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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—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 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.

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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.

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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 3hr 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 $1–2\times10^5$ cells/well with 1.5×10^4 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.

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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 0.7cm; range 0.3cm 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

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circulating MCPyV-specific T cells in MCC patients. Since culture can alter protein expression patterns, specimens were phenotyped directly *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 3). 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. 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 *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 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 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; 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 (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. 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-

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specific IFN-γ response even in the presence of blocking antibodies (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). Although these results are encouraging, this study could only be carried out in a single patient due to 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 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

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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 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 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 (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 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, 29–31), and in melanoma, PD-L1 expressing tumors cells are often localized immediately next to tumor infiltrating lymphocytes(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) (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 several 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 MCC patients and the diversity of MCPyV-specific CD8 T cells that can be characterized.

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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. 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, 34) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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.

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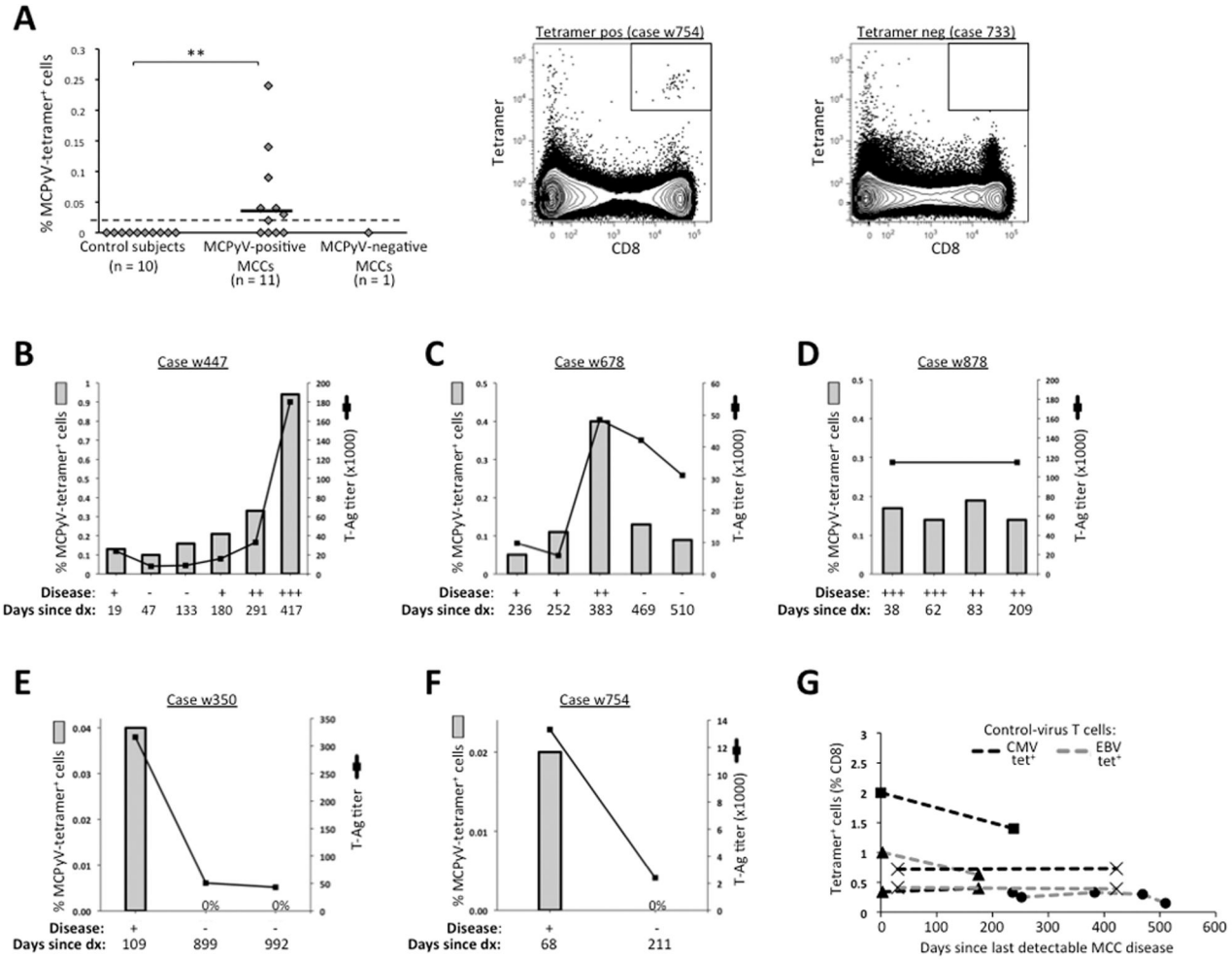

Figure 1. CD8 T cells specific for MCPyV were detected in the majority of MCC patients and tracked with tumor burden and with anti-T-Ag antibodies

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.

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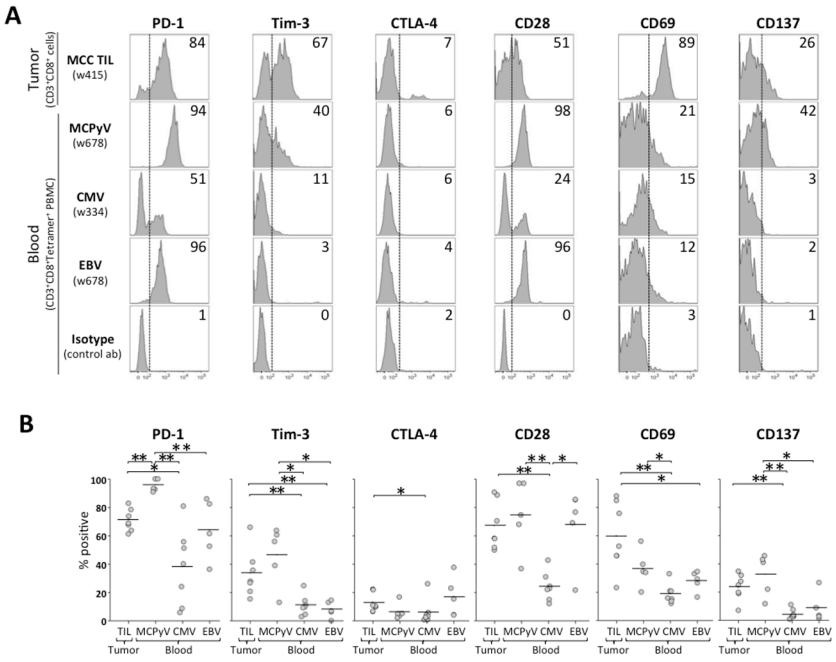

Figure 2. MCPyV-specific T cells and MCC TIL express multiple inhibitory receptors and activation markers

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. Summary data for all MCC patients' samples analyzed: tumor infiltrating lymphocytes (TIL) from MCC tumors (n=7); tetramer-positive PBMC specific for MCPyV (n=5), CMV (n=7), EBV (n=5). The horizontal line indicates the mean. 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.

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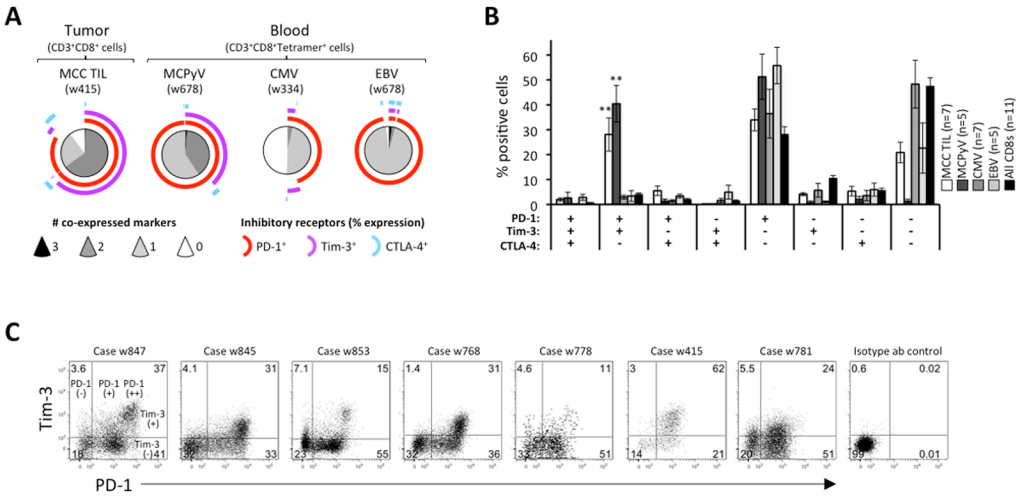

Figure 3. Co-expression of PD-1 and Tim-3 inhibitory receptors is elevated among MCPyV-specific T cells and MCC-infiltrating lymphocytes

A. Co-expression of inhibitory receptors from four representative samples analyzed with SPICE software (35). 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, Tim-3 and CTLA-4 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). 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 expression by flow cytometry. 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.

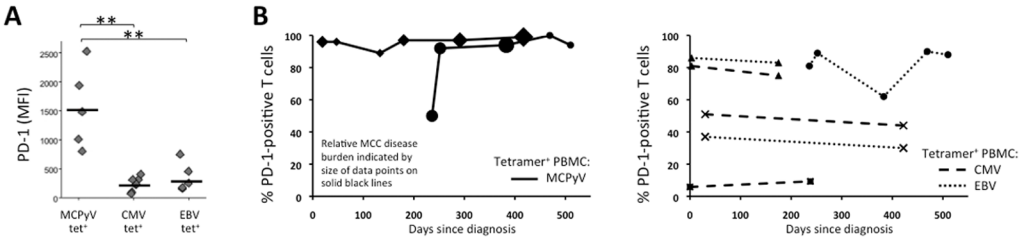

Figure 4. PD-1 is highly expressed on MCPyV-specific T cells and its expression is maintained throughout the MCC disease course

A. Median fluorescence intensity (MFI) of $CD3^+CD8^+PD\text{-}1^+$ 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.

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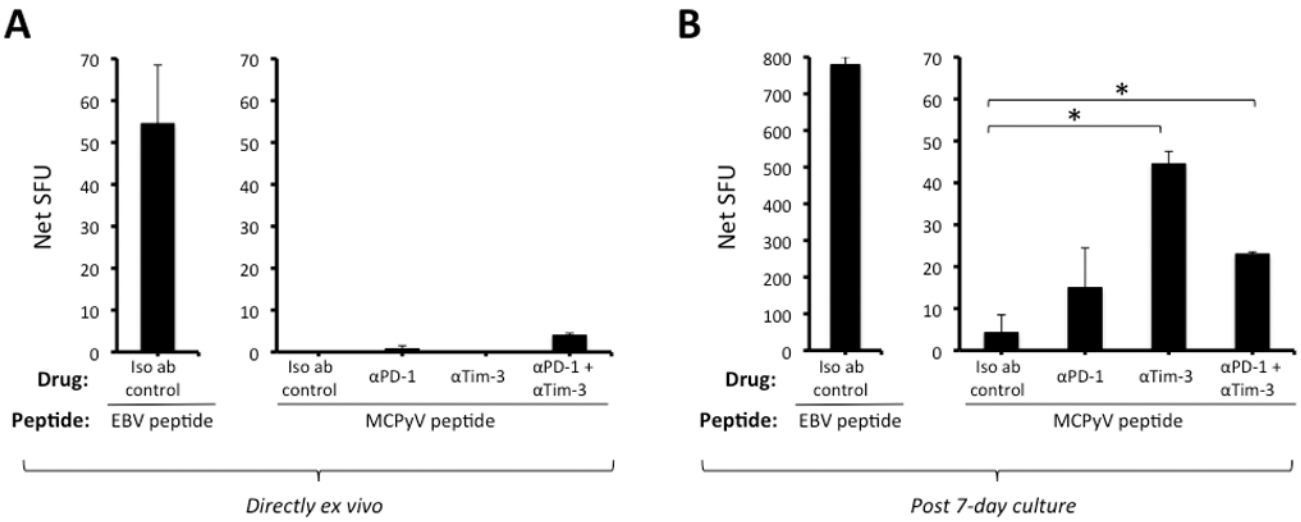

Figure 5. Culture with inhibitory receptor blocking agents augments MCPyV-specific T cell function

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.

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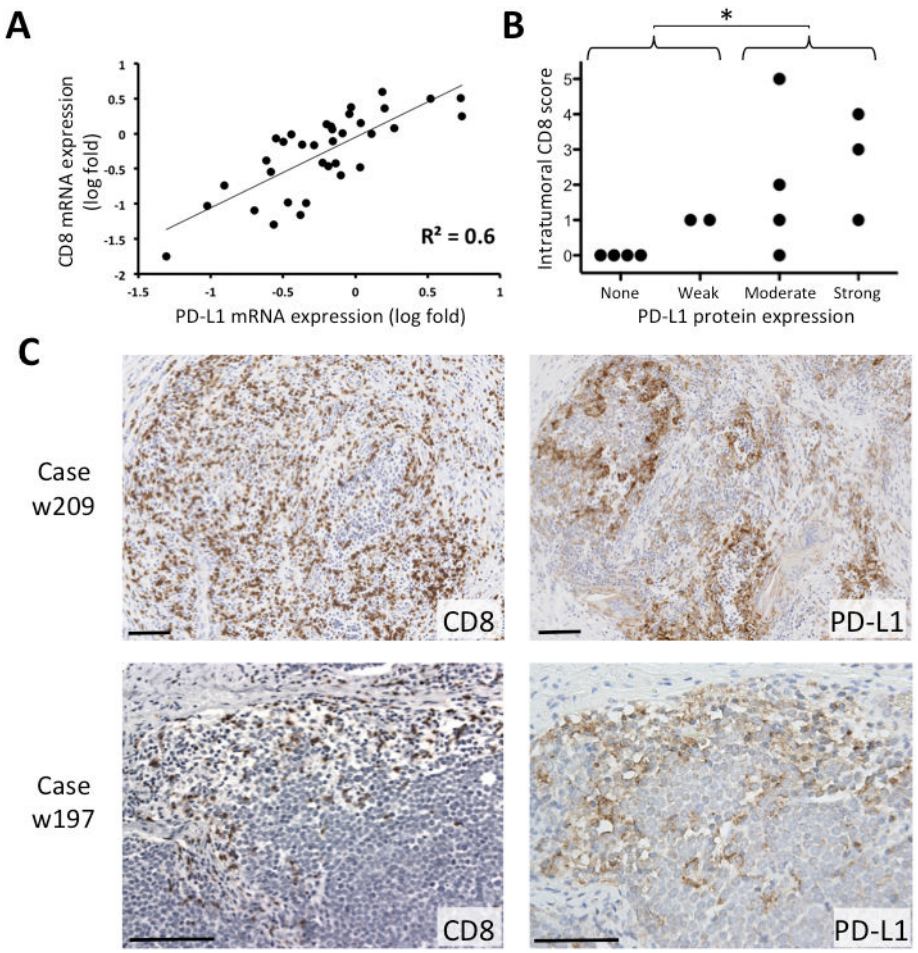

Figure 6. PD-L1 expression in MCC tumors correlates with CD8 lymphocyte infiltration

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.