in vitro in MCC cell lines. In vivo, in a retrospective case series, intralesional interferon-beta injection was associated with MHC-I upregulation and CD8+ T cell infiltration. Furthermore, 8 of 9 treated patients demonstrated tumor shrinkage in response to intralesional interferon therapy.
INTRODUCTION:

Merkel cell carcinoma (MCC) is a skin cancer with 46% disease-associated mortality(1) and increasing impact. Several lines of evidence point to a key role for T cell immunity in preventing and controlling MCC. Multiple forms of T-cell immune suppression (including immunosuppressive medications, HIV/AIDS, and lymphoid malignancies) have been associated with increased risk of MCC(2), and T-cell immune suppressed patients have poorer outcomes(3-5). Conversely, robust intratumoral CD8+ and CD3+ lymphocyte infiltration is associated with excellent MCC patient survival, however, most tumors lack these responses(6, 7). Greater than 90% of MCC patients have no clinically appreciable systemic immune suppression suggesting that T-cell evasion may instead be local and tumor-driven.

In 2008, MCC was associated with a novel but highly prevalent polyomavirus(8), the Merkel cell polyomavirus (MCPyV or MCV). Viral oncoproteins (T-antigens) are expressed in at least three-quarters of MCCs(9, 10) (11) and their persistent expression is necessary for MCC cell division(12). Furthermore, these non-human oncoproteins are targets for adaptive immune responses in MCC patients, with humoral(13) and more importantly cellular (including CD8+ T-cell) responses demonstrable in the blood and tumor microenvironment(14). Therefore, these viral antigens suggest that the tumor is immunogenic and must have specifically avoided CD8+ T cell recognition. They further represent compelling targets for MCC-specific immune therapy, including adoptive T cell therapies.

Nucleated cells express major histocompatibility complex class I (MHC-I), a requirement for presenting peptides from intracellular proteins to CD8+ T cells. Multiple viruses(15) and virus-associated cancers (e.g. Kaposi’s sarcoma(16), cervical cancer(17)) are known to directly or indirectly downregulate MHC-I as a mechanism of immune escape. We hypothesized MCC tumors would also frequently exhibit poor expression of MHC-I by viral or cellular mechanisms as a mechanism of immune escape. We further investigated potential avenues of reversal of MHC I downregulation in MCC tumors as a means for improved exposure of tumor antigen to native and therapeutic immune
responses. In particular, we focused on clinically available modalities including cytotoxic chemotherapy and radiation therapy. Interferons were of special interest as they promote anti-viral immune responses, have been reported to have anti-polyomavirus (18, 19) and anti-MCC activity (18, 20), have upregulation of MHC class I being one of their classical functions, and are broadly clinically available in the United States with current indications for antiviral, immunomodulatory, and anticancer applications.
PATIENTS AND METHODS:

Patients and tumors: All materials and data were obtained from the MCC Data and Tissue Repository at the University of Washington/Fred Hutchinson Cancer Research Center (IRB approval #6585). 123 patients were included, with 94 enrolled from the United States, 28 from Germany, and 1 from Japan. Certain details of the Japanese case have been previously reported(21). All patients had MCC as assessed by two or more pathologists. Diagnoses occurred between the years of 1985-2011.

Tissue microarrays: 114 tumors from 114 distinct patients were represented on at least one of 5 tissue microarray slides comprised of 0.6 mm cores of formalin-fixed, paraffin embedded tumors. 77 (67%) were primary lesions, 19 (16%) were nodal metastases, 2 (2%) were recurrences, 8 (7%) were skin metastases, and 8 (7%) lesions were from undetermined sites.

MHC class I immunohistochemistry: The EMR8-5 antibody (MBL International, Woburn, MA) was utilized to determine MHC-I expression. Tonsil cores and tumor stroma provided on-slide positive tissue staining controls. Normal mouse serum (NMS) was run as a negative isotype control. Further supporting the adequacy of staining were within-tumor controls: strong membranous MHC class I staining was observed as expected on stromal cells and tumor infiltrating lymphocytes but not on erythrocytes.

Specimens were assessed for tumor cell membrane staining by three observers who were blinded to the identity of the samples. Tissue microarrays were scored using the Allred method(22) as follows: a score between 0 and 8 is determined from the sum of a proportion score (0-5 scale reflecting the fraction of cells with any stain), and a staining intensity score (0-3 scale reflecting the strength of staining among the positive cells). The median of the observers’ scores was utilized in analyses. In the event that scorers disagreed by more than two points on the 0-8 scale, scores from an independent pathologist were used instead, or the specimen was eliminated if the pathologist deemed the sample quality to be inadequate. If a patient had more than one lesion represented, a
single lesion was included based on priority: primary > nodal metastasis > recurrence > regional skin metastasis > distant metastasis.

**MCC cell lines**: MCC cell lines were maintained in RPMI with 10% fetal calf serum, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). MCC cell lines MKL-1(23), WaGa(12), UISO(24), MCC13 (25), and MCC26 (26) were utilized.

**Flow cytometric detection of MHC-I expression**: Flow cytometry was performed using the w6/32 antibody(27) which detects MHC class I on the cell surface. K562 cells, which lack cell surface expression of MHC class I, served as negative controls. A known MHC-positive lymphoblastoid cell line served as positive control. Cells were treated with XRT, etoposide, carboplatin, or one of three recombinant interferons IFN-α-2b (Intron A, Merck, Whitehouse Station, NJ), IFN-β-1b (Betaseron; Bayer, Montville, NJ), or IFN-γ-1b (ActImmune, InterMune, Brisbane, CA).

**MCPyV immunohistochemistry**: The largest two tissue microarrays, representing 82 patients, had previously been stained for MCPyV T antigen(13). An Allred score of 2 was used as MCPyV positive.

**CD8 immunohistochemistry**: CD8 infiltration data were available and previously reported for 77 tumors(6). For the additional treated cases, CD8+ immunohistochemistry was performed using clone 4B11 (Novocastra, Newcastle, UK) at 1:200 after heat induced epitope retrieval at pH 8.

**mRNA expression data**: mRNA array expression data from a data set representing 35 MCC tumors from 34 distinct patients were utilized(6), GEO accession number GSE22396.

**B2M reverse-transcription quantitative PCR**: RNA was isolated from MKL-1 cells by RNeasy (Qiagen, CA). RNA quality was confirmed by spectrophotometry. cDNA was generated using the
Applied Biosystems High Capacity Reverse Transcription Kit (Applied Biosystems, CA). B2M and 18s (control) transcript quantities were determined by TaqMan® PCR using commercially available reagents (Applied Biosystems, CA) on an ABI 7900 platform in 384 well format (Applied Biosystems, CA) as per manufacturer’s instructions.

**Statistical analysis:** Student’s T-test was utilized in Figure 1B. Linear regression was utilized for two way comparisons in Figures 3A-3B. CD8+ cell infiltrates between MHC-I expressing tumors and non-MHC-I expressing tumors were compared using Fisher’s exact test. A P value less than 0.05 was considered significant. Analyses were performed using Stata version 11.0 (StataCorp, College Station, TX).
RESULTS:

**MHC class I is downregulated in the majority of Merkel cell carcinomas.**

MHC expression was determined by immunohistochemistry (Figure 1a) in MCC tumors from 114 patients. 84% of MCC tumors demonstrated MHC-I downregulation on tumor cells as compared to stroma (Figure 1b).

Approximately 80% of MCCs express Merkel cell polyomavirus (MCPyV) derived oncoproteins, and these oncoproteins have been demonstrated to be CD8+ T cell substrates(14). We hypothesized these tumors would be particularly likely to have lost MHC class I expression. Indeed, MHC class I expression was lower in MCCs with detectable virus (median score of 4 vs. 5.5, Figure 1b, p <0.01).

To determine whether MHC-I expression was associated with intratumoral CD8+ lymphocyte infiltration, we compared CD8+ infiltration with MHC expression for 77 MCC cases with both data types available. Although there was a trend toward improved CD8+ infiltration among MHC-expressing tumors, this did not reach statistical significance (p = 0.33 by Fisher’s exact test).

**MHC class I expression can be restored in MCC cells in vitro**

We tested baseline MHC-I expression on 5 established MCC cell lines. Merkel cell polyomavirus (MCPyV) positivity for MKL-1, WaGa, UISO, MCC13 has been previously reported and was determined based on PCR assay and Southern blot(12), MCC26 was determined to be negative for MCPyV by the same methods. At baseline, neither virus-positive line expressed MHC-I (MKL-1(23) and WaGa(12)) while two of three virus-negative lines expressed MHC-I at baseline (UISO(24), MCC13(25), MCC26(26)) (Figure 2A).

Interferons (IFN) are well-characterized mediators of antiviral immune responses, with upregulation of MHC class I being one of their classical functions. We therefore determined whether interferon(s) could reverse class I downregulation in MCC cell lines. Treatment with interferon-γ resulted in significantly increased expression of MHC class I in both MCPyV+ cell lines (Figure 2A).
A modest increase was observed in the UISO cell line, while MHC-I was already present at high levels at baseline in the remaining two cell lines.

We further investigated whether other clinically available treatments also could reverse MHC class I downregulation. Interferons beta (**Figure 2B**) and alpha (data not shown) each strongly induced MHC class I in a dose dependent fashion, although higher dosages were needed to achieve the same effect as for interferon-gamma. Etoposide, a standard MCC chemotherapeutic, also induced MHC class I expression (**Figure 2C**), while the platins (cisplatin and carboplatin) did not (data not shown). Finally, XRT resulted in modest MHC upregulation (**Figure 2D**), and this effect was dose dependent (data not shown).

**Mechanism of MHC-I downregulation and interferon-mediated reversal**

Delivery of class I MHC onto the cell surface requires not only expression of the relevant MHC class I heavy chain gene, but also of beta-2-microglobulin. Antigen processing and peptide loading are required for heavy chain/β2M assembly and transport to the cell surface for most MHC I alleles. Therefore, there are several potential steps at which this process could be defective. Among 35 MCCs(6), expression levels of MHC mRNAs were highly correlated to those of beta-2-microglobulin and genes involved in peptide processing and presentation (**Figure 3A, 3B**). This implies simultaneous downregulation of multiple components of this pathway in MCC tumors. Furthermore, interferon treatment of MKL-1 cells was associated with upregulated mRNA expression of pathway components other than HLA genes (eg. beta-2-microglobulin, **Figure 3C**), suggesting the effects of interferon on MHC-I expression in MCC are not limited to upregulating MHC class I heavy chain genes.

To determine the importance of these non-HLA components on the observed upregulation of MHC-I on the surface of MCC tumor cells, MKL-1 cells (genetically HLA-A*2402 negative) were transfected with HLA-A*2402 driven by a constitutive CMV promoter (**Figure 3D**). Transfection of HLA-A*2402 alone was not sufficient to restore MHC class I expression on the surface of MKL-1
cells. However, when IFN-beta-1b was added to the HLA-A*2402 transfection, surface HLA-A*2402 expression was observed (Figure 3D).

To test the role of MCPyV T antigen expression on MHC class I expression in MCC tumor cells, we attempted shRNA mediated knockdown of T-antigen in virus positive cell lines. However, poor cell viability post-knockdown in these virus-dependent lines precluded meaningful data interpretation.

In a retrospective case series, intralesional injection of interferon-β into human MCC tumors is associated with induction of MHC-I expression, infiltration of CD8+ lymphocytes, and lesional regression

Two cases have been previously reported in which intralesional interferon-β injection has been successful as primary therapy for MCC(21, 28). We report an additional eight cases that have been treated with interferon-β, along with other therapies including surgery, radiation, and chemotherapy (Table 1). As this is retrospective case review, these were not part of a standardized protocol, and tumors injected represented a mix of primary tumors and refractory disease. Among the eight new cases, seven of eight had partial or complete responses of the injected lesion to interferon therapy prior to receiving additional therapy. Interestingly, in cases #2 and #4 (both with interferon-associated lesional regression) enlargement of the draining lymph node developed following interferon injection. Importantly, on sentinel lymph node biopsy, the enlarged nodes were negative for MCC tumor cells by histology and immunohistochemistry, instead suggesting nodal enlargement was due to lymphocyte proliferation and expansion. Finally, in case #8, regression of numerous uninjected lesions occurred in addition to regression of the injected lesion (Figure 4a).

Tumor tissues from before and after interferon treatment were available for two of the newly reported cases and one previously reported case. We hypothesized that intralesional IFN-β injection would be associated with increased MHC-I expression on the tumor cells and also increased infiltration of CD8+ lymphocytes consistent with an immunostimulatory role for interferon in addition to
any direct anti-cancer effect. Before treatment, MHC class I on tumor cells was strikingly lower than on surrounding tissues, and there were virtually no intratumoral CD8+ lymphocytes (Figure 4B). However, after treatment, strong induction of MHC class I was observed, as well as a large influx of intratumoral CD8+ lymphocytes. Two of these three tumors were MCPyV positive at baseline, one demonstrated reduced MCPyV T antigen expression in the residual tumor (Allred score 6 to 4) and the other enhanced expression (Allred score 7 to 8).
DISCUSSION:

Merkel cell carcinoma (MCC) is an often-lethal skin cancer associated with a persistent requirement for expression of viral oncoproteins (T-antigens)(8, 12). Although CD8+ lymphocyte responses are associated with excellent disease-specific outcomes(6) and viral T antigens have been demonstrated to elicit specific CD8+ T cell responses in MCC patients(14), the majority of tumors lack intratumoral CD8+ infiltration suggesting cytotoxic T cell avoidance. Here we demonstrate the majority of MCC tumors exhibit poor expression of class I MHC, which represents an obstacle to native immune responses and to adaptive immunotherapies. However, we further establish that this downregulation may be reversed with clinically available therapies, suggesting that there are multiple possible adjuvants to immune therapies that may be rationally employed.

The most effective in vitro treatment for MHC upregulation that we tested were interferons. Of the interferons, interferon-beta intralesionally has shown promise in MCC in several prior case reports, whereas systemic interferon alpha has not(29-31). In retrospective case review of evaluable tumors, we find that interferon injection is associated with enhanced MHC-I expression and improved CD8+ influx. Among eight cases that had been treated with intralesional interferon-beta, tumor regression was noted in seven of the eight MCC patients.

Type I interferons have previously been reported to inhibit the growth of MCC cell lines(18, 20) and this inhibition appeared to be due at least in part to down-regulation of the MCPyV T antigen. In prior reports and our experience, some dose-dependent reduction in MCC tumor viability is observed with interferon-beta treatment but a significant population of viable cells remains even at the highest dosages in vitro. Also, detectable T antigen expression persists both in vitro and in vivo after interferon treatment. The complete interferon-beta mediated clearance of MCC tumors in vivo in several patients suggests immune effects likely synergize with growth inhibition. Further support for immune contributions of interferon-beta to MCC regression is suggested by two cases with lesional regression who developed enlargement of the draining lymph node following interferon injection. As no MCC was found in these nodes upon excision, such nodal enlargement might be due to
lymphocyte proliferation and expansion. Furthermore, in case #8, regression of numerous uninjected lesions occurred, potentially consistent with immune mediated clearance of neighbor lesions. Further studies are indicated on this issue, and the findings provide the rationale for a clinical trial of the use of interferon in MCC.

In summary, Merkel cell carcinoma is an aggressive skin cancer with persistent expression of immunogenic viral oncoproteins. Clinically, improved CD8+ type immune responses are associated with excellent outcomes. Given this, MCC is an appealing target for novel and established immunotherapies. MHC class I downregulation represents one mechanism of immune evasion employed by a majority of MCCs. This presents an obstacle to both native immune responses and T cell or vaccine-based immunotherapies, but may be reversed with multiple clinically available treatments. Therapies aimed at restoring T-cell responses represent a promising avenue for MCC treatment.
FIGURE 1: Major histocompatibility complex class I downregulation is frequent in Merkel cell carcinoma. A) Immunohistochemistry demonstrating MHC-I expression on normal epidermis and representative MCC tumors. Expression of MHC-I was maintained on stromal structures and TIL in MHC-I negative MCC tumors. B) MHC-I protein expression among 114 MCC tumors as determined by IHC. MHC-I was downregulated on 84% of MCCs. Scores reported on Allred scale, with interpretations provided above. In a subset of tumors (n=82), Merkel cell polyomavirus T antigen expression information was available. Virus positive tumors had significantly poorer MHC-I expression as compared to virus negative tumors (p<0.01).
FIGURE 2: MHC-I downregulation is reversible in vitro with multiple treatment modalities.
A) Effect of IFN-γ on MHC expression among 5 MCC cell lines. Merkel cell polyomavirus status is indicated by the (+) or (-) sign below each treatment bar. Cells were treated with 2000 IU/mL IFN-γ for 72 hours. B) Dose-dependent IFN-γ and IFN-β induction of MHC-I expression on the MKL-1 MCC cell line. Surface MHC-I measured by flow cytometry. Day 7 data are shown; partial induction was seen as early as treatment day 1. C) Etoposide induced induction of MHC-I on the MKL-1 cell line. Partial effects were seen as early as day 1, day 4 is shown. IFN-B = 3000 IU/mL. D) Radiation induced induction of MHC-I on the MKL-1 cell line. Day 2 is shown as there were few viable cells thereafter. IFN-B = 300 IU/mL.
FIGURE 3: Mechanism of MHC downregulation in MCC tumors.
A and B) mRNA expression of MHC class I HLA genes was highly correlated to mRNA expression of B2M and antigen processing genes among 35 MCC tumors (B2M example is shown in panel c). Values in panel d represent R-squared values for linear correlation comparing relative expression of gene at left to gene at top (B2M example is indicated with a double box). C) Treatment of MKL-1 MCC cells with IFN is associated with induction of B2M mRNA expression as determined by real-time, reverse transcription PCR. D) Transfection of HLA-A24 under a constitutive promoter (CMV) was insufficient to restore expression of MHC class I in MKL-1 cells suggesting deficiencies in surface MHC expression were not solely due to poor HLA gene expression. However, surface expression of MHC class I was induced when HLA-A24 was combined with interferon.
FIGURE 4: Treatment of human MCC tumors with intralesional interferon is associated with MHC upregulation, CD8+ lymphocyte influx, and tumor regression. a) Clinical images demonstrating treatment response in patients #1 and #8. Clinical details of patient 1 (not including immunologic studies) were previously reported(21). After interferon monotherapy, patient 1 subsequently experienced 8+ years disease-free survival. For patient #8, the two lesions indicated by arrows were injected with interferon and the others were not directly treated. A complete response was obtained with regression of all lesions. b) Marked induction of MHC-I expression on tumor cells and increased CD8 influx after 19 days of interferon treatment in patient #1 using immunostains as indicated. Dot-like cytokeratin 20 (CK20) is a characteristic feature of MCC tumor cells and is included to aid in differentiating tumor from stroma. c) In cases 2 and 3, which also had available before/after specimens, similar increases in MHC expression were observed after intralesional interferon, CD8+ lymphocyte infiltration was also noted (data not shown).
Table 1: Summary of 9 MCC patients treated with intratumoral interferon-beta. Each intratumoral injection ranged from 1.5-3 million international units of interferon beta. The number and timing of injections varied by patient as indicated. Patient 1 was treated with interferon-beta monotherapy; certain non-immunologic details of this case have been previously reported(21). In all other patients, interferon injections were followed by best available therapy including surgery, radiation, and/or chemotherapy; responses were assessed after interferon but prior to initiating any other treatment. Patients 2 and 7 developed complete clinical responses but had minimal residual disease present microscopically on tumor excision shortly after interferon was discontinued. Numbers in parentheses represent MCC Repository ID numbers.

<table>
<thead>
<tr>
<th>Pt #</th>
<th>Injected MCC lesions</th>
<th>Number of injections</th>
<th>Response to treatment: injected lesions</th>
<th>Response to treatment: uninjected lesions</th>
<th>Draining node swelling</th>
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<tbody>
<tr>
<td>1 (JPN3)*</td>
<td>3 in-transit metastases</td>
<td>27 per each of 3 lesions over 3.7 days.</td>
<td>Complete response (pathologic)</td>
<td>N.A.</td>
<td>Unknown</td>
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<tr>
<td>2 (w419)</td>
<td>Primary tumor</td>
<td>14 over 14 days.</td>
<td>Partial response (pathologic)</td>
<td>N.A.</td>
<td>Yes, no tumor by IHC</td>
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<tr>
<td>3 (w360)</td>
<td>2 recurrences</td>
<td>15 injections per each of 2 lesions over 15 days.</td>
<td>Partial response (pathologic)</td>
<td>N.A.</td>
<td>No</td>
</tr>
<tr>
<td>4 (w438)</td>
<td>Primary tumor</td>
<td>9 over 9 days.</td>
<td>Complete response (pathologic)</td>
<td>N.A.</td>
<td>Yes, no tumor by IHC</td>
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<tr>
<td>5 (w425)</td>
<td>Recurrence</td>
<td>15 over 15 days.</td>
<td>Progressive disease</td>
<td>N.A.</td>
<td>No</td>
</tr>
<tr>
<td>6 (w390)</td>
<td>Recurrence</td>
<td>12 over 24 days.</td>
<td>Partial response (pathologic)</td>
<td>N.A.</td>
<td>No</td>
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<tr>
<td>7 (w131)</td>
<td>Recurrence</td>
<td>12 over 15 days.</td>
<td>Partial response (pathologic)</td>
<td>N.A.</td>
<td>No</td>
</tr>
<tr>
<td>8 (w732)</td>
<td>Multiple recurrences</td>
<td>21 over 31 days.</td>
<td>Complete response (clinical)</td>
<td>Complete response</td>
<td>No</td>
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<tr>
<td>9 (w668)</td>
<td>2 nodal metastases</td>
<td>10 over 14 days</td>
<td>Partial response (clinical)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
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REFERENCES


104
Title:
Regression of metastatic Merkel cell carcinoma following HLA class-I upregulation and transfer of polyomavirus-specific T cells.

Authors: Aude G. Chapuis1*, Olga Afanasiev2*, Jayasri Iyer2, Upendra Parvathaneni3, Joo Ha Hwang4, Ivy Lai1,5, Ilana M. Roberts1,6, Heather L. Sloan1, Kendall Shibuya1, Cindy Desmarais7, David M. Koelle8, Cassian Yee1,5,**, Paul Nghiem2,**.

Affiliations: 1Program in Immunology, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, USA. 2Departments of Medicine (Dermatology) and Pathology, University of Washington Medical Center (UWMC), Seattle, WA, USA. 3Division of Radiation Oncology, UWMC, Seattle, WA, USA. 4Division of Gastroenterology, UWMC, Seattle, WA, USA. 5Current address: Melanoma Medical Oncology, MD Anderson Cancer Center, Houston, TX, USA. 6Current address: Surgery Branch, NIH, Bethesda, MD, USA. 7Adaptive Biotechnologies, Seattle, WA, USA. 8Vaccine and Infectious Disease Division, FHCRC, Seattle, WA, USA. *Contributed equally. **To whom correspondence should be addressed.

Corresponding Authors:
Paul Nghiem, MD, PhD. Cassian Yee, MD
Professor, Professor,
Division of Dermatology UWMC Dept. of Melanoma Medical Oncology
850 Republican Street Dept. of Immunology Unit 0430
Box 358050 MD Anderson Cancer Center
Seattle, WA 98195 1515 Holcombe Blvd. Unit: 904
Tel: +1 206 221 26 32 Tel: +1 713 563 5272
Fax: +1 206 221 43 94 Fax: +1 713 563 3424
e-mail: pnghiem@uw.edu e-mail: cyee@mdanderson.org
Abstract:
Merkel cell carcinoma (MCC) is an aggressive skin cancer that typically requires the persistent expression of Merkel cell polyomavirus (MCPyV) oncoproteins that could thus serve as targets for immune therapy. Several immune evasion mechanisms have been identified as being active in MCC including frequent down-regulation of HLA class-I expression on tumor cells and MCPyV-specific CD8 T cell dysfunction. To overcome these obstacles, here we report the combination of local and systemic immune therapies in a 67-year-old man who developed metastatic MCPyV-expressing MCC. To reverse the down-regulation of HLA-I expression noted in his tumors, intralesional IFNβ-1B or targeted single dose radiation were administered as a pre-conditioning strategy to make the tumors more susceptible to T cell lysis. This was followed by the adoptive transfer of ex vivo expanded polyclonal, polyomavirus-specific T cells as a source of reactive anti-tumor immunity. The combined regimen was well tolerated and led to persistent up-regulation of HLA-I expression in the tumor and a durable complete response in two of three metastatic lesions. Relative to historical controls, the patient experienced a prolonged period without development of additional distant metastatic disease (535 days compared to historic median of 200 days, 95% confidence interval = 154-260 days). The transferred CD8+ T cells preferentially accumulated in tumor tissue, remained detectable and functional for >140 days, persisted with an effector phenotype, and exhibited evidence of recent in vivo activation and proliferation consistent with the maintained ability to respond to persistent antigen exposure. The combination of local and systemic immune stimulatory therapies was well tolerated and is a promising approach for treating virus-driven cancers.
Introduction:
Merkel cell carcinoma (MCC) represents a highly aggressive neuroendocrine skin malignancy with a high disease-associated mortality, early metastatic disease, and a high propensity for recurrence after initial treatment. The cause–specific mortality ranges from 23-80% at five years, thus making it three times as lethal as melanoma\(^1,^2\). The Merkel cell polyomavirus (MCPyV) is clonally integrated into at least 80% of MCC tumors and produces T-antigen (T-Ag) oncoproteins that are persistently expressed by MCC and are necessary for the survival and proliferation of tumor cells\(^3\text{-}^5\). Compared to mammalian tumor-associated antigens that all have some degree of expression within normal tissue, MCPyV T-Ag expression is restricted to MCC, is a foreign antigen, faces no issues of tolerance and is thus an optimal target for immunotherapy. Since no viral particles are formed in the tumor cells, antiviral agents are inefficient\(^4\).

As adoptive transfer has demonstrated clinical benefit for both viral and endogenous tumor antigens\(^6\text{-}^8\), we sought to apply the use of antigen-specific T cells to target the MCPyV large T-Ag (LT-Ag) oncoprotein. The HLA-A*2402-restricted MCPyV LT-Ag\(_{92\text{-}101}\)–specific T cells (hereafter referred to as MCPyV-specific cells) were identified in a patient with metastatic MCC\(^9\). These MCPyV-specific T cells when isolated from tumors or PBMC of MCC patients are largely dysfunctional and exhibit an immune inhibitory (PD1+/Tim3+) phenotypic profile\(^{\{\text{Afanasiev, manuscript submitted to CCR}\}}\). We hypothesized that ex vivo generation of polyclonal MCPyV-specific T cells may augment the probability of including and expanding cells that had an increased potential for proliferation, function and persistence after transfer as evidenced in murine and non-human primate models\(^10,^11\).

Similar to observations in other virus-associated cancers, HLA class-I (HLA-I) downregulation is an immune escape mechanism present in the majority of MCC tumors\(^{\{\text{Paulson, et al, manuscript in preparation}\}}\)^12.13. Single dose low-dose radiation has been show to up-regulate HLA-I expression as a result of the increased degradation of intracellular proteins\(^{\{\text{Reits, JEM, 2006}\}}\)^14.15. In addition, mouse model data suggest that single dose radiation is more effective in promoting tumor immunity and subsequent RT fractions suppress the function of lymphocytes that are recruited to the tumor. Furthermore, interferons (IFN) mediate antiviral immune responses by direct up-regulation of HLA-I\(^{\{\text{Boss et al, 1997}\}}\), and intralesional IFN-beta has
been observed to promote immune responses in MCC {Nakajima et al, 2009; Paulson et al, in preparation}.

Here we investigated whether adoptive transfer of polyclonal MCPyV-specific CD8\(^+\) T-cells preceded by HLA-I upregulation strategies (intralesional IFN-beta or local, single dose radiotherapy) could safely establish persistent anti-MCC responses, migrate to tumor tissue and induce regression of MCPyV-positive, HLA-I-deficient MCC metastases.

**Case Report:**
A 67-year-old man presented with a 1.6 cm Merkel cell carcinoma lesion on his left upper thigh and a negative sentinel node biopsy (stage IA MCC). He underwent a wide local excision followed by 50 Gy of fractionated local radiation to the primary site. Past medical history was significant for renal cell carcinoma for which he received a nephrectomy at the age of 50 and ensuing chronic renal failure. Eight months later while still asymptomatic, he presented with a 2.9 x 1.8cm FDG-avid lesion adjacent to the pancreatic head on a follow-up whole body positron emission tomography (PET) scan. Two additional metastases in the pancreatic head and neck appeared in the 137 days leading up to start of treatment. No other sites compatible with tumor uptake were detected prior to treatment.

Both the primary MCC tumor and pre-treatment metastasis were confirmed to be classical MCC by dot-like cytokeratin-20 staining (data not shown) and to be MCPyV-positive by T-Ag expression (Fig. 2). HLA-I expression was absent or sparse in the primary and pre-treatment metastasis. We used an HLA-A24-restricted MCPyV-specific tetramer \(^9\) to identify tetramer-positive CD8 T cells among tumor-infiltrating lymphocytes and peripheral blood (Suppl. Fig. S1). The patient was enrolled in a single patient clinical trial of autologous T-cell therapy for MCC at the Fred Hutchinson Cancer Research Center (FHCRC) in Seattle (protocol #2558).

The patient underwent a total of three treatments (Fig. 1). As noted above, as HLA-I expression on tumor cells was low, HLA-I upregulating intralesional IFN\(\beta\)-IB (3 x 10\(^6\) IU) was administered to the largest of the three detectable pancreatic metastases (designated as metastasis A). After 24 hours, the patient received an infusion of 10\(^{10}\)/m\(^2\) MCPyV tetramer-specific polyclonal CD8\(^+\) T cells. Low-dose subcutaneous (s.c.) IL-2 (2.5 x 10\(^5\) IU/m\(^2\) BID) was administered twice daily
for 14 days as a means to increase T-cell persistence\textsuperscript{19}. This was followed 37 days later by 8 Gy radiation to all three remaining lesions (designated as metastases 1, 2, and 3) 24 hours before a second infusion and low-dose s.c IL-2 administration. On day 148 since the first infusion, metastasis B was injected with IFN\(\beta\) followed by a third infusion and another low-dose two week IL-2 regimen two days after. The patient experienced transient, <72 hours grade 2 Cytokine Release Syndrome (CRS) and lymphopenia after each infusion, consistent with expected immediate toxicities associated with T cell infusions\textsuperscript{20,21}. No changes in end organ function, inflammation- or autoimmune-related complications were observed.
Methods:

Clinical protocol and patient characteristics. All clinical investigations were conducted according to the Declaration of Helsinki principles. The single patient protocol #2558 was approved by the FHCRC Institutional Review Board and the U.S. Food and Drug Administration. The patient provided written informed consent.

Isolation and expansion of MCC-specific CTLs:
Peripheral blood mononuclear cells (PBMC) were collected by leukapheresis and all ensuing ex vivo manipulations involving processing of products destined for infusion were performed in the cGMP Cell Processing Facility (CPF) of the FHCRC. PBMC were depleted of CD25+ T-cells to eliminate regulatory T-cells (Miltenyi Biotec Inc.), and stimulated twice for 7-10 days with HLA-A*2402-restricted MCPyV LT-Ag92-101 peptide (CPC Scientific)-pulsed autologous dendritic cells (DC). Each stimulation was supplemented with the γc-chain cytokines IL-2 (10 IU/ml), IL-7 (5 ng/ml) and IL-21 (30 ng/ml). Cultures that contained >5% specific CD8+ T-cells assessed by tetramer stains were clinically-grade sorted (BD Influx cell sorter, BD Biosciences) before expansion to sufficient numbers for infusion as previously described. Cell products bound the MCPyV LT-Ag92-101 peptide-HLA tetramer, secreted IFNγ and lysed MCPyV LT-Ag92-101 -pulsed FUJI (A24+ cell line) pulsed with 10µg/ml peptide (Suppl. Fig. 2).

T-cell tracking by HLA-peptide tetramers. Tetramers (produced by the FHCRC immune monitoring core facility) were used to detect transferred MCPyV LT-Ag92-101-specific CD8+ T-cells in PBMCs collected after infusions. The sensitivity of the tetramer is 0.05% of total CD8+ T-cells, below which the capacity to distinguish between transferred cells and background staining is diminished. Persistence after infusions was calculated as the last time-point at which tetramer+ T-cells were detected at 2x background levels or ≥0.05%.

Flow Cytometry. Cells that bound tetramer were analyzed by flow cytometry after staining with fluorochrome-conjugated mAbs to CD14, CD16, CD19 (dump channel), CD8, CD4, CD137, CD28, CD127, CD62L, CCR7, PD-1 and TIM-3 (BD-pharminen). Intracellular cytokine production of IFNγ, TNFα and IL-2 by CTL responding to in vitro stimulation with MCPyV LT-Ag92-101 peptide for 4-5 hours were performed as described. Intranuclear expression of Ki-67 was assessed after permeabilization (eBioscience). Cells were analyzed on an LSRII (Becton
Tracking polyclonal T-cells using high-throughput TCR DNA sequencing. A pool of primers to all V and J pairs specifically designed to amplify the complete VDJ junction region, were designed such that only the minimal region (60 nucleotides) containing the antigen-specific nucleotide information for each TCRβ CDR3 could be amplified and sequenced within an individual. Using genomic DNA isolated from blood and tumor samples as template, this method was used to capture the frequency of individual TCRs in biologic samples with accurate reproducibility and a sensitivity of 1/100,000 TCR-containing lymphocytes. DNA from blood and tumor samples was prepared using the DNeasy Blood & Tissue Kit (Qiagen). Source of cells for DNA extraction included: cryopreserved PBMC derived using Ficoll isolation, phytohemagglutinin and IL-2 expanded TIL isolated from the primary tumor and freshly isolated cells from the pancreatic metastatic tumor biopsy following immune therapy treatment. Results of individual TCRs are expressed as a percent of all CD4+ and CD8+ TCRs sequenced.

Immunohistochemistry. Anti-CD8 clone 4B11 (Novocastra, Newcastle, UK), anti-MCPyV T-Ag, and anti-HLA-I clone EMR8-5 (MBL International, Woburn, MA) were used at 1:200, 1:4000 and 1:200 dilutions respectively after heat induced epitope retrieval. Specimens were assessed for MCPyV T-Ag and HLA-I expression and scored using the Allred method. An Allred score of 0-1.5 was considered negative (0), 2-5.5 weak (+), 6-7.5 moderate (++), and 8 strong (+++). Peritumoral and intratumoral CD8 infiltrates were scored separately as previously described. The numbers of CD8+ cells were assessed and expressed on a 0 to 5 scale with an average of 0, 90, 306, 508, 675, 732+ CD8s/ mm², respectively. Intratumoral CD8 lymphocytes were surrounded by tumor cells only with no visualized contact with stroma.

Statistical Analysis. Kaplan-Meyer curves were based on the observed time between first metastasis and second metastasis for 49 patients used as historic controls. All of these patients presented with local or regional disease (stage I, II, or III) and later developed distant metastatic disease, for which they then received treatment. We only included a highly analogous patient population from our historical controls. Cases that would not be eligible for the trial were eliminated, including those with profound immune suppression, and those who only had a single subsequent skin metastasis (these are often independent primary lesions or represent a
very benign subset of patients). Statistical tests were performed with the Graph-Pad prism software version 3.0 or with the R-package for statistical analysis (http://www.r-project.org).
Results:
HLA-I upregulation followed by adoptive transfer of MCPyV-specific T cells induces regression of Merkel cell carcinoma.

Pre- and post-treatment tumor tissue immunohistochemistry was used to evaluate viral oncoprotein expression, HLA-I expression and CD8 lymphocyte infiltration (Fig. 2A). As anticipated, the primary tumor and pre-treatment biopsies were positive for the MCPyV T-Ag oncoprotein, but had weak or no HLA-I expression. Importantly, MCPyV T-Ag expression was persistently maintained and HLA-I was strongly up-regulated on the post-treatment metastasis (metastasis B, biopsied 146 days after the first treatment cycle). Scattered CD8+ T-cell infiltrates were detected in the primary tumor, as well as in the pre- and post-treatment biopsies (Fig. 2A).

Tumor burden and clinical efficacy was monitored using MRI and PET/CT scans (Fig. 2B). Just prior to beginning T cell therapy, the patient’s initial pancreatic metastasis had expanded, and 2 adjacent pancreatic tumors were newly detected as compared to the prior scan 146 days before. Restaging 30 days after the first treatment showed that the largest lesion (injected with IFNβ-IB) decreased in size, but the other two lesions remained stable or increased in size. We observed the best response in all three lesions following the second treatment (localized, single dose 8 Gy radiation prior to T cell infusion and low-dose s.c IL2). After the third treatment, 2 of 3 lesions continued to regress and one lesion remained refractory to treatment.

To determine systemic effects of adoptively transferred T cells, the time for new metastasis to develop after treatment was assessed in comparison to historical matched controls undergoing standard therapy, which was typically cytotoxic chemotherapy (Fig. 2C). The patient demonstrated no additional systemic metastasis until 535 days after the appearance of the first metastasis, which greatly exceeds the median time of 200 days (95% confidence interval: 154-260 days) for development of new distant metastatic disease in historic controls (49 MCC patients). The scan performed 535 days after initial metastasis revealed new brain lesions that the patient declined to work up. These likely represented MCC metastases, however the patient also had a history of remote renal cell carcinoma. Overall these data suggest the treatment induced regression in 2 of 3 metastases and may have contributed to a delay in the development of new distant disease.
Prevalence and characteristics of virus-specific CD8 T cells before and after infusion

The frequency and persistence of MCPyV-specific CD8 T cells in peripheral blood was assessed by their ability to bind the HLA-A*2402-restricted MVPyV-TAg92-101 tetramer (Fig. 3A, solid circles). The frequency of these cells peaked 4-7 days after infusions (up to 8.8% of total CD8+ T-cells) and remained detectable at the last assessment (245 days after treatment initiation) at frequencies of ~1% which corresponded to a >3-fold increase compared to pre-infusion levels (0.26%).

As an increased percentage of virus-specific T cells may indicate an enhanced potential to divide, we investigated whether these T cell expressed Ki-67, a marker of proliferation34. Prior to infusion, less than <1% of MCPyV-specific T cells were Ki-67-positive, indicating minimal proliferative potential. Cell products harvested for infusion on day 14 of the stimulation cycle expressed 92.4% of Ki-67 consistent with recent in vitro activation. Although most transferred cells maintained in vivo Ki-67 expression early after transfer (>80% on day 1), Ki-67 expression gradually decreased after each infusion, but remained well above baseline levels (Fig. 3B, solid diamonds). Ki-67 expression on MCPyV-specific CD8 T cells was overall significantly higher (p=<0.005) than Ki-67 expression of host tetramer-negative CD8+ T-cells (Fig. 3B, open diamonds), suggesting the infused cells continued to proliferate in response to persistent antigen.

We next assessed if the virus-specific T cells persisting after treatment exhibited augmented function as assessed by IFNγ secretion in response to cognate peptide. Prior to treatment, <0.05% of T cells secreted IFNγ in response to MCPyV peptides, suggesting these cells were non-functional. However, the frequency of CD8 T cells that were secreted IFNγ in response to MCPyV peptide increased after each infusion (5.1, 4.5, and 3% after infusion 1, 2 and 3 respectively) and remained detectable at frequencies of ≥1% of total CD8+ T-cells up to 245 days after the first infusion (Fig. 3A, open circles). Importantly, the frequency of MCPyV peptide-responsive CD8 T cells correlated closely with the frequency of MCPyV tetramer-positive cells, suggesting that most of the persisting virus-specific cells remained functional in vivo.

In order to further validate the functional status and determine the differentiation profile of pre- and post-treatment virus-specific CD8 T cells, we evaluated surface marker phenotypic expression. Consistent with the functional data above, both PD-1 (a marker of activation/exhaustion) and TIM-3 (a marker of exhaustion) were expressed on MCPyV-specific
cells prior to immune therapy (90% and 78% respectively) suggesting the endogenously produced cells were dysfunctional and exhausted (Fig 3C and 3D). As expected, following ex vivo expansion, the infusion product also expressed PD-1 (34%) and TIM-3 (93%). However, once infused into the patient, the persistent cells had high expression of PD-1 and undetectable expression of Tim-3, consistent with a functional phenotype (Fig. 3C). Furthermore, seven days after transfer, 53% of MCPyV LT-Ag-specific cells transiently expressed CD137 (4-1BB), a marker associated with very recent activation (Fig. 3E). Prior to infusions, endogenous MCPyV-specific T cells in the peripheral blood and had absent expression of activation markers CD28 and CD127. However, a subset of the infusion product generated for the patient expressed CD28 (78%) and CD127 (19%), consistent with the use of IL-21 in ex vivo cultures. Further analysis of the phenotype of the circulating infused cells in vivo showed that persisting cells maintained CD28 expression (Fig. 3F) and up-regulated CD127 (Fig. 3G). CD62L and CCR7 were not detected on all cells analyzed (data not shown) suggesting the cells persisted with an effector-memory phenotype.

Overall, adoptive transfer of polyclonal, MCPyV-specific T cells was associated with several key characteristics suggestive of an enhanced ability to respond to antigen: 1) markedly increased frequency of tetramer-positive cells that persisted in vivo, 2) increased fraction of IFNγ-producing, virus-reactive T cells, and 3) persistent expression of activation (CD137, CD28, CD127, PD1) and proliferation (Ki-67) markers on MCPyV-specific T cells.

**High throughput TCR clonotyping from blood and tumors**

To capture the number and characteristics of MCPyV-specific CD8+ T-cell clones, we analyzed infusion products, tumor infiltrating lymphocytes (TIL) and PBMC for individual TCRβ CDR3 using high-throughput TCR DNA sequencing. MCPyV-specific TCRs were sequenced and identified from tetramer-sorted samples. The infusion product was polyclonal and consisted of 502 individual virus-specific clonotypes. Interestingly, three clonotypes were predominant and represented 99% of MCPyV-specific TCR reads (all three clones shared V-beta7-9 and differed by their J-beta subunits as depicted in Fig. 4A-B). Prior to treatment, these three J-beta variant clones were present among primary tumor TIL (0.03% J-beta1-5, 0.06% J-beta1-1, 2.8% J-beta2-3), suggesting these pre-existing clonotypes had been expanded in the PBMC-derived infusion product (Fig. 4C, left panel). Analysis of the post-treatment metastasis 146 days after infusion 1 demonstrated that clone Jb1-1 now
represented 4.7% of all sequences, and the clones Jb1-5 and Jb2-3 were absent (Fig. 4C, right panel). We then investigated whether additional clonotypes that were present in the post-treatment metastasis were also present within the low-prevalence MCPyV-specific clonotypes (<1%) in the infusion product. Indeed, this analysis identified an additional MCPyV-specific clonotype that was present at 0.6% of all TCR reads in the post-treatment metastatic biopsy. Overall, MCPyV-specific CD8 T-cell clonotypes present in the infused product increased from 2.85% in the primary tumor to 5.3% in the post-infusion metastasis (Fig. 4). Two clones account for the increase in MCPyV-specific cells present in the biopsy metastasis following immune treatment compared to the primary lesion. No increase in frequency of these two clones was observed in the peripheral blood before and after immune treatment (Suppl. Fig. 3). In summary, the fact that the infusion product contained clones that were present individually and in aggregate at a higher percentage in the metastatic lesion than the primary lesion, suggests there was preferential localization of virus-specific T cells to the metastatic tissue.
Discussion:
The purpose of this study was to investigate the efficacy and safety of HLA-I upregulating agents in combination with polyomavirus-specific adoptive T cell therapy in the setting of metastatic Merkel cell carcinoma. Although MCPyV-specific T cells were detectable in this patient at baseline, they were unable to prevent the development of metastatic disease. We addressed two key factors that may have contributed to the inefficiency of endogenous virus-specific T cells. To reverse the observed down-regulated HLA-I expression on tumor cells (necessary for the display the MCPyV viral peptides) and render the cells accessible to specific CD8+ T cell lysis, either intralesional IFNβ-1B and tumor-targeted, single-dose ionizing radiation (8 Gy) was administered 1-2 days before each T cell infusion. The infusion of polyclonal ex vivo expanded MCPyV-specific CD8 T-cells persistently increased the frequency of detectable and functional CD8 T cells for over three months in vivo and preferentially reached tumor tissue as evidenced by clonotypic analysis. The combination of HLA-I upregulation followed by the infusion of MCPyV-specific CD8 T cells was well tolerated and safe and mediated tumor regression in 2 of 3 detectable metastases consistent with T cell mediated lysis. The absence of new metastatic disease in this patient for a prolonged period of time compared to historic controls also suggests the treatment may have delayed or prevented the progression of distant metastatic disease. Although the patient refused definitive workup, it appears he developed metastatic disease in the brain, a site with perhaps limited lymphocyte accessibility.

There are several known mechanisms that may have contributed to immune escape in some lesions in the patient. However, two specific evasion mechanisms could be eliminated as having played a role in the escape of pancreatic lesion. Specifically, the pancreatic metastasis maintained HLA-I and MCPyV T-Ag expression, eliminating these means of immune escape. There are known several mechanisms that we were not able to test, but which may have contributed to immune escape in some lesions in the patient. Amongst other factors, the presence and number of local regulatory T-cells, which was not assessed in the tumor tissue due to limitation of the tissue sampled, have been demonstrated to impact survival and tumor progression in many cancers. Additionally, a recent report suggests that vascular E-selectin within MCC tumors is important in mediating intratumoral CD8 T cell infiltration (Afanasiev, JID, 2013); however, the available pancreatic metastasis needle biopsies were inadequate for assessment of intratumoral vascular architecture. Furthermore, PD1 expression has been
shown to be higher in the tumor infiltrating CD8$^+$ T cells and is associated with disease progression $^{37-39}$. Although transferred cells were PD1$^+$ and functional in the peripheral blood, the detailed phenotype of the CD8$^+$ T-cells infiltrating the tumor could not assessed. In the future, systemic immune stimulating strategies (such as CTLA-4 or PD-1/PD-L1 targeting agents) may be effectively combined with adoptive immune therapy $^{43,44}$.

This study had several limitations. This was a single patient trial, with lesions that were difficult to access and acquire for research purposes. Specifically, regressing lesions in the pancreas could not be biopsied and the escaping lesion was amenable to a limited fine need aspirate. Therefore, no direct comparison of the immune milieu or TCR clonotypic profile between the responding and non-responding lesions could be made. In addition, the patient declined further workup of new distant disease in the brain. While the combinatorial approach employed in this study targeted specific mechanisms of immune escape in MCC, it made it difficult to tease apart the direct local (IFN-beta, radiotherapy) and systemic (T cell infusions) effects on anti-tumor efficacy.

MCC, similar to other virus-driven cancers, is an attractive target for non-cross reactive immunotherapy because non-self antigens are constitutively expressed and necessary for tumor survival $^4$. The results of this single patient study suggest that immune-mediated antitumor activity can be established with a very limited side effect profile. A multi-patient clinical trial is currently underway (NCT01758458) to further test this combinatorial immunotherapy approach using additional viral epitopes in a broader cohort of MCC patients.
Acknowledgements:
We would like to thank Dr. James A. DeCaprio for providing the MCPyV Large T-Ag antibody Ab3.
Figure Legends:

Figure 1: Timeline for disease presentation and immune therapy. Events as indicated relative to immune therapy start date. The patient received a total of three treatments that consisted of HLA-I upregulation (either intralesional injection of $3 \times 10^6$ IU IFNβ-IB injection or 8 Gy radiotherapy) followed by administration of $10^{10}/m^2$ MCPyV-specific polyclonal CD8$^+$ T cells and 14 days of twice daily subcutaneous injections of IL-2 ($2.5 \times 10^5$ IU/m$^2$). XRT: radiotherapy.

Figure 2: Response of individual MCC metastases after combined immune therapy. (A) Columns from left to right: Hematoxylin and Eosin stains, Immunohistochemistry for MCPyV T-Ag, HLA-I and CD8 (red arrows) of the primary tumor, pre-treatment (metastasis A) and post-treatment (metastasis B) tumors. Scale bar = 50µm. (B) MRI imaging (except the first datapoint, day -146, which was obtained by PET/CT) of individual metastases that included a peri-pancreatic lesion adjacent to the anterior duodenum (metastasis A, red), a pancreatic neck lesion (metastasis B, green) and a pancreatic head lesion (metastasis C, blue). The longest diameter in cm (y axis) of each metastasis is graphed over time (x axis). Representative images of pre- and post-treatment PET/CT scans are shown above the graph. Timing of each treatment is indicated by arrows. The corresponding RECIST 1.1 criteria are indicated below the graph. (C) Kaplan Meyer curve of the probability of developing a second distant metastasis after the first detected metastasis (plotted at day zero) in 49 patients with MCC who developed distant disease. The 95% confidence interval is indicated in red.

Figure 3: Persistence, function and phenotype of transferred MCPyV-specific CD8$^+$ T-cells. (A) Assessment of tetramer$^+$CD8$^+$ T-cells (% solid circles, solid line) and IFNγ-reactive CD8$^+$ T-cells (% open circles, dashed line) in PBMC collected at baseline (137 days and immediately prior to the first treatment) and indicated timepoints after treatments. (B) Intranuclear Ki-67 expression on baseline and post-treatment CD8$^+$tetramer$^+$ cells (solid diamonds, solid line), and CD8$^+$tetramer$^-$ cells (open diamonds, dashed line). Arrows indicate timing of treatments as in (A) above. (C-G) Expression of PD-1, TIM-3, CD137, CD28 and CD127 on the infusion product, tetramer$^+$CD8$^+$ PBMC collected at baseline and after treatment as indicated. A two-tailed paired t-test was used for statistical analysis.

Figure 4: TCR clonotypic analysis for infused T cells and TIL within MCC tumors. (A)
Legend for the four Vb7-9 TCR clonotypes that were MCPyV-specific as assessed by HLA-A24-restricted MCPyV-specific tetramer binding. (B) Pie-chart indicating the individual TCRβ CDR3 clonotypes composing the CD8^+^ tetramer^+^ MCPyV-specific T cells isolated from the infusion product. Gray indicates other clonotypes found in the tetramer-sorted infusion product. (C) Pie-charts indicating the prevalence of the individual TCRβ CDR3 clonotypes present in the infusion product among all TCRβ CDR3 clonotypes isolated from expanded tumor infiltrating lymphocytes from the primary tumor before treatment (left panel) and directly ex vivo from metastasis B after treatment (right panel). The relative percentages of the individual clones are indicated. Gray indicates all other clonotypes found in the biopsy sample.
Figure 1

**Treatment #1:**
- Day 0: IFNβ injection (metastasis 1)
- Day 1: T cell infusion
- Day 1-14: IL-2 administration

**Treatment #2:**
- Day 37: Single dose XRT (all lesions)
- Day 38: T cell infusion
- Day 38-53: IL-2 administration

**Treatment #3:**
- Day 148: IFNβ injection (metastasis 2)
- Day 150: T cell infusion
- Day 150-163: IL-2 administration

**Key Points:**
- Primary lesion & initial therapy
- Metastatic disease
- Immune therapy start
Figure 2

A

<table>
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<th>Primary tumor</th>
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<th>MCPyV T-Ag</th>
<th>HLA-1</th>
<th>CD8</th>
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B

<table>
<thead>
<tr>
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<th>Post-treatment (Day 71)</th>
<th>Post-treatment (Day 219)</th>
</tr>
</thead>
</table>

Graph: Longest diameters (cm) vs. Days after 1st treatment

- Metastasis A
- Metastasis B
- Metastasis C

RECIST 1.1:

SD, PR, SD, PD

C

Graph: Probability of New Met vs. Days after Initial Metastasis

- Present case develops new met at day 535

95% CI for standard tx
Figure 3

(A) %Tet.⁺ CD8⁺ T cells
- %Tet.⁺ CD8⁺ T cells
- %IFNγ⁺ CD8⁺ T cells

(B) %Ki67⁺ Tet.⁺ T cells
- %Ki67⁺ Tet.⁺ T cells
- %Ki67⁺ Tet.⁻ T cells

(C) Gated on Tet.⁺ CD8⁺ T cells
- Pre-treatment
- Infusion Product
- Day 45
- Day 112

(D) PD-1

(E) Tim-3

(F) CD137

(G) CD28

CD127
Figure 4

A

**TCR clonotype proportion:**
- Vb7-9, Jb1-1 (CASSLGSTEAFF)
- Vb7-9, Jb2-3 (CASSFGTDTQYF)
- Vb7-9, Jb1-5 (CASSLGSGPQHF)
- Vb7-9, Jb1-1 (CASSFGSTEAFF)
- Other

(% of all productive reads is indicated)

B

Infusion product (tetramer sorted)

- 37 %
- 17 %
- 45 %
- 2.6 x 10^6 %
- 1 %

99%

C

**Tumor: pre-treatment**

- 0.06 %
- 2.8 %
- 0.03 %
- Not present
- 97.1 %

2.9%

**Tumor: post-treatment**

- 4.7 %
- 0.6 %
- Not present
- Not present
- 94.7 %

5.3%
Supplementary Fig. S1: Detection of MCPyV-specific CD8 T cells among tumor infiltrating lymphocytes (TIL) from an MCC tumor (left) and PBMC (right). Cells were stained with A24/MCPyV.LT.92-101 tetramer and CD8.
Supplementary Fig. S2: Cell products destined for infusion bound the MCPyV LT-Ag\textsubscript{92-101} peptide-HLA tetramer (left), secreted IFN\textsubscript{γ} (middle) and lysed MCPyV LT-Ag\textsubscript{92-101} -pulsed FUJI (A24\textsuperscript{+} cell line) and autologous PBMC pulsed with 10\textmu g/ml peptide (right).
Supplementary Figure 3

**Supplementary Fig. S3**: Prevalence of the individual TCRβ CDR3 clonotypes present in the infusion product within all TCRβ CDR3 clonotypes isolated from PBMC pre- (day -1) and post- (Days +8, +45 and +146) treatment.
References:

Emerging Prognostic and Therapeutic Approaches

Olga Afanasiev and Paul Nghiem

Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer with increasing incidence, high mortality, and limited treatment options for progressive disease [1, 2]. The recent discovery of the Merkel cell polyomavirus (MCPyV) and its causal association with MCC [3, 4] has provided insight into MCC pathogenesis and underscored the importance of disease-specific immune responses. This chapter will explore emerging approaches to harness antitumor and antivirus immunity to track and treat MCC.

Association with Immune Suppression Leads to Merkel Cell Polyomavirus Discovery

Numerous lines of evidence suggest that the immune system is critical in preventing and controlling MCC. Epidemiologic data suggest that patients who are chronically immune suppressed by HIV infection, chronic lymphocytic leukemia, or medications after solid organ transplant have a 3–30-fold increased risk of MCC [5]. Although these cases represent fewer than 10 % of MCC patients, most patients have an apparently normal immune system. Instead, as discussed below, localized tumor-specific defects are likely at play. In addition, 29 cases of complete spontaneous regression of MCC have been reported [6] representing 1.4 % of all reported cases of MCC [7]. Many of these regressions followed improvement in immune function [8–10], thus suggesting a sudden recognition by the immune system leading to the clearance of MCC. This evidence raised the possibility of an infectious etiology for MCC. The discovery of Merkel cell polyomavirus in 2008 provided a missing link between MCC and immune suppression [3]. As detailed in Chap. 2, MCPyV is integrated into the host genome in approximately 80 % of MCC tumors, with a cumulative average among multiple studies from around the world recently summarized as 77 % (924 of 1,198 MCCs) [11].

Although infection with MCPyV is common (80 % seroprevalence [12], and the prevalence of MCPyV DNA isolated from cutaneous swabs is 40–100 % [12, 13]), the rarity of MCC can be explained by the requirement for several uncommon mutagenic events as well as escape from immune surveillance (Fig. 17.1). Ultraviolet (UV) radiation or other environmental mutagens may promote virus integration into the host genome and “oncogenic truncation” of the large...
If infection with Merkel cell polyomavirus (MCPyV) is so common, why is Merkel cell carcinoma very uncommon? Early childhood infection with MCPyV is extremely common, not known to be symptomatic, and induces antibody and cellular immune responses. Ultraviolet (UV) radiation and/or other mutagens likely contribute to virus integration into the host genome and large T (LT)-antigen truncation mutations. Persistent T-antigen expression (brown stain with IHC anti-LT antibody, CM2B4) plays a key role in Merkel cell carcinoma (MCC) pathogenesis. Furthermore, immune evasion at the local, systemic, or tumor-specific levels is also likely required for developing MCC. Immune biomarkers such as anti-T-antigen antibody levels and T cell infiltration into MCC tumors may aid in disease monitoring and prediction of outcome (adapted from Bhatia S. Immunobiology of Merkel Cell Carcinoma: Implications for Immunotherapy of a Polyomavirus-Associated Cancer. Current Oncology Reports 2011;13(6): 488–97. With permission from Springer Science + Business Media)
T-antigen. Such T-antigen truncation events are very common in MCC pathogenesis and important in that they preserve critical cell-cycle progression functions but eliminate cell-lethal viral DNA replication activities [14]. These rare genetic events result in persistent T-antigen expression that plays a key role in MCC pathogenesis [15]. Elimination of T-antigen from tumor cell lines results in inhibited growth or tumor cell apoptosis [4, 16]. Thus, the viral protein that drives MCC progression is an appealing target for disease tracking and therapeutic manipulation. As discussed below, disease progression can be effectively monitored via immune biomarkers such as anti-T-antigen antibody levels [17], and disease outcome can be predicted by the extent of tumor infiltration by CD8+ lymphocytes [18] that are presumably capable of eliminating MCC tumor cells.

Tumor Antigens: A Prerequisite for Immunologic Tracking and Treatment of MCC

All cells present “antigens” or molecules that can be surveyed by the immune system. The term was originally derived from the molecule’s ability to be an “antibody generator” but now also refers to molecular fragments that can be recognized by T cells when presented by major histocompatibility complex (MHC, also known as human leukocyte antigen, HLA). The immune system is generally tolerant to “self” antigens but neutralizes “non-self” antigens (foreign and potentially harmful) via antibodies or kills infected cells via cytotoxic CD8 T cells. Tumor-associated antigens (TAAs), or molecules that can generate an antitumor immune response, were first described in mouse models in the 1950s [19] but have been intensively studied and therapeutically exploited in humans in the past two decades. A milestone in the history of human tumor immunology was the molecular characterization of the melanoma-associated antigen (MAGE) and its recognition by T cells [20].

The ever-increasing list of tumor antigens is unfortunately balanced by a lengthy list of reasons why their efficacy in eliciting an antitumor response leading to tumor shrinkage is still clinically unsatisfactory [23, 24]. However, unlike most cancers, MCC is causally associated with the expression of viral antigens that can serve as specific targets for the immune system.

MCPyV T-antigens are (1) foreign to the host, (2) permanently integrated into the cancer genome (3) persistently expressed, and (4) necessary for MCC tumor progression [21]. These concepts allow the immune system to battle MCC via its evolutionarily designated job of killing virus-infected cells. Importantly, MCC’s “addiction” to viral oncoproteins makes it unlikely that immunologic pressure will cause the escape of virus-independent tumor cell variants. Indeed, unlike the oncoproteins, viral capsid proteins (VP1, VP2/3) offer no advantage for tumor progression and are typically lost in MCC tumors.

For the 20 % MCC tumors with no virus association, other nonviral proteins such as survivin [22], HIP1 oncoprotein that interacts with c-KIT [23] or CD56 might be considered as tumor-associated antigens. However, these TAAs would not be ideal targets for immunotherapy for several reasons. First, like most TAA, these are self-antigens that are not specific to the tumor tissue. For example, CD56 (also known as neural cell adhesion molecule) is expressed on many normal tissues including neurons, skeletal muscle, and natural killer immune cells. As such, during thymic education, T cells that are highly reactive to self-antigens are eliminated to prevent autoimmune disease development. Second, survivin, a protein that inhibits apoptosis and been found to be expressed in up to 100 % of MCCs [22], is also expressed on activated T cells, including those that themselves recognize survivin. Promoting a T cell response against survivin in fact leads to elimination of survivin-specific T cells (“fratricide”) [24]. In order to overcome these potential barriers, approaches are being developed to create drugs or even engineered T cells that are activated only by tumor microenvironment-specific cues such as tumor-induced hypoxia [25]. The discovery of MCC-specific antigens can lead to innovative advances in tracking MCC disease progression and creating novel rational therapies as discussed below.
The antibody response is critical in preventing many viral infections and is often an important component of infection resolution. When infected with MCPyV, antibodies are produced against both the outer viral capsid proteins (such as VP1, VP2/3) [12, 30, 31] and against the virus-encoded oncoproteins (such as the T-antigens) [17]. Interestingly, although the prevalence of antibodies to viral capsid proteins is high in the general population, the titer of antibodies to MCPyV capsid proteins is higher still in MCC patients (Fig. 17.2a) [12, 26, 31, 32]. Importantly, MCC patients and control subjects have no difference in antibody levels to capsid proteins derived from other polyomaviruses [26]. Increased MCPyV capsid antibody levels in MCC patients as compared to controls are not due to increased MCPyV viral capsid antigen production by tumor cells because MCC tumor cells typically do not express viral capsid proteins [14]. The higher capsid antibody titers may instead be due to:

**Fig. 17.2** Unlike capsid antibodies that are highly prevalent, antibodies against T-antigen oncoproteins are specific to MCC patients and are a useful biomarker of MCC disease burden. (a) Although antibodies against MCPyV capsid protein VP1 are higher in MCC patients compared to control subjects, these antibodies are prevalent in the general population. In contrast, seropositivity to T-antigen is highly specific to MCC patients. (b) T-antigen titers are dynamic and reflect the extent of disease burden. Each line represents a different MCC patient and each data point represents the T-antigen titer at the indicated time after diagnosis. NED no evidence of disease, MET metastasis/progression (adapted from [17, 26])
a higher burden of wild-type Merkel polyomavirus in MCC patients, which has been documented [13]. It remains to be determined if higher virus burden in patients was a predisposing factor for MCC development or, alternatively, if MCC results in MCPyV-specific immune tolerance leading to higher virus levels in MCC patients.

In contrast to antibodies to viral capsid proteins, antibodies to MCPyV T-antigen oncoproteins are rarely detected in the general population (<1 %) but appear to be present in a substantial proportion (~40 %) of patients with MCC (Fig. 17.2a) [17]. The apparent correlation between the humoral response to T-antigens and MCC disease burden is not surprising given the differences in T-antigen expression and biology between the normal MCPyV life cycle and its role in MCC tumors. In the normal virus life cycle, T-antigen oncoproteins are expressed in the infected cell nucleus only transiently, limiting exposure of this protein to the immune system. In contrast, T-antigen oncoproteins are persistently expressed in MCC tumor cells that rapidly proliferate and die, triggering antibody responses to the released intracellular proteins. Importantly, the antibody titers to T-antigen oncoproteins fluctuate dynamically in response to changing MCC disease burden (Fig. 17.2b). The antibody titer rapidly drops (~eightfold per year) after successful treatment of MCC tumors but rises with tumor progression (oftentimes prior to clinical detection or development of symptoms) [17]. Thus, antibody titers against T-antigen oncoproteins can serve as a biomarker of MCC disease burden and have indeed been used to detect occult MCC recurrences [18].

**Cellular Immune Responses Against MCC Predict Survival and Can Be Used for Therapy**

Cytotoxic T lymphocytes (also known as CD8 T cells) have the primary function of eliminating infected or damaged cells. The surprising high number of cases [29] of complete spontaneous regression of MCCs given its rarity [34, 35] supports the notion that MCC tumor cells may be susceptible to T-cell-mediated immunologic attack. Histologic analyses of MCC tumors revealed a variable presence of tumor-infiltrating lymphocytes (TILs) among MCC tumors [18]. Indeed, in a pattern similar to other cancers [27, 28], intratumoral (but not peritumoral) infiltration of CD8+ lymphocytes is an independent predictor of improved survival among MCC patients. Patients with robust CD8+ intratumoral infiltration (n=26) had 100 % MCC-specific survival as compared to 60 % survival among patients with sparse or no CD8+ intratumoral infiltration (n=120) (Fig. 17.3) [18]. These findings underscore the importance of the cellular immune response in the natural history of MCC and help explain the increased incidence of MCC in patients with cellular immune suppression.

Some of the key players mediating the antiviral and antitumor response likely include MCPyV-specific T cells. In fact, recent studies have identified virus-specific CD8 and CD4 lymphocytes present in the blood and tumors of MCC patients [29]. These viral epitopes could serve as tools to (1) isolate and track virus-specific T lymphocytes in MCC patients using an HLA/peptide tetramer (Fig. 17.4), (2) characterize immune evasion mechanisms and MCPyV-specific T cell functional status, and (3) develop tumor-specific therapies such as peptide vaccines or adoptive immunotherapy. Importantly, despite the association of infiltrating CD8 lymphocytes with improved survival and the presence of virus-specific T cells in blood and tumors of MCC patients, clinically apparent disease is likely the result of immune evasion by MCC tumors. Future research and clinical efforts will focus on immune system activation and reversal of tumor-mediated immune evasion.

**Rational Treatment Strategies**

Although surgery and/or radiation therapy (RT) may be curative for patients with locoregional MCC without clinically detectable distant metastases, relapses are common and often incurable [1]. The discovery of MCPyV and the importance of the immune system in cancer control support...
the pursuit of rational biology-driven therapies for MCC. The goals of the therapies discussed below are to stimulate the immune system and to establish a tumor microenvironment that favors immune system activation to mediate tumor regression.

**Rethinking “Conventional” Therapies**

Although radiotherapy and chemotherapy can effectively debulk tumors, they may, more importantly, mediate immunologic “side effects” that are critical in igniting the immune response to eliminate residual or therapy-resistant disease [30]. Even the mere removal of the tumor bulk can reverse cancer-induced immune tolerance to restore immune responses [31]. Radiation therapy can favor CD8-mediated cytotoxicity by upregulating HLA class I molecules and tumor antigens on the tumor cell surface [32], as well as by upregulating adhesion molecules (ICAM-I) and death receptors (Fas) [33]. Chemotherapy that leads to immune suppression associated with lymphopenia may mediate depletion of tumor-protective T regulatory cells and repletion of immune effectors that contribute to an antitumor response [30]. These antitumor therapies may also cause “immunogenic cell death” in which dying cells release antigens and produce immunostimulatory molecules [39, 42].

The dosing, fractionation, schedule, and selection of drugs may need to be optimized in order to best engage the immune system with conventional tools. For example, standard radiation therapy (RT) for MCC involves repeated administration of relatively low doses. Interestingly, studies in mice provide compelling evidence that single high-dose (“ablative”) RT results in the rejection of local and distant tumors by engaging the immune system [34]. Specifically, these studies suggest that ablative RT activates myeloid dendritic cells in the primary tumor that ultimately result in vigorous priming and expansion of effector T cells in the draining lymph node. Importantly, these effects are abrogated by current fractionated radiotherapy perhaps because infiltrating T cells are eliminated by subsequent cytotoxic radiation doses. Notably, however, RT that involves high doses to multiple lymph node chains can lead to a decrease in nonspecific

**Fig. 17.3** Intratumoral CD8 lymphocytic infiltration is predictive of survival. Tumors with robust CD8+ infiltration are associated with excellent survival, as compared to those with cells that are stalled at the tumor-stroma border or those that lack CD8+ cells. CD8 lymphocytes are schematized as black dots or stained brown on IHC (adapted from Paulson KG, Iyer JG, Tegeder AR, et al. Transcriptome-Wide Studies of Merkel Cell Carcinoma and Validation of Intratumoral CD8+ Lymphocyte Invasion as an Independent Predictor of Survival. J Clin Oncol, 2011;29(12):1539–46. With permission from American Society of Clinical Oncology)
17 Emerging Prognostic and Therapeutic Approaches

Immune system responses that may remain suppressed several months following treatment [35]. Similarly, while sentinel lymph node biopsy is a sensitive test for detecting the spread of MCC [45], it could be important to preserve the sentinel lymph node, which constitutes the designated site of antigen priming.

Chemotherapeutics not only have variable clinical efficacy but also have broad effects on the immune system. Immunogenic cell death that favors immune activation can be induced by some chemotherapeutic agents (such as anthracyclines and oxaliplatin), while others (such as alkylating agents and cisplatin) fail to trigger such an immune reaction [36]. Furthermore, similar to radiotherapy administration, while a single cycle of chemotherapy-induced lymphodepletion may favor the repletion of T cells stimulated by tumor antigens, repeated cycles may deplete the expanding population of tumor-specific lymphocytes. These findings (rigorously studied in preclinical animal models) have important implications for the rational treatment of cancer in humans.

Overview of Immunotherapies

The immune system is extremely complex, multifactorial, and redundant; therefore, it is unlikely that a single silver bullet immunomodulatory therapy will cure the majority of MCC tumors. Nevertheless, we will introduce single modality therapies and then will discuss advantages of combination therapies that may offer durable cancer-specific effects.

Antigen-Targeted Immunotherapies

Adoptive T cell therapy uses a person’s own T cells that have been selected, expanded, and sometimes even genetically manipulated to produce immune cells with augmented antitumor immune responses (Fig. 17.5). In contrast to tumor vaccination strategies, adoptive T cell therapy can offer far greater control over the magnitude and avidity of the targeted response by appropriate manipulation and selection in vitro of the T cells used for therapy [37]. In melanoma, adoptively transferred T cells persisted in vivo in response to low-dose IL-2, preferentially localized to tumor sites, and mediated an antigen-specific immune response characterized by minor, mixed, or stable responses in 8 of 10 patients for periods of 2–21 months [38]. T cell therapy may especially hold promise for MCC, a cancer that is highly dependent on immune control. As discussed above, 80% of MCCs express a specific viral tumor antigen that can serve as a target for therapeutic T cells. Indeed, MCPyV-specific T cells, such as those that have been recently identified [29], can likely be used for T cell therapy in MCC patients. Virus-specific peptide-HLA tetramers can be used for isolation, enrichment, and monitoring of MCPyV-specific...
O. Afanasiev and P. Nghiem

**Before:** low # of dysfunctional tumor-specific T cells

**Select tumor-specific T cells & expand in vitro**

**Optional patient pre-conditioning**

**T cell reinfusion +/- combination therapy**

**After:** high # of functional tumor-specific T cells

![Image of immunotherapy process]

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**Fig. 17.5** Adoptive immunotherapy. In this approach, leukapheresis is commonly used to collect a patient’s white blood cells. These white blood cells are then selected for antigen specificity (using a peptide-HLA tetramer for example) and expanded in tissue culture conditions that can skew or preserve a desired T cell phenotype. The patient can then undergo a preconditioning regimen prior to T cell infusion (such as chemotherapy to lymphodeplete and make “space” for infused T cells or radiotherapy to decrease tumor bulk and enhance tumor immunogenicity). After T cell infusion, additional treatments can be initiated.

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T cells among tumor-infiltrating lymphocytes and PMBC in MCC patients before, during, and after immunotherapy [29].

Importantly, MCC seems to be an immunogenic cancer regardless of whether or not it harbors viral T-antigen oncoproteins. Although intratumoral CD8+ lymphocyte infiltration is an independent prognostic factor in MCC, no relationship was observed between CD8 infiltration and virus status of the tumor [18]. In the 20 % of virus-negative MCCs with unknown tumor antigens, tumor-specific T cell therapy is still an option. Similar to promising reports in metastatic melanoma [39], tumor-infiltrating lymphocytes could be isolated directly from tumors, selected only for the CD8 marker (regardless of specificity), expanded to clinically useful numbers, and reinfused into the patient with appropriate pre-infusion conditioning (such as nonmyeloablative chemotherapy). In melanoma, this approach resulted in 19 of 33 patients (58 %) having an objective response by RECIST criteria, including three complete responders [39].

A recent immunotherapy breakthrough has been in the field of genetically modified T cells. One promising approach has been to engineer T cells to stably express binding moieties that recognize a tumor cell surface antigen, as well as various co-stimulatory and signaling molecules that enhance T cell activation, persistence, and antitumor responses. Using this chimeric antigen receptor (CAR) technology to target B cell surface protein CD19 that is expressed on malignant (and normal) B cells in chronic lymphocytic leukemia resulted in persistent expression of CAR T cells and complete remission of disease in two of three treated patients [40]. Healthy B cells were also targets of attack, resulting in hypogammaglobulinemia that could be mitigated with IVIG therapy. MCC also has potential targetable surface proteins (such as CD56), but activation of T cells targeting ubiquitously expressed self-proteins would need to be restricted to the tumor microenvironment.

Vaccines may help boost antigen-specific anti-tumor cellular immune responses by mediating T cell activation outside of the immunosuppressive tumor microenvironment. In HPV-associated vulvar intraepithelial neoplasia (VIN), synthetic long-peptide vaccination with incomplete Freund’s adjuvant resulted in a 79 % (15 of 19) clinical response rate and a 47 % (9 of 19) complete response rate in women with HPV-16-positive high-grade VIN [41, 42]. This clinical response was attributed to efficient dendritic cell targeting to induce therapeutic CD8 and CD4 T cell responses.

Another approach increasingly used in a clinical setting involves preloading autologous dendritic cells (DC) ex vivo with appropriate tumor antigens. Such therapy (sipuleucel-T) has been approved by the FDA in 2010 to be used for the...
treatment of metastatic castration-resistant prostate cancer. Several phase III studies showed that treatment with sipuleucel-T resulted in a benefit in overall survival of about 4 months and improvement in the rate of 3-year survival (31.7% for patients receiving sipuleucel-T, as compared with 23.0% for those receiving placebo) [43, 44].

Taking Off the T Cell Brakes

The cellular CD8 lymphocyte response has evolved to fight virus-infected cells. As discussed above, in MCC, the presence of CD8 lymphocytes is associated with survival benefit [18]. Thus, MCC likely requires an immunosuppressive environment to progress. While clinically apparent immune dysfunction is present in 10% of MCC patients, in the remaining 90%, a wide spectrum of local immune evasion mechanisms may play a role. Some types of tumors effectively hide from the immune system by downregulating antigen presentation via MHC class I molecules, which is associated with worse prognosis [45]. Fortunately, this mechanism can potentially be therapeutically reversed using radiotherapy as discussed above [32] or cytokines (such as interferons and TNF-alpha) [46]. In contrast, chronic antigen stimulation can lead to T cell exhaustion characterized by progressive loss of T cell function [47]. In fact, the high antigenic burden of MCPyV proteins persistently expressed by MCC tumors strikingly resembles chronic infection of mice with lymphocytic choriomeningitis virus (LCMV). This mouse model was key in identifying the mechanisms (PD-1/PD-L1 pathway) by which antiviral T cell responses are circumvented in the context of chronic antigen exposure [48]. As mentioned above, it remains to be resolved if the high wild-type viral load in the skin of MCC patients contributes to T cell exhaustion predisposing to MCC pathogenesis or if it is the consequence of poor immunologic control by tumor-mediated T cell exhaustion.

There are several pathways that are important in immunoregulation of T cell responses (Fig. 17.6). Inhibitory pathways include programmed death (PD)-1 and its ligands PD-L1 and PD-L2, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoglobulin-, and mucin-domain-containing molecule-3 (Tim-3). Drugs inhibiting these pathways are already being investigated for their clinical utility in cancer. CTLA-4 receptor blocking agents such as ipilimumab are FDA approved for metastatic melanoma. These drugs, alone or in combination with other therapies, may be promising in enhancing T cell function in MCC patients.

Promoting T Cell Activation and Memory

An alternative or complementary approach to taking off the brakes to enhance T cell function is to provide pro-immunogenic T cell activation signals. Immunostimulatory cytokines such as interleukin (IL)-2, IL-12, IL-15, and IL-21 or interferons could be delivered systemically or intratumorally to promote T cell activation or to counteract immune evasion strategies employed by MCC tumors. A phase II trial (NCT01440816) using intratumoral delivery of IL-12 plasmid DNA followed by in vivo electroporation of MCC tumors designed to lead to intratumoral persistent expression is underway. Other therapeutic agents that are appealing to investigate for MCC treatment include drugs targeting the co-stimulatory 4-1BB (CD137) pathway that can preferentially target CD8+ cells to enhance proliferation, survival, and cytokine production [49, 50].

“Treat Locally, Cure Systemically”: Tumor-Targeted Drug Delivery

The immune system is under tight regulation. Disturbing the balance between immune cell activation and inhibition may result in intended anticancer effects in combination with undesirable off-target effects. Delivering therapy (chemotherapy, immunotherapy, etc.) directly to the tumor site offers an attractive alternative to systemic treatment that may allow for higher therapeutic doses to the tumor with fewer systemic side effects. Intratumoral and locoregional therapies have been used with many drugs (ranging...
from chemotherapeutics to immunotherapeutics) in a wide number of tumors [51], including MCC. For example, intralesional bleomycin, a chemotherapeutic with pro-immunogenic properties, has been used in MCC in conjunction with conventional therapy with promising results [52]. Systemic treatment may be ineffective due to insufficient drug delivery to the tumor site or due to compensatory systemic immunoregulation. For instance, systemic interferon, often used as a broad-spectrum antiviral agent, has not been reported to be effective in inducing MCC regression [53]. However, two cases have been previously reported in which intralesional interferon-β injection has been successful as primary therapy for MCC [54, 55]. Similarly, cytokines have been delivered intratumorally in various cancers [51].

Many therapies are given systemically because metastatic tumors are often numerous, widely disseminated, and difficult to access. However, advanced techniques developed by interventional radiologists are limiting the inaccessibility of tumors. As discussed above, in mouse models, radiation treatment itself can provide an immunogenic stimulus to reduce or eradicate not only primary tumors but also distant metastases in a CD8+ T cell-dependent manner [34]. For tumors that are difficult to access, next-generation tumor-targeting molecules are being developed. For example, soluble T cell antigen receptor (STAR) reagents combine a T cell receptor with an exchangeable “warhead” to allow for tumor-specific immunohistochemistry (by coupling to biotin/peroxidase) or immunotherapy (by coupling drugs or cytokines) (Fig. 17.7) [56]. STAR molecules employ the TCR’s ability to specifically recognize MHC-restricted, peptide-specific antigen targets on virus-infected or cancerous cells.
With T cell clones recognizing specific MHC-peptide complexes already identified, this approach has the potential to offer “off-the-shelf” targeted drug delivery to MCC tumors. Local tumor targeting with treatments that can elicit an appropriate systemic antitumor immune response is promising in eliminating both metastatic and microscopic disease. The complexity of the immune system will require the use of multiple treatment modalities to trigger antitumor responses and reverse tumor-specific immune escape mechanisms. Importantly, conventional anticancer strategies can be combined with novel therapeutic advances for optimal treatment of patients. For example, while radiotherapy (RT) can be used to control local tumor growth, it is an ineffective tool to use for micrometastatic (undetectable) disease deposits. However, RT, even as monotherapy, is sometimes capable of mediating an “abscopal” effect (when local radiation has antitumor effects at a distant nonirradiated site) likely via activation of the immune system [58, 59]. RT in conjunction with immunotherapy may further synergize to exploit (1) radiation-induced tumor cell death as a source of antigens for immunotherapy and (2) postirradiation tumor cell modulation (i.e., increasing MHC-I, providing pro-immunogenic “danger” signals) that enhances immune cell access and T cell-mediated killing [33]. Similarly, chemotherapy has been combined with immunotherapies such as vaccines with promising results [60].

Future directions in clinical research and patient management will include the optimization of synergistic therapies with maximum efficacy, minimum toxicity, and integration with tumor-specific immune evasion strategies.

**Conclusion**

Merkel cell carcinoma (MCC) is an aggressive skin malignancy with an increasing incidence and high mortality rate. In the majority of MCC tumors, Merkel cell polyomavirus (MCPyV)
oncoproteins appear to drive tumor progression and their ongoing expression is likely required. Thus, MCPyV is a promising tumor-associated target for disease tracking and immunotherapy. Indeed, virus-specific humoral and cellular immune responses are detectable in MCC patients, are linked to the natural history of the disease, and can be used for prognosis and early detection of disease recurrence. Learning how to therapeutically exploit the immune system to generate long-lasting and effective antitumor responses will be an ongoing challenge, but as our knowledge of the synergistic effects of therapies increases, the translational use of these strategies will become more feasible for MCC patients.

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Conclusions

Summary of research findings

As outlined in the Introduction, the studies in this dissertation focus on the central theme of cellular immune responses and immune evasion mechanisms in Merkel cell carcinoma and the associated Merkel cell polyomavirus. The first three chapters served as introduction and overview of the clinical and biological concepts relevant for MCC. The first section of original research (Chapter 4) identified specific MCPyV-encoded oncoprotein regions that are recognized by T cells in tumors and blood of MCC patients and control subjects. Studies presented in Chapter 5 demonstrated that while MCC-specific T cells were shown to expand and contract with tumor burden, these cells are dysfunctional and express high levels of inhibitory receptors. Chapters 6 and 7 provide insight into additional immune evasion mechanisms, E-selectin and HLA class-I downregulation, respectively, that likely play a role in diminishing lymphocyte entry into and recognition of MCC tumors. Finally, Chapter 8 presents details of the first MCC patient to be treated with adoptively transferred MCPyV-specific T cells in combination with immune stimulating agents.

Research in Chapter 4 demonstrates that the persistently expressed MCPyV oncoproteins are especially immunogenic to T lymphocytes. This was the first study to report circulating and tumor infiltrating T cell responses to specific regions of MCPyV proteins (26 epitopes). The presence of these T cells in the blood and tumors of MCC patients suggested that several immune evasion mechanisms may be important in preventing effector T cells from controlling MCC.

Indeed, several mechanisms of immune escape are explored in Chapters 5 through 7. First, studies in Chapter 5 show that while MCC-specific T cells are capable of expanding in response to increase tumor burden, they lack appropriate cytokine production in response to cognate antigen and co-express markers of exhaustion, PD-1 and Tim-3, at far higher levels.
than T cells specific for other common human viruses. Furthermore, these studies also identify that the relevant PD-1 ligand (PD-L1) is expressed by the tumor microenvironment. Importantly, inhibitory receptor blocking agents were able to functionally augment the response of MCC-specific T cells to the cognate peptide in vitro.

The previously reported lack of protective CD8 lymphocytic infiltrates into MCC tumors prompted us to investigate if specific mechanisms of T-cell migration may be commonly disrupted in MCC tumors. Studies in Chapter 6 show that, indeed, 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 with improved MCC-specific survival. Furthermore, we provide evidence that metabolic pathways leading to production of nitrotyrosines are associated with E-selectin downregulation and with poor CD8 T cell infiltration into MCC tumors.

An additional mechanism of immune evasion, described in Chapter 7, involves the loss of antigen presentation by downregulation of human leukocyte antigen class I (HLA-I, or major histocompatibility complex class I, MHC-I) in the majority of MCC tumors. While this mechanism of T cell immune evasion may pose obstacles for effective immunotherapies, these studies also revealed that HLA-I downregulation is reversible with approved interferons in MCC cell lines and in vivo.

Finally, in Chapter 8, we have uniquely combined the tools and immune escape reversal approaches outlined above to treat a patient with metastatic virus-dependent MCC. Specifically, we used an HLA/peptide tetramer identified in Chapter 4 to expand polyclonal, tumor-specific cytotoxic T cells that were infused into the patient following a pre-conditioning regimen with interferon or radiotherapy to reverse local immune evasion mechanisms. This combination resulted in the elimination of 2 of 3 treated metastatic tumors and yielded a distant metastasis free survival of 535 days (compared to 200 days in historic controls, 95% CI: 154-260 days).
Immunologic responses included persistence of MCPyV-specific T cells at several-fold above baseline and improved antigen-specific T cell responses that persisted beyond 100 days after treatment. Importantly, this therapy was well tolerated without significant side effects.

In summary, these studies characterized key cellular immune responses to the polyomavirus-associated cancer, identified pertinent immune evasion mechanisms and pioneered a new paradigm for immune therapy in MCC. These translationally relevant findings suggest promising therapeutic targets and approaches for Merkel cell carcinoma, which are further detailed below.

Clinical significance of these studies

As outlined in the Introduction, there is unmet need for biology-driven and disease-specific therapies in the virus-associated Merkel cell carcinoma. The studies in this dissertation help advance our understanding of the interplay between MCC and the immune system and point towards rational treatment modalities to control this cancer.

Studies presented in Chapter 4 identify specific MCPyV epitopes that provide tools to 1) isolate both antigen- and tumor-specific T lymphocytes from blood and tumors of MCC patients 2) characterize immune evasion mechanisms 3) develop tumor-specific therapies such as adoptive immunotherapy, “off-the-shelf” tumor targeting molecules, or peptide vaccines 4) track T-cell responses during tumor progression or clinical trials. Indeed, a specific epitope identified in these studies was critical in the generation of autologous T cell therapy described in Chapter 8. The clinical and immunologic data from treatment of metastatic MCC with autologous, polyclonal, MCC-specific T cells in combination with immune stimulatory adjuvants provided compelling evidence that this therapy should be further tested in a larger cohort of MCC patients. We have developed an NIH-funded Phase I/II trial to test the safety and efficacy of therapy with polyclonal antiviral T cells infused following HLA-I upregulating pre-treatment. Although adoptive T cell therapy is promising and currently feasible, it requires costly, patient-
specific isolation and expansion of autologous antigen-specific cells that may be rare or absent in a given patient. Importantly, MCC-specific HLA-peptide complexes identified in Chapter 4 also facilitate the generation of “off-the-shelf” recombinant T cell receptor (TCR)-based therapies, such as MCPyV-specific transgenic TCR expression in autologous lymphocytes or soluble T cell antigen receptors (STAR). STAR reagents rely on technologies that fuse α and β chains from a T cell receptor into one polypeptide that can recognize a specific peptide-HLA complex and can be coupled to a reagent of choice. Thus, these versatile tools could be readily developed into tumor-targeted delivery vehicles for stimulatory cytokines or radioactivity emitters.

Importantly, immune evasion mechanisms presented in Chapters 5-7 may help explain why sometimes endogenous and adoptively transferred T cells fail to eliminate seemingly immunogenic tumors. Fortunately, the aforementioned immune barriers can be overcome with existing therapies (interferons for HLA downregulation {Paulson, in preparation}; anti-CTLA-4 blockers, angiostatins (20) and imiquimod (21) for E-selectin upregulation) as well as emerging therapeutic agents (anti-PD-1 (22), anti-PD-L1 (23) and anti-Tim-3 blockers (24)). Thus multi-pronged and synergistic therapies will likely be indicated for optimal management of MCC. For example, the blockade of the PD-1/PD-L1 pathway has been recently shown to effectively induce durable tumor regression and stabilization of disease in a subset of several diverse types of cancer (22, 23). A similar phase II trial to test the effectiveness of PD-1 blockade in Merkel cell carcinoma is currently being developed for collaborative, multi-center studies.

In summary, these findings suggest that although robust tumor-specific immune responses can be elicited by MCC, several escape mechanisms prevent the immunologic control of this cancer among those tumors that become clinically evident. Importantly, the presented studies provide a biology-driven foundation for the development and use of rational immune stimulatory therapies for Merkel cell carcinoma.
Future directions

The unique viral association with Merkel cell carcinoma lends itself to many exciting future directions for MCC research. While the focus of this dissertation was on the adaptive immune response to MCC, it is likely that the innate immune system also plays a key regulatory role in MCC.

Natural killer (NK) cells are at the boundary between innate and adaptive immune systems, and have a critical role in tumor immune surveillance. The downregulation of HLA-I in MCC (Chapter 7) should induce NK cell cytotoxicity (the so-called "missing self" hypothesis of Karre (25)). The conundrum that MCC tumors lacking HLA-I are able to escape NK-mediated killing, suggests that NK immune evasion is particularly relevant in MCC. One may hypothesize that tumor escape from NK surveillance may be evoked by systemic lack of NK cells, inaccessibility of the tumor by NK cells, functional impairment caused by either increased inhibition or decreased activation signaling. Interestingly, preliminary data from our lab indicated that stress-induced ligands, MICA and MICB, recognized by the NK activating receptor NKG2D are highly upregulated on MCC tumor cells. This poses an interesting conundrum that MCC tumors persist in spite of diminished HLA surface expression (thus eliminating its inhibitory signal for NK cells) and high MICA/B expression (activating signal for NK cells). A mechanism of MIC-induced NKG2D endocytosis and degradation has been observed in other cancers (26), and may play an important role in NK evasion in MIC positive MCCs. Further studies and understanding of these immune escape mechanisms may facilitate future immune modulatory intervention.

Natural Killer T (NKT) cells have recently gained attention from the cancer immunotherapy field for their anti-tumor properties, which include the production of interferon-γ to activate NK and CD8+ T cells and activation of dendritic cells to secrete IL-12. Preliminary data from our lab suggest that MCC patients have MCPyV-specific, HLA-Cw0202-restricted NKT cells (CD3+CD16+CD56+ phenotypic profile) in their tumors and blood. NKT cell signaling
can occur through T cell receptors (TCR) or killer cell immunoglobulin-like receptors (KIR). It will be important to elucidate if the virus peptide/HLA complex signals through the TCR or KIR. Signaling through KIRs can be stimulatory or inhibitory, with the latter being a potential pathway of tumor immune escape. If NKT cells are determined to have anti-cancer properties in MCC, NKT immunotherapy using KIR blockers currently in clinical development may hold promise for MCC management in the future.

Macrophages are also known to play a role in determining the nature of the immune response to tumors (27) and can be polarized into two categories: M1 (associated with immune stimulation) or M2 (associated with immune dampening, tissue repair and cancer). In some virus-associated tumors, macrophages (more commonly the M2 subtype) have been shown to suppress the anti-tumor CD8 T-cell response (28) known to be important for survival in MCC (13). Furthermore, tumor-associated macrophages secrete many cytokines, chemokines and proteases, which promote tumor angiogenesis, growth, metastases and immune suppression (29). Thus, exploring the role of macrophages and the associated molecular pathways regulating macrophage polarization within the MCC tumor microenvironment may hold great promise for anti-cancer therapy and immunity.

Myeloid derived suppressor cells (MDSCs) play a major role in cancer-related immune suppression (30). Recent evidence suggests that there is a strong link between vascular E-selectin downregulation and nitric oxide production by MDSCs in squamous cell carcinomas (SCC) (31). Similarly, MDSCs may play a role in regulating T cell trafficking into MCC tumors. It is plausible that similar mechanisms of E-selectin regulation are at play in MCC. Indeed, we observed that nitrotyrosine, a surrogate marker of nitric oxide and reactive nitrogen species production, is associated with E-selectin downregulation and deficient CD8 lymphocyte infiltration in MCC tumors. Several additional mechanisms are implicated in the MDSC-induced suppression of adaptive and innate immunity. The production of arginase and ROS, the nitration of the TCR, cysteine deprivation, and the induction of T regulatory cells inhibit T cell activation.
Innate immunity is dampened by the down-regulation of macrophage-produced IL-12, the increase in MDSC production of the immunosuppressive IL-10, and the suppression of NK cell cytotoxicity (32). Thus, regulation of MDSC signaling to enhance the anti-tumor response may have a role in MCC treatment paradigms in the future.

While most MCC tumors appear to harbor the tumor-promoting MCPyV oncoproteins, little is known about the pathogenesis of virus negative MCCs. Previous studies have consistently reported that MCPyV is integrated into the host genome in approximately 80% of MCC tumors, with a cumulative average of 77% (924 of 1198 of MCC) (33). These data have been recently challenged by a report that used improved tools to detect the persistently expressed viral oncoproteins in 97% (56 of 58) of MCCs by immunohistochemistry and in 100% (60 of 60) of MCCs by PCR (19). While this study remains to be validated, it is likely that several molecular and genetic risk factors contribute to MCC pathogenesis independent of MCPyV oncoprotein expression. It is possible that in low copy DNA positive tumors, MCPyV oncoprotein expression played a role in tumor initiation with subsequent selection for less immunogenic MCPyV oncoprotein non-expressing MCC tumor subclones. Indeed, the heterogeneity of MCPyV DNA or T-antigen expression levels in MCC tumors supports immune selection within the tumors and is consistent with the “hit and run” hypothesis for tumorigenesis in MCPyV-negative MCC tumors. In addition, several molecular signaling pathways commonly involved in cancer pathogenesis have also been reported to be deregulated in MCCs. The tumor suppressor retinoblastoma protein (an inactivation target of MCPyV oncoproteins) was observed to be expressed at lower levels in virus negative MCCs compared to virus positive MCCs (34), suggesting there may be genetic deletion of the Rb locus that predisposes one to MCC. Furthermore, Merkel cell carcinoma patients show genetic and epigenetic loss-of-function mutations in the tumor suppressor Atonal homolog 1 (also essential for cell fate commitment of Merkel cells in skin), which have been shown to promote formation and progression of tumors in mice and human cell lines (35). It is plausible that such mutations would be more likely to be
found among virus-negative MCCs. In addition, a recent report showed that the oncogenic phosphatidylinositol 3-kinase (PIK3CA) activating mutations and presence of MCPyV was mutually exclusive in MCC tumors (with one exception) (36). Importantly, future investigations could unveil the possibly different clinical behavior and therapeutic responses of virus negative MCCs compared to virus positive ones. Thus, while much of the recent research and therapeutic efforts have been focused on MCPyV associated MCCs, understanding the molecular pathways underlying MCCs that are not dependent on the virus will be important for the management of this subset of tumors.

In conclusion, Merkel cell carcinoma (MCC) is a highly aggressive neuroendocrine skin cancer associated with the Merkel cell polyomavirus (MCPyV), immune suppression, UV exposure and age over 50. Our studies help to establish the immunogenicity of persistently expressed MCPyV oncoproteins, identify the presence of virus- and tumor-specific T cell responses, and characterize several immune evasion mechanisms. Furthermore, we have demonstrated the translational relevance and clinical efficacy of these studies in humans in vivo, the results of which hold promise of new therapeutic directions for MCC. Learning how to therapeutically exploit the immune system to generate long-lasting and effective anti-tumor responses will be an ongoing challenge. However, in the recent years, huge progress has already been made in the understanding of biology-driven therapy for MCC (detailed herein) and for cancers more generally (approval of immune stimulatory ipilimumab/anti-CTLA-4, with approval for anti-PD-1 and anti-PD-L1 drugs certainly to follow). The concept of immune therapy for cancer has gone from a dream (a status it had for decades) to a reality in a subset of patients with various cancers. It is almost certain to be significantly expanded in the coming years to benefit a far greater proportion of cancer patients. As our knowledge of key immune regulatory pathways and the synergistic effects of therapies increases, the translational use of these strategies will be become more feasible for patients with MCC and other cancers more generally.
References (from Introduction and Conclusion chapters):


Appendix:

I. List of additional publications authored during period of dissertation research