Human CD4⁺ T Cells Specific for Merkel Cell Polyomavirus Localize to Merkel Cell Carcinomas and Target a Required Oncogenic Domain

Natalie V. Longino, Junbao Yang, Jayasri G. Iyer, Dafina Ibrani, I-Ting Chow, Kerry J. Laing, Victoria L. Campbell, Kelly G. Paulson, Rima M. Kulikauskas, Candice D. Church, Eddie A. James, Paul Nghiem, William W. Kwok, and David M. Koelle

Abstract

Although CD4⁺ T cells likely play key roles in antitumor immune responses, most immuno-oncology studies have been limited to CD8⁺ T-cell responses due to multiple technical barriers and a lack of shared antigens across patients. Merkel cell carcinoma (MCC) is an aggressive skin cancer caused by Merkel cell polyomavirus (MCPyV) oncoproteins in 80% of cases. Because MCPyV oncoproteins are shared across most patients with MCC, it is unusually feasible to identify, characterize, and potentially augment tumor-specific CD4⁺ T cells. Here, we report the identification of CD4⁺ T-cell responses against six MCPyV epitopes, one of which included a conserved, essential viral oncopgenic domain that binds/disables the cellular retinoblastoma (Rb) tumor suppressor. We found that this epitope (WEDLT209-228) could be presented by three population-prevalent HLA class II alleles, making it a relevant target in 64% of virus-positive MCC patients. Cellular staining with a WEDLT209-228-HLA-DRB1*0401 tetramer indicated that specific CD4⁺ T cells were detectable in 78% (14 of 18) of evaluable MCC patients, were 250-fold enriched within MCC tumors relative to peripheral blood, and had diverse T-cell receptor sequences. We also identified a modification of this domain that still allowed recognition by these CD4⁺ T cells but disabled binding to the Rb tumor suppressor, a key step in the detoxification of a possible therapeutic vaccine. The use of these new tools for deeper study of MCPyV-specific CD4⁺ T cells may provide broader insight into cancer-specific CD4⁺ T-cell responses.

Introduction

Merkel cell carcinoma (MCC) is a rare but deadly skin cancer with a relative mortality rate of 46%, making it approximately three times as deadly as malignant melanoma on a per-case basis. The current annual U.S. incidence of ~2,500 cases per year is projected to climb to ~3,200 by the year 2025. This predicted increase is due to the unusually strong association of age with MCC and the aging of the “Baby Boomer” generation. In the United States, the majority of MCCs (~80%) are etiologically linked to the Merkel cell polyomavirus (MCPyV; ref. 3), a small (~5.4 kilobase genome) double-stranded DNA virus that encodes oncogenic T-antigens, including large T (LT) and small T (sT; ref. 3). Importantly, elimination of the C-terminal region of LT necessary for viral DNA replication is an invariant requirement for oncogenesis. In contrast, MCCs retain the N-terminal region, which promotes cell-cycle progression and contains the “LxCxE motif” (4, 5). The LxCxE-binding motif is conserved in LT among human polyomaviruses and several other DNA viruses, suggesting that manipulation of retinoblastoma (Rb) biology is critical for viral pathogenesis (6). Interestingly, the LxCxE domain in several DNA viruses was recently shown to mediate direct binding to STING and, thus, antagonize the cyclic GMP-AMP synthase (cGAS) innate antiviral interferon pathway (7), which may further increase the selective pressure to maintain this sequence. Consequently, the MCPyV T-antigens, which are mechanistically involved in tumor formation, are both attractive targets for the study of tumor-specific T-cell responses and rational candidates for specific immunotherapy (8, 9).

The importance of immune cell function in MCC is highlighted by the fact that MCC patients exhibit high response rates to PD-1 blocking agents (10–12). Robust CD8⁺ T-cell intratumoral infiltration is associated with 100% disease-specific survival, independent of stage at diagnosis (13, 14). However, this favorable
pattern is only observed in 4% to 18% of patients (13, 14). In light of this link between local CD8\(^+\) T cells and MCC survival, we sought to explore mechanisms that could regulate the CD8\(^+\) T-cell response, including the adequacy of CD4\(^+\) T-cell help. CD4\(^+\) T cells are known to provide crucial support for CD8\(^+\) T-cell function in a variety of settings. Cellular therapies utilizing CD4\(^+\) chimeric antigen receptor (CAR) T-cell products (15), autologous CD4\(^+\) T cells (16), or T-cell receptor (TCR)–transgenic CD4\(^+\) T cells (17, 18) have mediated cancer regression. Therapeutic cancer vaccines primarily targeting CD4\(^+\) T cells can induce epitope spreading of CD8\(^+\) T-cell responses (19) and have shown promise in treating melanoma (20, 21). These findings suggest that CD4\(^+\) T cells significantly contribute to cancer-immune-based therapies. However, the low frequencies of antigen-specific CD4\(^+\) T cells within peripheral blood (several logs lower than antigen-specific CD8\(^+\) T cells) combined with a lack of specific tools to identify these cells have hindered their study. Consequently, we optimized and used a suite of complementary methods to identify MCPyV-specific CD4\(^+\) T cells with the goal of enabling detailed tetramer-based characterization of CD4\(^+\) T-cell responses against MCPyV and of contributing to the development of novel therapeutic strategies for MCC patients.

**Materials and Methods**

**Subjects and specimens**

Studies were approved by the Fred Hutchinson Cancer Research Center (FHCRC) and the Benaroya Research Institute (BRI) Institutional Review Boards and conducted according to the Declaration of Helsinki principles. Informed written consent was received from all participants. Subjects were HLA class II typed via polymerase chain reaction (PCR)–based methods at Bloodworks Northwest (Seattle, WA), by high-throughput next-generation sequencing at Scisco Genetics (FHCRC) as described (22) or by real-time PCR at BRI as described (23). We screened 89 MCC patients for appropriate HLA types from our repository of over 1,400 patients. Of those, 5 patients with available tumor material had HLA types we could study. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood from MCC patients and healthy donors with Lymphocyte Separation Medium (Corning) and cryopreserved in freezing medium [50% human serum (Valley Biomedical), 40% RPMI (Thermo Fisher), and 10% DMSO (Thermo Fisher)]. Fresh MCC tumor material and punch biopsies (>1 cm\(^2\)) were processed into single-cell digests by mincing them into small pieces with sterile forceps and scissors, followed by incubation in 20 mL of digestion medium composed of RPMI plus 0.002 g DNAse ( Worthington Biochemical), 0.008 g collagenase ( Worthington Biochemical), and 0.002 g hyaluronidase ( Worthington Biochemical) in a 10-cm dish at 37°C with frequent, gentle swirling. After 3 hours of digestion, cells were strained through a 70- μm filter, centrifuged, resuspended in freezing medium [50% human serum (Valley Biomedical), 40% RPMI (Thermo Fisher), and 10% DMSO (Thermo Fisher)].

**MCC cell lines**

MCC cell lines WaGa, MKL-1, and MCC-13 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Atlantic Biologicals), 200 nmol/L L-glutamine, and penicillin–streptomycin (100 U/mL). WaGa cells were a gift from Dr. Juergen Becker, German Cancer Research Center, Heidelberg, Germany (8), MKL-1 cells from Masa Shuda (26), and MCC-13 cells from Helen Leonard (27). All cells, except COS-7, were confirmed authentic with short-tandem repeat analysis (STR; ATCC) and were Mycoplasma negative via Plasmofest (InvivoGen). COS-7 cells (ATCC, CRL-1651, 2005) were maintained in DMEM supplemented with 10% FBS, 200 nmol/L L-glutamine, and penicillin–streptomycin (100 U/mL), and were free of Mycoplasma.

**Epitope determination**

MCPyV oncoprotein CD4\(^+\) T-cell epitopes were identified using three approaches: tetramer-guided epitope mapping (TGM), intracellular cytokine staining (ICS), and carboxyfluorescein succinimidyl ester (CFSE) dilution.

For TGM studies (28), PBMCs obtained from 24 healthy donors expressing at least one of eight discrete HLA class II allele types were cultured in TCM in the presence of pools of five MCPyV 20-mer peptides (GenScript; Supplementary Table S1) at 1 μg/mL final concentration of each peptide for 10 to 14 days. Subsequently, expanded PBMCs were subjected to two rounds of tetramer staining (first by tetramers loaded with a pool of five consecutive overlapping peptides and then with individual peptide-loaded tetramers corresponding to positive pools) as previously described (28). Tetramers were generated at the BRI for the following alleles: HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB1*0404, HLA-DRB1*0701, HLA-DRB1*1501, HLA-DRB5*0101, and HLA-DQB1*0602. Flow cytometry stains identified live CD4\(^+\) cells (Biologend, #344618) cells containing tetramer-pool-positive cells and excluded CD8\(^+\) cells and excluded CD8\(^+\) (Invitrogen, MHC08050), CD14\(^+\) (Biologend, #301828), and CD19\(^+\) (Biologend, #302232) cells. Cultures were then stained with single tetramers containing relevant peptides within the positive pool (Table 1). Four HLA alleles yielded MCPyV epitopes (HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB5*0101, and HLA-DQB1*0602), and for each allele type, four individuals were tested (Fig. 1). The fraction of patients with a given allele in which tetramer-positive cells were detected is denoted in Table 1. For ICS assays focusing on IFNγ secretion, peptide pools composed of 17 to 27 peptides (13-mers) at final concentrations of 1 μg/mL of each peptide were incubated with 2.5 × 10\(^5\) to 5 × 10\(^5\) TILs and an equal number of autologous PBMCs as antigen-presenting cells (APC) for 16 hours as previously described (24). Data were acquired on a Canto RUO cytometer (Becton Dickinson). Flow cytometry analysis evaluated the percentage of 200 nmol/L L-glutamine, penicillin–streptomycin (100 U/mL), 55 μmol/L 2-Mercaptoethanol (Thermo Fisher), and 1 mmol/L sodium pyruvate (Thermo Fisher) as described (24).
Table 1. Newly identified CD4 T-cell responses against MCPyV oncoproteins

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live IFNγ<sup>+</sup> (BD Pharmingen, 554701) CD4<sup>+</sup> cells and excluded dead (LIVE/DEAD Fixable Violet Dead Cell Stain, Invitrogen, #L34955), CD8<sup>+</sup>, CD14<sup>+</sup>, and CD19<sup>+</sup> cells.

For CFSE dilution (29), PBMCs from 3 MCC patients were stained with CFSE (Vybrant CFDA SE cell tracer kit, Invitrogen cat. #V-12883) following the manufacturer’s instructions and incubated in RPMI-1640 containing 10% human serum, 2 mmol/L L-glutamine (HyClone), and 1% penicillin/streptomycin (HyClone) at a concentration of 4 × 10<sup>6</sup> cells per well in a 24-well plate. Two peptide pools composed of 24 peptides (20-mers overlapping by 12 AA; GenScript; Supplementary Table S1) were incubated with CFSE-labeled PBMCs at final concentrations of 1 μg/mL for each peptide for 5 days. Flow cytometry analysis evaluated the percentage of CFSE dilution among CD3<sup>+</sup> (ECD; Beckman Coulter; cat. #IM27051) CD4<sup>+</sup> cells (PE; BioLegend; cat. #357404), excluding dead (7-AAD<sup>+</sup>; BD Biosciences; cat. #559925).

In each of these methods, positive signals using peptide pools were confirmed through follow-up assays using individual peptides to define epitopes. The MCPyV/CD4 epitopes discovered and validated through these complementary methods are summarized in Table 1.

For 1 patient in whom WEDLT209-228 specificity was based on IFNy secretion (Fig. 2A), generated T-cell clones (described in the next section) were then tested for production of IFNγ following exposure to antigen in the presence of HLA class II locus-specific blocking mAbs (30) in order to determine HLA class II allele restriction (Fig. 2B). The IFNγ flow cytometry release assay was the same protocol as described above, with the exception that APCs were incubated for 10 minutes at room temperature with HLA blocking antibodies, L243 (HLA-DR), and SPVL3 (HLA-DQ) prior to the addition of responder cells with antigenic peptide. Antibodies pan-specific for blocking all HLA-DR (clone L243), HLA-DP (clone B7.21), or HLA-DQ (clone SPVL3) allelic variants were generated from murine hybridoma cell lines (31) and stored as crude supernatants at −80°C. Supernatants were used at a final 1:8 dilution for blocking.

T-cell sorting

WEDLT209-228-specific CD4<sup>+</sup> T cells were sorted using tetramer-based single-cell sorting, IFNy capture, or CFSE dilution staining. Clones were derived from either expanded TIL cultures or peptide-stimulated PBMCs expanded as previously described (24, 28).

For tetramer-based sorting, PBMCs or TILs (5 × 10<sup>6</sup>–20 × 10<sup>6</sup>) were thawed, phosphate-buffered saline (PBS) washed, and incubated with 100 nmol/L dasatinib (SelleckChem, #S1021) for 10 minutes at 37°C in TCM (RPMI, 8% human...
serum, 200 nmol/L L-glutamine, and 100 U/mL penicillin–
streptomycin). Cells were washed and resuspended in 50 µL
tCM, stained with 2 µL of DQB1*0401-WEDLT209-221 PE tetra-
mer (BRI), and incubated for 1 hour in the dark at room
temperature. Cells were washed with 3 mL of 4% FCS, 1% BSA in
PBS (PBS-A), collected by centrifugation, and resuspended in
500 µL of 1:2,000 diluted LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen, #L34955), and incubated at 25°C for 20 minutes. Cells were then washed in 2 mL of 4°C PBS-A, resuspended in 95 µL of Fc block
(STEMCELL Technologies, #18551) and stained with anti-
CD4–AlexaFluor 488 (BioLegend, #344618), anti-CD8–APC
(Invitrogen, MHCD0805), anti-CD14–PacBlue (BioLegend,
#301828), and anti-CD19–PacBlue (BioLegend, #302232).

Following a 25-minute incubation on ice, cells were washed
2× with ice-cold PBS-A and resuspended in 150 µL of 2% human serum in RPMI. Viable CD4⁺ T cells (negative for CD8/CD14/CD19) in the lymphocyte forward/side scatter region were sorted into 96-well plates containing 100 µL tCM. For single-cell sorting using IFNγ capture, the IFNγ secretion enrichment kit (Miltenyi Biotec, #130-054-201) was used as per the manufacturer’s instructions. T cells were sorted on an Aria II cell sorter (Becton Dickinson), and data were collected using Becton Dickinson FACSDiva software (v6.1.1). For sorting using CFSE dilution, cells were stained and stimulated with peptide as described above.

**T-cell expansion**

Cells sorted using one of the three methods described above were expanded for 2 weeks in 96-well plates with allogeneic irradiated feeder PBMCs (150,000 cells/well) and

**Figure 2.**

The WEDLT209-228 epitope is presented by HLA-DRB1*0301 alleles. A, ICS screening of expanded TILs from an MCC patient biopsy incubated with four MCPyV T-antigen 13-mer peptide pools (two shown). Reactive pool 3 was broken down into individual 13-mer peptides (two shown). The percentage of viable CD4⁺ IFNγ⁺ T cells (CFSE-labeled APCs excluded and negative for CD8/CD14/CD19) in the lymphocyte forward/side scatter region is denoted. Negative control: TILs stimulated with DMSO and the peptide “TYGWEDLFCDES,” which is 4 AA N-terminal to the “WED” peptide. Positive control: TILs stimulated with SEB. B, A WEDLT209-228-specific clone: anti-HLA class II locus-specific blocking mAbs established HLA-DQ as the restricting locus. The percentage of viable CD4⁺ IFNγ⁺ T cells (CFSE-labeled APCs excluded and negative for CD8/CD14/CD19) in the lymphocyte forward/side scatter region is denoted. Negative control: DMSO stimulated; positive control: SEB stimulated. C, HLA-DQB1*0301 restriction was established using LCLs from 3 different patients (“w678,” “w683,” and “w420”) with defined HLA-DQ genotype with a WEDLT209-228-specific CD4⁺ T-cell clone, peptide (“WED”), and inclusion of anti-
HLA-DQ mAb (“DQ-Block”). The percentage of viable CD4⁺ IFNγ⁺ T cells (negative for CD8/CD14/CD19) in the lymphocyte forward/side scatter region is denoted. DMSO and SEB were used as negative and positive controls, respectively. Plots shown represent one of two replicates.
Flow cytometric analysis was done on viable IFNγ+CD4+ cells (negative for CD8/CD14/CD19) in the lymphocyte forward/side scatter region. These cells were designated as responder cells in ICS assays.

**IFNγ ELISA assays**

WEDLT209-228-specific T-cell functional avidity was determined by co-incubating HLA-DRB1*0401–restricted WEDLT209-228 T-cell clones and autologous PBMCs (1:1 ratio, 2 × 10^5 cells/well each) in 200 µL TCM with 10-fold serial-diluted antigenic peptides at final concentrations of 10^{-6} to 10^{-14} g/mL for 16 hours. Cross-reactivity against homologous regions of 12 additional human polyomaviruses was assessed by incubating HLA-DRB1*0401–restricted WEDLT209-228 T-cell clones and autologous PBMCs (1:1 ratio, 2 × 10^5 cells/well each) in 200 µL TCM with 13-mer peptides (Supplementary Table S3) at a final concentration of 1 µg/mL for 16 hours. To determine the reactivity of HLA-DRB1*0401–restricted WEDLT209-228 T-cell clones against wild-type versus S220-phosphorylated WEDLT209-228, HLA-DRB1*0401–restricted WEDLT209-228 T-cell clones were incubated with autologous PBMCs (1:1 ratio, 2 × 10^5 cells/well each) in 200 µL TCM with 20-mer wild-type WEDLT209-228 or S220-phosphorylated WEDLT209-228 (GenScript) peptides at a final concentration of 1 µg/mL for 16 hours. Concentrations of secreted IFNγ in cell culture supernatants were determined by ELISA (Affymetrix, #88-7316-76). To calculate EC50, IFNγ concentrations versus peptide concentrations were analyzed via nonlinear regression using Prism version 7.0 (GraphPad Software, Inc.).

**LT antigen production and site-directed mutagenesis**

The expression plasmid for the LT antigen fusion protein (pDEST103-GFP-LT) was created using Gateway recombinational cloning technology (Thermo Fisher Scientific) to insert LT from pCMV-MCV156 into pDEST103 (33). The resulting plasmid expresses the eGFP-LT fusion protein under the control of the CMV promoter. Site-directed mutagenesis (New England Biolabs, #E0554S) was performed to generate LT S220A and LT E216K.
mutants with the following forward sets: forward primer 5′-GCGATGACACCTCTCCGCTCTGCTC-3′ and reverse primer 5′-AAAGTGGATATCCCGTGAGGTTG-3′ for S220A and forward primer 5′-CGAAGATCTCCTCCGACGCTCC-3′ and reverse primer 5′-CAGAAGATCTCCTCCGACGCTC-3′ for E216K. Mutations at the specific sites were confirmed by sequencing. Protein was expressed by COS-7 cells (ATCC, CRL-1651, 2005). COS-7 cells were plated the day before transfection at 75,000 cells/well in 12-well plates in 0.5 mL of DMEM + 10% FBS, 200 mmol/L L-glutamine, and penicillin–streptomycin (100 U/mL), and pDEST103-GFP-LT was transfected into COS-7 cells using FuGENE HD (Promega, #E2311) as per the manufacturer’s instructions. Seventy-two hours after transfection, eGFP-positive cells were sorted, and lysates were generated as described above. Clarified supernatants were used as the antigen in ICS assays as described above.

T-cell receptor beta sequencing and analysis of dextramer-sorted WEDLT209-228-specific cells

At least 3 million cells derived from fresh tumor digest of three HLA-DRB1*0401-positive MCC patients were stained with HLA-DRB1*0401-WEDLT209-228-PE dextramer (Immudex), and the same mAb cocktail as described for tetramer sorting plus LIVE/DEAD Fixable Violet (Invitrogen, #L34955). Viable T cells that recognized WEDLT209-228 were detected by TGEM and HLA-DRB1*0401-WEDLT209-228 dextramer, as dextramers are reported to have improved sensitivity compared with tetramers (36). Screening of peripheral blood samples using this dextramer indicated that WEDLT209-228 specific CD4+ T cells could be visualized directly ex vivo in the majority of HLA-DRB1*0401 patients (14 of 18, 77.8%) and healthy donors (8 of 10, 80%; Table 1).

The initial discovery of WEDLT209-228 using TILs from an HLA-DRB1*0401+ and HLA-DRB1*0301-negative patient indicated that this peptide could also be presented by a non-HLA-DRB1*0401/DRB1*0301 allele (Fig. 2A). To determine this additional allele, PBMCs from the TIL donor in which WEDLT209-228 was initially identified were expanded in the presence of WEDLT209-228 peptide and sorted using IFNy capture to generate clones (37). After confirming WEDLT209-228 specificity, T-cell clones were tested for production of IFNy following exposure to antigen in the presence of HLA class II locus-specific blocking mAbs (ref. 30; Fig. 2B). Only anti–HLA-DQ blocked responses, indicating HLA-DQ restriction. The subject in which the WEDLT209-228 response was initially identified is heterozygous for HLA-DQB1*0301 and HLA-DQB1*0501. LCLs from HLA-genotyped donors (Fig. 2C) partially matched to the restricting DQB1 alleles were used as APCs. Only APCs bearing HLA-DQB1*0301 induced IFNy responses from T-cell clones, and responses were blocked by anti–HLA-DQ. These data collectively indicated that WEDLT209-228 could be presented by DRB1*0401, DRB1*0301, and DQB1*0301. Among the MCC patients in our cohort for which we have class II HLA typing data (DRB1 locus, n = 131; DQB1 locus, n = 53), 64% expressed at least one of these three alleles. Consequently, this epitope is immunologically relevant for the majority of patients with MCPyV-positive tumors.

The epitope within WEDLT209-228 encompasses the LxCxE motif CD4+ T-cell epitopes typically range from 9 to 22 AA in length (38). We sought to determine the core sequence necessary for T-cell recognition within WEDLT209-228 in the context of HLA-DRB1*0401, the most prevalent allele of the three restriction elements in the general population that accommodated this epitope. HLA-DRB1*0401-expressing APCs were loaded with 11-mer peptides overlapping by 10 AA spanning WEDLT209-228 and incubated with WEDLT209-228-specific, HLA-DRB1*0401-restricted CD4 clones from 3 individuals (Supplementary Table S2) to identify its minimal epitope (Fig. 3). WEDLT209-228-specific clones responded to each 11-mer spanning LT-210-222, which share the 9 AA core LFDCESLLS (LT-212-220; Fig. 3).
Stimulation with this 9-mer did not elicit a significant response in three DRB1*0401-restricted WEDLT209-228-specific clones from the 3 individuals (Supplementary Fig S5A), suggesting that flanking residues may stabilize and improve the strength of TCR stimulation and/or HLA binding as has been described (39). Importantly, this epitope encompasses the conserved LxCXe-binding motif that is critical for MCPyV LT binding to Rb. The minimal epitope presented by DQB1*0301 was found to be LT-210-219 (Supplementary Fig. SSB), whereas the minimal epitope presented by DRB1*0301 was not determined. Because the LxCXe motif is conserved among many DNA viruses, including the other described human polyomaviruses, four HLA-DRB1*0401–restricted WEDLT209-228 T-cell clones were incubated overnight with 13-mer peptides spanning the LxCXe motif of 12 additional human polyomaviruses with homologous regions (Supplementary Table S3). IFNγ secretion from CD4+ T cells clones was detected against the MCPyV peptide (LT-210-222), as well as the homologous sequence from the human polyomavirus 9 (HuPyV9) for one clone and human polyomavirus 12 (HuPyV12) for three of the four clones (Supplementary Fig. S5C). These data indicated that the T-cell receptors expressed by these three clones could tolerate different degrees of variance and that cross-reactivity against other polyomaviruses was possible.

CD4+ T cells recognize the WEDLT209-228 epitope in the context of MCC tumors

Phosphorylation of serine residue 220 adjacent to the MCPyV LT LxCXe motif is required for binding to Rb (ref. 40; Fig. 4A). To test whether WEDLT209-228–specific T cells tolerated this posttranslational modification, five HLA-DRB1*0401–restricted T-cell clones were assayed for recognition of wild-type (WED-WT) or phosphorylated-S220 (S220p) 20-mers (Fig. 4B). Functional avidity, expressed as EC50, was calculated for the five DRB1*0401-restricted clones from 4 donors (2 MCC patients and 2 healthy controls; Fig. 4C). Functional avidities ranged over two orders of magnitude for WED-WT and one order of magnitude for S220p. Phosphorylation of S220 did not significantly change the EC50 for WEDLT209-228–specific CD4+ T-cell clones collectively. However, the two clones derived from healthy donors did show a decrease in functional avidity against WED-S220p relative to WED-WT. Evaluation of a much larger number of clones from additional subjects would be necessary to determine whether this trend is significant. To evaluate whether the WEDLT209-228 epitope can be processed from the LT protein in MCC tumor cells, APCs (autologous PBMCs) and DRB1*0401-restricted WEDLT209-228–specific CD4+ T cells (data from MCC patient z1107 clone 1 depicted, representing replicate data from 3 tested individuals) were incubated with lysates from two MCPyV-positive MCC cell lines, MKI-1 and WaGa, and an MCPyV-negative cell line, MCC-13. WEDLT209-228–specific CD4+ T-cell clones responded specifically to the MCPyV-positive lysates (Fig. 4D). These data indicated that the LT protein was present in an immunogenic context in virus-positive MCC cell lines and suggest that WEDLT209-228 is naturally processed in the virus-positive MCC tumor context in vivo.

Virus-specific CD4 T cells are enriched within tumors and have diverse TCR repertoires

To determine if WEDLT209-228–specific cells infiltrate MCC tumors, we stained tumor digests from 3 MCC patients with the HLA-DRB1*0401-WEDLT209-228 dextramer. The percentage of WEDLT209-228–specific cells within the total tumor-infiltrating CD4+ T cells ranged from 0.03% to 0.72% (median 0.56%; Fig. 5A). In contrast, PBMCs from 13 MCC patients were stained with the HLA-DRB1*0401-WEDLT209-228 dextramer, and the median frequency of WEDLT209-228–specific CD4+ T cells within the periphery was significantly lower at 0.0022% of total CD4+ T cells (Fig. 5B). These frequencies closely aligned with prior reports of peripheral virus-specific CD4+ T-cell frequencies (41). These results indicated an enrichment of WEDLT209-228–specific CD4+ T cells within MCC tumors compared with the periphery, suggesting active recruitment of these cells into the tumor microenvironment.

Next, we evaluated the clonal diversity of WEDLT209-228–specific CD4+ T cells within MCC tumors by next-generation sequencing of the TRB from dextramer-sorted cells isolated from these tumor digests as previously described (33). Analysis of the TRB complementary determining region 3 (CDR3) sequences yielded 366 unique TRB AA sequences from the three tumors (Fig. 5C). Of the 366, two sequences were shared between patients “w876” and “w1056” (exploded, starred pie slices; Fig. 5C). These were identical at the AA but not nucleotide level, suggesting convergence. In order to estimate immunodominance within each tumor’s WEDLT209-228–specific CD4+ T-cell population, clonality scores were calculated (see Materials and Methods). The clonality score of the dextramer-sorted samples ranged from 0.111 to 0.137, indicating that although the TRB repertoires of infiltrating T cells within these MCC tumors were diverse, a few dominant clonotypes were expanded, with the top clonotype occupying 6.3% to 12.5% of the populations (Fig. 5C).

The S220A mutation within WEDLT209-228 retains immunogenicity

The data presented thus far suggest that the WEDLT209-228 epitope could be a therapeutically useful target, either for adoptive cellular therapy or in the context of a therapeutic cancer vaccine. However, inclusion of the LxCXe motif in a cancer vaccine is not straightforward due to the Rb-binding, oncogenic activity of this domain. This issue has been addressed in human papillomavirus (HPV) vaccines by mutating the LxCXe motif to render tumors more immunogenic (42). Two mutations within the LxCXe domain of MCPyV LT have been reported to inhibit oncogenic activity in vitro, E216K (4) and S220A (40). To test whether immunogenicity of the WEDLT209-228 epitope could be retained with either of these mutations, we created an antigen by transfecting COS-7 cells with wild-type LT (LT-WT), or the LT-S220A or LT-E216K mutants (33). As an experimental control, we also tested a mutant that has all three critical LxCXe residues mutated (LT-VsSxD). Lysates from LT-expressing cells were tested as antigen with autologous PBMCs as APCs and a WEDLT209-228–specific, HLA-DRB1*0401–restricted CD4+ T-cell clone (wb688 clone 1,3). WEDLT209-228–specific clones responded to wild-type and MCPyV LT-S220A (Fig. 6A) but not to the "detoxified" E216K and VsSxD variants. To further compare the strength of response against LT-WT and LT-S220A, HLA-DRB1*0401–restricted CD4+ T-cell clones were stimulated with serial dilutions of WED-WT and WED-S220A peptides (20-mers). The S220A variant stimulated clones derived from MCC patients more potently than WED-WT, as measured by EC50 for each.
responder CD4 clone, whereas little change was observed in the measured EC50 of clones derived from healthy donors (Fig. 6B). It will be important to test additional donors and MCC patients to validate whether this difference in response is consistently found between these two donor sources. However, these data do suggest that the LT-S220A mutant (reported not to bind Rb) retained and may even strengthen immune recognition of this region in the context of HLA-DRB1*0401. These data suggest that this mutation may be clinically useful in the design of a therapeutic cancer vaccine.
CD4+ T-cell Responses against the Merkel Cell Polyomavirus

Discussion

Here, we report six MCPyV CD4+ T-cell epitopes, including a conserved and immunogenetic WED1209-228 epitope within a key oncogenic region of the MCPyV LT antigen. The WED1209-228 epitope was recognized in the context of at least three discrete, population-prevalent HLA allelic variants. Within our HLA-typed MCC cohort, 64% expressed at least one of these alleles, indicating that this epitope was immunologically relevant in most MCC patients with MCPyV-positive tumors. Further supporting this notion, the WED1209-228 epitope encompasses the LxCeE motif, which is a critical binding site of the tumor suppressor Rb (4, 40). Persistent expression of this region is required for MCC tumor development and growth (4), and this sequence is conserved in MCC tumors. Indeed, of 99 MCPyV tumor--associated LT sequences in GenBank, only one has a coding variation within LT-212-220 (S219F; GenBank KJ128376.1). WED1209-228-specific T cells infiltrated each of three tested MCC tumors from DRB1*0401-positive patients, and WED1209-228-specific T cells were 250-fold enriched within these tumor samples compared with blood, as assessed by MHC-peptide multimer staining. These WED1209-228-specific T cells exhibited diverse TCR repertoires that include expanded clonotypes, suggesting in vivo antigen recognition and, consequently, that these T cells could home appropriately into tumor tissue and have therapeutic potential.

To date, tumor immunology research has largely focused upon studying and improving CD8+ (rather than CD4+) T-cell responses. This is due to the lower relative frequencies of antigen-specific CD4+ T cells compared with CD8+ T cells (~100–1000-fold; refs. 41, 43), making detailed characterization of tumor-specific CD4+ T cells more difficult. The generation of HLA class II tetramers for the isolation and study of antigen-specific CD4+ T cells poses numerous technical challenges (44). However, a growing body of evidence suggests that...
harnessing tumor-specific CD4⁺ T cells can improve immune-based therapies and sustain CD8⁺ T-cell responses (44). Specifically, infusion of autologous TILs, which contain both CD4⁺ and CD8⁺ T cells, has shown higher response rates (50%–70%) in malignant melanoma than have CD8⁺ T cells alone (45). A study has described the case of a stage IV acral melanoma patient who experienced a complete response after TIL therapy, which included CD4⁺ T cells specific for BRAF-V600E. These tumor-specific CD4⁺ T cells increased in frequency within the blood after therapy and persisted long term, suggesting that these cells were of clinical significance and augmented the antitumor immune response (46). Administration of autologous cancer-specific CD4⁺ T cells has mediated regression of distant metastatic disease in epithelial cholangiocarcinoma (17) and complete clinical remission in another patient with metastatic melanoma (16). The use of genetically engineered CD4⁺ T cells targeting melanoma-associated antigen-A3 (MAGE-A3) yields objective responses among patients with esophageal cancer, urothelial cancer, and osteosarcoma (18), indicating that cellular therapy utilizing CD4⁺ T cells has powerful therapeutic potential in a wide array of solid cancers. Clinical trials utilizing neoantigen-based therapeutic vaccines for melanoma have yielded impressive clinical responses that correlate with robust CD4⁺ T-cell stimulation, particularly in the setting of combined PD-1 blockade therapy (20, 21). Against this backdrop, our identification of CD4⁺ T-cell epitopes and the generation of four HLA
class II multimers (tetramers/dextramers) facilitated the characterization of MCPyV-specific CD4+ T-cell responses and the subsequent development of CD4-targeted therapies for MCC, including a therapeutic cancer vaccine.

Vaccination approaches targeting MCPyV oncoproteins have clinical appeal for several reasons. Viral antigens are shared among MCC patients and, therefore, could be used to generate "off-the-shelf" vaccines that are not patient specific. Indeed, MCPyV LT and ST are immunogenic, containing at least 35 known T-cell epitopes (28 CD8 and 7 CD4), including WEDLT209-228, and are restricted by diverse HLA class I and II alleles, suggesting that an MCPyV oncoprotein vaccine has the potential to induce T-cell immunity in most MCC patients (24, 47–50). To date, two groups have described potential MCPyV therapeutic vaccine strategies utilizing both in vitro (50) and ex vivo models (51). Both studies indicate the ability to induce MCPyV-specific immunity following vaccination with the MCPyV LT antigen. However, additional work is required to translate these findings to the clinic.

Importantly, vaccination with MCPyV T-antigens in humans raises safety concerns due to the known oncogenic activity of these proteins. Approaches to "detoxify" oncogene-based cancer vaccines have shown promising safety profiles and efficacy in treating HPV-induced premalignant lesions (52). These "detoxified" vaccines harbored mutations within the LxCxE motif of the HPV-E7 oncoprotein, suggesting that a similar approach in the context of MCPyV LT may be possible (52). One possible "detoxification" strategy would be to simply delete the LxCxE region. However, this would result in loss of the immunogenic WEDLT209-228 CD4+ epitope. Notably, two point mutations within the LxCxE region at residue 216 (E216K mutation) or 220 (S220A mutation) have been reported to prevent Rb binding, thereby blocking oncogenic activity at this site (4, 5, 40). However, we showed that of these two mutations, only S220A retained the relevant epitope that allowed recognition by HLA-DRB1*0401–restricted WEDLT209-228–specific T-cell clones. Collectively, these data indicate that the WEDLT209-228 epitope is immunogenic, expressed within MCC tumors, and could be "detoxified" through mutating S220 without disrupting antigenicity.

There are several limitations of our studies. First, it is unlikely that we identified all relevant CD4 epitopes within the MCPyV oncoproteins. Study of additional MCC patients, as well as other methods of epitope identification, may yield additional disease-relevant epitopes (53). The peptide lengths used in our initial mapping studies were 13mers, but minimal CD4+ T-cell epitopes can sometimes be longer, and indeed, other groups have reported using different lengths or RNA-transfected APCs encoding the antigen of interest for CD4 epitope mapping studies (54). Consequently, alternative antigen formats could be utilized to more comprehensively map CD4 epitopes within the MCPyV T-antigens. The characterization of WEDLT209-228 reported here was largely carried forward in the context of the HLA-DRB1*0401 allele. Therefore, additional research is required to fully characterize the response to WEDLT209-228 in the context of HLA-DRB1*0301 and HLA-DQB1*0301.

The findings reported here have key implications for future investigation and clinical applications. The ability to deeply probe the immunobiology of this disease using the tools described herein provides a powerful opportunity to understand tumor-specific T-cell responses against a shared tumor antigen. Although checkpoint inhibitors have revolutionized cancer therapy, only half of MCC patients experience durable responses (10–12). Therefore, the development of novel therapies including cancer vaccines and/or CD4+ T-cell therapy may provide much-needed adjunctive therapeutic strategies for MCC patients and cancer utilizing.

**Disclosure of Potential Conflicts of Interest**

K.G. Paulson reports receiving other commercial research support from Merck-SITC. P. Nghiem is a consultant/advisory board member for EMD Serono and Merck. D.M. Koelle reports receiving a commercial research grant from Immunomics Therapeutics. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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