Merkel Polyomavirus-Specific T Cells Fluctuate with Merkel Cell Carcinoma Burden and Express Therapeutically Targetable PD-1 and Tim-3 Exhaustion Markers

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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:** Multiparameter flow cytometry panels and HLA/peptide tetramers were used to identify and characterize T cells from tumors (n = 7) and blood (n = 18) of patients with MCC 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 samples of 7 out 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 with 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 express high levels of immune checkpoint receptors PD-1 and Tim-3. Reversal of these inhibitory pathways is therefore a promising therapeutic approach for this virus-driven cancer. *Clin Cancer Res;* 19(19); 5351–60. © 2013 AACR.

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; ref. 1). MCC is increasingly common with an estimated 1,600 cases per year in the United States (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 more than 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 antitumor 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 shown 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 patients with MCC 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.

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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 patients with MCC. 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 patients with MCC. These findings have implications for the use of existing and emerging agents that may augment immune responses in this virus-associated cancer.

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), costimulation, and activation (CD28, CD69, CD137). Many of these molecules are the targets of therapeutic agents that are FDA-approved (ipilimumab for CTLA-4) or in clinical 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 antitumor responses.

Materials and Methods

Human subjects and samples

This study was approved by the Fred Hutchinson Research Center Institutional Review Board and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Blood was obtained from HLA-A*0201, 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 real-time PCR (RT-PCR) for MCPyV T-Ag immunohistochemistry (CM2B4 antibody, Santa Cruz Biotechnology) 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 0.1 mg/mL DNAse-I, 0.4 mg/mL collagenase-IV, 0.1 mg/mL hyaluronidase (all from Worthington Biochemical) in serum-free RPMI for 3 hours at 37°C, then passed through a 70 μm nylon cell strainer. Isolated lymphocytes were incubated for 30 minutes at 37°C with allopeycoyanin (APC)-conjugated tetramers specific for MCPyV (11), cytomegalovirus (CMV), or Epstein–Barr virus (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 minutes at 4°C, and cells were stained for 30 minutes at 4°C with: CD3-Qdot605 (Invitrogen), CD8-V500 (BD), PD-1-BrilliantViolet421 (BioLegend), Tim-3-PE (R&D Systems), CTLA-4-FTIC (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 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 with external controls (tonsil). As previously described, grades 3 and 2 were grouped as high-expressor cases and grade 1 or 0 were defined as low-exposer cases (18). Tumor-infiltrating CD8 lymphocytes were scored as previously described (9). MCPyV T-antigen staining was done using CM2B4 (Santa Cruz; ref. 19) and/or Ab3 (8) antibodies.

mRNA profiling

mRNA profiling and analysis was carried out as previously described (9) and relevant expression data were 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 to 2 x 10^5 cells per well with 1.5 x 10^4 autologous peripheral blood mononuclear cells (PBMC) used as APC, and stimulated in duplicate wells with peptides specific for MCPyV (MCPyV.LT-92-101, 10 μg/mL), EBV (BMLF1.280-288, 10 μg/mL), or media (negative control). Blocking anti-Tim-3 (10 μg/mL, Biologend) and anti-PD-1 (10 μg/mL, R&D Systems), or isotype control monoclonal antibodies (10 μg/mL) were added. For assays conducted directly ex vivo, cells were plated directly onto 96-well multi-screenIP plates (Millipore) precoated with anti-INF-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 T cell medium and 20 U/mL IL-2 (Chiron Corporation), 20 ng/mL IL-7 (R&D Systems), and 10 ng/mL IL-15 (R&D Systems) added on days 2, 4, and 6. On day 7, cells were transferred to IFN-γ precoated 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), and verified for quality control. Representative experiments are shown, with each experiment conducted 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 conducted as previously described (11) and is detailed in Supplementary Methods.

Statistical analysis

For quantitative comparisons, Fisher exact test, Wilcoxon rank sum test, or Student t test was conducted 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

To investigate the prevalence of MCPyV-specific T cells found in the blood of patients with MCC and control subjects, we used an HLA-peptide tetramer (HLA-A24: MCPyV.LT.92-101) in a direct 

\[ \text{ex vivo} \] screen of HLA-compatible PBMCs from first available blood draw. Viral oncoprotein-specific CD8 T cells were not detectable in blood from any of 10 HLA-A24 control subjects in this assay (detection sensitivity of ~0.01% of CD8 T cells). In contrast, 64% of HLA-compatible patients with MCC (7/11; P<0.01) had MCPyV-specific T cells in their blood (range: 0.03%–0.24% of CD8 T cells; Fig. 1A, Supplementary Fig. S1). Patients with detectable virus-specific T cells had a significantly greater disease burden (average 3.7 cm in the longest dimension; range 1.8–5.5 cm; n = 6), compared with those

![Figure 1](https://www.aacrjournals.org/content/19/19/5353/F1.large.jpg)

Figure 1. CD8 T cells specific for MCPyV were detected in the majority of patients with MCC and then tracked relative to tumor burden and 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 from cases that were tetramer-positive or tetramer-negative. **P<0.01, Fisher 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 patients with MCC at indicated times in their disease course. Days since diagnosis (dx) 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 patients with MCC (circle, w678; X, w334; triangle, w672; square, w131) at the indicated times.
without detectable T cells (average 0.7 cm; range 0.3–1.0 cm; n = 3; P < 0.05). Furthermore, among the 7 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 before completion of treatment. In contrast, in the 4 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 (as assessed by CM2B4 or Ab3 antibody immunohistochemistry, data not shown; refs. 8, 19), 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 show that circulating MCPyV-specific T cells are more likely to be found among patients with MCC with larger MCPyV-associated tumors.

**MCPyV oncoprotein-specific T cells fluctuate with tumor burden and antiviral antibodies**

The greater likelihood of detecting MCPyV-specific T cells among patients with large MCC tumors and in the ones with 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 with 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 (Fig. 1). In contrast, MCPyV-specific T-cell frequencies varied dramatically over time, correlating directly with tumor burden (Fig. 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 (Fig. 1A) and longitudinal (Fig. 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 coexpress high levels of immune checkpoint receptors PD-1 and Tim-3**

To determine the functional status of MCC-targeting T cells, we used a multiparameter flow cytometry phenotyping panel to characterize TILs and circulating MCPyV-specific T cells in patients with MCC. Because culture can alter protein expression patterns, specimens were phenotyped directly ex vivo for markers associated with costimulation (CD28, CD137), activation (CD69, CD137), and T-cell inhibition (PD-1, Tim-3, CTLA-4; Fig. 2 and Supplementary Fig. S2). T cells specific for CMV or EBV were used as controls. Activation and costimulation 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 with other viruses (Fig. 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 with T cells specific for CMV and EBV (Fig. 2B). Tim-3 was also significantly more likely (>3-fold) to be expressed on TILs from MCCs (34 ± 17%, n = 7) and MCPyV-specific T cells from PBMC (46 ± 21%, n = 5) as compared with control virus-specific T cells (Fig. 2B). Surface expression of another inhibitory molecule, CTLA-4, was generally low among TIL and CD8 T cells specific for MCPyV, CMV, and EBV (Fig. 2B).

Because 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 coexpressed key inhibitory receptors among TIL and PBMC specific for EBV, CMV, or MCPyV in patients with MCC (Fig. 3). The combination of PD-1 and Tim-3 coexpression 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; Fig. 3B). More than 90% of those Tim-3+ cells coexpressed PD-1. Furthermore, Tim-3 expression was most often observed among TIL with high-positive PD-1 levels as compared with cells with intermediate-positive PD-1 levels (Fig. 3C). MCPyV-specific PD-1+ cells had a significantly higher median fluorescence intensity (MFI; 4-fold) compared with the PD-1+ T-cell subset specific for CMV or EBV (Fig. 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, whereas there was minimal fluctuation in T cells specific for CMV or EBV (Fig. 4B).

To test function, we assayed the IFN-γ response of MCC-infiltrating lymphocytes and MCPyV-specific PBMC. Because none of the available TILs were from HLA-A24-positive patients, we used phosphor myristate acetate (PMA) and ionomycin to stimulate the cells. Of the four tested TIL samples, we failed to produce IFN-γ when stimulated with PMA/ionomycin directly 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 Fig. S2). 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 Materials and Methods. In this patient, the baseline number of MCPyV-specific CD8 T cells that secreted IFN-γ was markedly lower than would be expected on the basis of the number of virus-specific cells that were plated (Fig. 5; 1 × 10^5 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 1 × 10^5 CD8 T cells), these cells produced a more robust IFN-γ response to the cognate antigen (Fig. 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 whether blocking these inhibitory receptors could improve the function of MCPyV-specific T cells. CD8 T cells were exposed to cognate peptide and antibodies that functionally block PD-1, Tim-3 alone or in combination. After a short (20-hour) ex vivo stimulation, there was minimal peptide-specific IFN-γ response even in the presence of blocking antibodies (Fig. 5A). In contrast, when CD8 T cells were preincubated 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 with similarly cultured cells to which blocking antibodies were not added (Fig. 5B). Although these results are encouraging, this study could only be carried out in a single patient because of experimental requirements including a high frequency of virus-specific T cells and a large starting blood volume.

In summary, we show that MCPyV-specific CD8 T cells from blood and MCC-infiltrating T cells predominantly coexpress 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 whether PD-L1 ligand, PD-L1, was present within MCC tumors and whether 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 ($R^2 = 0.6$; Fig. 6A). A nonoverlapping 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 ($P < 0.05$; Fig. 6B). Representative histopathologic photographs are provided in Fig. 6C. This pattern of PD-L1 staining suggests that tumor infiltrating PD-1+ T cells have a high

Figure 3. Coexpression of PD-1 and Tim-3 inhibitory receptors is elevated among MCPyV-specific T cells and MCC-infiltrating lymphocytes. A, coexpression of inhibitory receptors from four representative samples analyzed with SPICE software (35). Pie chart indicates number of coexpressed markers. Out 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 coexpress PD-1, Tim-3, and CTLA-4 in MCC CD8+ TIL ($n = 7$), PBMC specific for MCPyV ($n = 5$), CMV ($n = 7$), and EBV ($n = 5$), and all CD3+CD8+ T cells ($n = 11$). The mean and SEM are shown. **P < 0.01, Wilcoxon rank sum test. C, CD3+CD8+ TIL ($n = 7$) assessed for PD-1 and Tim-3 expression by flow cytometry. The appropriate isotype antibody controls are included in the rightmost panel. 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, 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 patients with MCC. Most patients with MCC only had detectable tetramer-positive T cells for one of these viruses. Line indicates median. Tet− = tetramer-negative. *, P < 0.05; **P < 0.01, Wilcoxon rank sum test. B, percent PD-1 expression among CD3+CD8+ T cells specific for MCPyV (solid lines, left), CMV (dashed lines, right), or EBV (dotted lines, right) measured in serial blood draws from patients with MCC (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 patients with MCC 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.
chance of encountering their relevant inhibitory ligand in the MCC microenvironment.

Discussion

The purpose of this study was to investigate the mechanisms that prevent MCPyV-specific T cells from controlling MCC. Here, we show that MCPyV-specific T cells: (i) dynamically correlate in frequency with clinical disease burden and with antibodies against the viral oncoprotein (T-antigen), (ii) coexpress therapeutically-reversible markers of exhaustion, PD-1, and Tim-3 at far higher levels than T cells specific for other common human viruses, (iii) 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 patients with MCC with variable disease burdens. In patient with MCC, 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 to obtain sufficient T cells for adoptive T-cell immunotherapy, it may be important to use PBMC acquired at times of higher tumor burden. In addition, 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 coexpressed, 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 likely 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 is 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 (28). 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 with 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 with control viruses, and speculate that this may be associated with decreased function. The relevant ligand, PD-L1, is often expressed within the tumor microenvironment (17, 29–31), and in melanoma, PD-L1-expressing tumor cells are often localized immediately next to TILs (32). 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 in MCC tumors with PD-1-expressing T cells, PD-L1, and PD-L2 expression is mostly restricted to a subset of dendritic cells and macrophages (but not the cancer cells themselves; ref. 33). 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 patients with diverse types of cancer (12, 34).

There are several limitations to this study. We focused on a single, well-established MCPyV-specific epitope (11), which may provide a limited representation of the total antigen-specific immune response to MCC. We were limited in the number of longitudinal studies and antibody blockade experiments that were possible because of the rare aggressive nature of MCC, as well as the limited number of patients who have T cells that can be identified by the currently available peptide/HLA tetramer. The development of new peptide/HLA tetramers will expand the number of patients with MCC and the diversity of MCPyV-specific CD8 T cells that can be characterized.

In summary, this study shows 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. Therefore, our data support the investigation of agents currently in clinical trials.
or preclinical trials, such as blockers of the PD-1/PD-L1 (12, 34) and of the Tim-3 axis (14, 15), or agonists of costimulatory molecules such as CD137 (13) in patients with advanced MCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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