**Human Cancer Biology**

**T-cell Responses to Oncogenic Merkel Cell Polyomavirus Proteins Distinguish Patients with Merkel Cell Carcinoma from Healthy Donors**

Rikke Lyngaa, Natasja Wulff Pedersen, David Schrama, Charlotte Albæk Thrué, Dafina Ibrani, Özcan Met, Per Thor Straten, Paul Nghiem, Jürgen C. Becker, and Sine Reker Hadrup

**Abstract**

**Purpose:** Merkel cell carcinoma (MCC) is a highly aggressive skin cancer with strong evidence of viral carcinogenesis. The association of MCC with the Merkel cell polyomavirus (MCPyV) may explain the explicit immunogenicity of MCC. Indeed, MCPyV-encoded proteins are likely targets for cytotoxic immune responses to MCC as they are both foreign to the host and necessary to maintain the oncogenic phenotype. However, to date only a single MCPyV-derived CD8 T-cell epitope has been described, thus impeding specific monitoring of T-cell responses to MCC.

**Method:** To overcome this limitation, we scanned the MCPyV oncoprotein large T and small T antigens and the virus capsid protein VP1 for potential T-cell epitopes, and tested for MHC class I affinity. We confirmed the relevance of these epitopes using a high-throughput platform for T-cell enrichment and combinatorial encoding of MHC class I multimers.

**Results:** In peripheral blood from 38 patients with MCC and 30 healthy donors, we identified 53 MCPyV-directed CD8 T-cell responses against 35 different peptide sequences. Strikingly, T-cell responses against oncoproteins were exclusively present in patients with MCC, but not in healthy donors. We further demonstrate both the processing and presentation of the oncoprotein-derived epitopes, as well as the lytic activity of oncoprotein-specific T cells toward MHC-matched MCC cells. Demonstrating the presence of oncoprotein-specific T cells among tumor-infiltrating lymphocytes further substantiated the relevance of the identified epitopes.

**Conclusion:** These T-cell epitopes represent ideal targets for antigen-specific immune therapy of MCC, and enable tracking and characterization of MCPyV-specific immune responses. Clin Cancer Res; 20(7); 1768–78. ©2014 AACR.

**Introduction**

Merkel cell carcinoma (MCC) is a rare, aggressive skin cancer with an estimated 1,600 cases per year in the United States (1). MCC is more frequently observed in the elderly or immunocompromised populations, suggesting a role for immune surveillance in the development of MCC. Indeed, in intratumorally immunocompromised patients, spontaneous regressions of MCC have been observed following cessation of immune suppression (2). Although the hazard ratio for MCC is above 10 for immunosuppressed individuals, more than 90% of patients with MCC are not obviously immune compromised (3). Moreover, intratumoral CD8 T-cell infiltration is strongly correlated to the survival of patients with MCC (4). Nevertheless, once metastasized, MCC has a very poor prognosis; the 5-year relative survival for patients with stage IV metastatic disease is 18% (1). To date, there is no established effective treatment to offer these patients. Due to the apparent function of the immune system to control this tumor, ongoing clinical trials are focusing on immune-modulating approaches such as immune checkpoint blocking antibodies or targeted delivery of interleukin (IL)-2 (e.g. http://www.immomec.eu).

In 2008, Merkel cell polyomavirus (MCPyV) was discovered in association with 80% of MCCs. Subsequent work confirmed this association and further indicated that most, if not all, MCCs are associated with this polyomavirus (5). The expression of MCPyV-encoded proteins in MCC may explain the explicit immunogenicity of MCC. In MCC, the MCPyV genome is clonally integrated into the host cell

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**Authors' Affiliations:** 1Center for Cancer Immune Therapy (CCIT), Department of Hematology, University Hospital Herlev, Herlev, Denmark; 2General Dermatology, Medical University of Graz, Graz, Austria; 3Departments of Medicine/Dermatology, Pathology, University of Washington; 4Fred Hutchinson Cancer Research Center, Seattle Cancer Center Care Alliance, Seattle, Washington; and 5Department of Oncology, University Hospital Herlev, Herlev, Denmark

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

J.C. Becker and S.R. Hadrup contributed equally to this article.

**Corresponding Author:** Sine Reker Hadrup, University Hospital Herlev, Herlev Ringvej 75, 6508, 2730 Herlev, Denmark. Phone: 0045-38689442; Fax: 0045-38684153; E-mail: sine.reker.hadrup@regionh.dk

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Translational Relevance

Merkel cell carcinoma is an often-lethal skin cancer of increasing interest due to the strong association with a polyomavirus. New data suggest that virtually all Merkel cell carcinoma cases are associated with expression of Merkel cell polyomavirus oncogenes. These oncogenes represent ideal targets for immunotherapy of Merkel cell carcinoma—being both specific and essential for the cancer cells, and foreign to the immune system. We have identified multiple T-cell epitopes in Merkel cell polyomavirus-encoded proteins, and show that T-cell responses to Merkel cell polyomavirus oncogenic versus capsid proteins clearly distinguish Merkel cell carcinoma patients from healthy donors. These epitopes can serve as important targets for immunotherapy, and will improve our ability to track immune responses in patients with Merkel cell carcinoma. Furthermore, polyomavirus-specific T-cell responses can be targeted by immune-modulatory agents such as anti–CTLA-4 and anti–PD-1 for treatment of Merkel cell carcinoma.

Materials and Methods

Samples

Peripheral blood lymphocytes (PBL) samples were collected from 30 healthy donors and 38 patients with the histologically diagnosis of MCC at various time points during their course of disease (Supplementary Table S1). No information on age, gender, or the presence of MCPyV DNA was available for the control subjects. From 2 patients, we analyzed both PBL and tumor-infiltrating lymphocytes (TIL) samples. In addition, we analyzed TIL cultures from another 17 patients with MCC. PBLs were isolated by density centrifugation on Lymphoprep (Axis-Shield PoC) and cryopreserved at −150°C in FBS and 10% dimethyl sulfoxide. Tumors were categorized as MCPyV-positive based on immunohistochemical staining for LTA and/or PCR-based detection of MCPyV DNA in the tumor (16). Tumors were categorized as MCPyV-negative if tumor material was available for testing but gave a negative result even though some of the patients were seropositive for antibodies recognizing LTA/STA, described by Paulson and colleagues (16). Tumors were not available for staining in 9 of 38 patients analyzed. Patient material was collected based on informed written consent according to the Declaration of Helsinki and with an approved Institutional Review Board (Fred Hutchinson Cancer Research Center; IR#6585). For the establishment of TIL cultures, tumors were removed, cut, and incubated in T-cell medium in 48-well plates with the addition of 3.4 μg/mL phytohemagglutinin. On the second day, IL-2 (50 U/mL) and IL-15 (20 ng/mL) were added. Media were refreshed every few days. TILs were cultured for 2 to 3 weeks and frozen before analysis. Healthy donor PBLs were obtained from buffy coats available from the central blood bank of the capital region of Copenhagen (RegionH), and isolated using the same procedure as for the patient PBLs.

The peptide library

For selection of LTA- and STA-derived peptides, the CCH 348 (Genebank ID FJ173809.1) sequence was used. For selection of VP1-derived peptides, the MKL-1 (Genebank ID FJ173815) sequence was used. Peptides were purchased from Pepscan Presto, with >70% purity. Predicted peptides were tested for experimental binding affinity by an MHC ELISA (22). Briefly, rescue of the MHC complex after UV-mediated cleavage of the conditional ligand depends on the affinity of the rescue peptide. The level of MHC rescue was determined by an MHC ELISA. Plates were coated with streptavidin (2 μg/mL;
Invitrogen; cat#S888) and biotinylated MHC complexes were added after the UV-mediated peptide exchange reaction (in 2.5 nmol/L, based on 100% rescue). Plates were incubated for 1 hour and washed, and correctly folded MHC molecules were detected by binding to horseradish peroxidase–conjugated β2m antibodies (1.4 μg/mL; Acris GmbH; cat# 604HRP). After incubation and washing, a colorimetric reaction was initiated by addition of ABTS peroxidase substrate (KPL; cat# 506606). HLA-binding peptides were selected based on the ability to rescue the MHC molecule after UV-mediated cleavage of the conditional ligand, compared with that of a virus-derived control. The threshold for selection was set to 60% of the virus control peptides.

**MHC monomer production and combinatorial encoding of MHC multimers**

The production of monomers was performed as described by Hadrup and colleagues (23). In brief, HLA heavy chain is expressed in bacterial BL21 (DE3) pLysS strain (Novagen; cat# 69451) and purified as inclusion bodies. After solubilization, inclusion bodies are refolded with β2m light chain and a UV-sensitive ligand (24, 25). Following refolding, monomers are biotinylated (Avidity; cat#BirA) and purified using size-exclusion column (Waters, BioSuite125, 13μm SEC 21.5 ×300 mm column) HPLC (Waters 2489). Specific MHC monomers were generated by UV-induced peptide exchange (25), and thereafter multimerized with fluorochrome-conjugated streptavidin.

The generation of combinatorially encoded MHC multimers and the gating strategy is described in detail by Andersen and colleagues (19). Briefly, UV exchange was carried out for all selected HLA ligands and each multimer was assigned with a unique color combination, such that a T-cell response was identified by two colors enabling the specific MHC multimers. For the screening of TILs, and phycoerythrin (405203) from Biolegend to encode the virus control peptides. The threshold for selection was set to 60% of the negative fraction during the separation and 5 × 10^6 anti-CD3/CD28-coated Dynabeads (Invitrogen; cat# 111.31. D). Enriched cells were cultured in 96-well plates, and medium was refreshed at least twice a week. After 2 to 3 weeks of culturing, antigen-specific T-cell responses were analyzed by flow cytometry using combinatorial encoding with MHC multimers.

**Cell sorting and culturing**

T cells were stained with relevant MHC multimers and sorted on a FACSAria (Becton Dickinson) into 96-well plates containing 10^5 irradiated feeder cells incubated with 2 μg/mL phytohemagglutinin overnight in X-vivo supplemented with 5% human serum, 500 U/mL IL-2 and 30 ng/mL anti-CD3 (eBioscience; clone OKT3; cat# 14-0037-82). Cultures were left untouched for 5 days; media were refreshed to high IL-2–containing media (3,000 U/mL of IL-2), which was maintained through the remaining of the culturing period. Established cultures were tested for antigen specificity by MHC multimer staining. MCC cell lines (MKL-2, WaGa, and MCC-13) and K562 cells carrying HLA-A1, -A2, -A3, -A11, and -B7 were cultured in RPMI-16 (Gibco; #72400-021), 5% FBS (Gibco; cat#10270), 100 μg/mL penicillin/streptomycin (Gibco; cat# 15140-122).

**Production of in vitro–transcribed mRNA**

The cDNA encoding–truncated LTA (Genebank no.: FJ 173809.1) was synthesized and cloned into pSP73-SphA64 (kindly provided by Professor E. Gilboa, Duke University Medical Center, Durham, NC) using 5’PvuII/3’KpnI restriction sites (Geneart/Life Technologies). Before serving as DNA template for in vitro transcription, pSP73-Sph/LTA/A64 was linearized with SpeI and purified using Wizard DNA Clean-Up System (Promega). The in vitro transcription was performed with the mMESSAGE mMACHINE T7 Ultra Kit (Ambion) and mRNA was purified with the MEGAClear Kit (Ambion) according to the manufacturer’s instructions. The mRNA length, concentration, and purity were evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies), using the RNA 6000 Nano LabChip Kit (Agilent Technologies),
according to the manufacturer’s instructions. Data analysis was performed with 2100 Bioanalyzer software (Agilent Technologies).

Electroporation of K562 cells
K562 cells stably transduced with the relevant HLA (A2, A11, and B7, kindly provided by Miriam Heemskerk, University Hospital Leiden, the Netherlands), were washed twice, suspended in PBS (Invitrogen), and adjusted to a final cell density of $40 \times 10^6$ cells/mL. The cell suspensions (400 µL) were preincubated in a 2-mm gap electroporation cuvette for 5 minutes on ice. Eight micrograms of mRNA-encoding LTA was transferred to the cuvette and K562 cells were pulsed using a BTX 830 Square Wave Electroporator (Harvard Apparatus). Electroporation settings were adjusted to 6 pulses, 560 V, 99 µs. After electroporation, K562 cells were rested in 37°C and subsequently transferred to preheated culture medium (RPMI-1640 containing 10% FBS). HLA-transduced K562 cells were analyzed for mycoplasma negativity, HLA expression, and expression of the LTA mRNA.

Cytotoxicity assays
To investigate the cytotoxic capacity of T-cell cultures, a VITAL-FR cytotoxicity assay was performed as previously described (26). In short, relevant K562 cells transfected with LTA were used as target cells, labeled with 10 µmol/L carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen; cat# C34554) for 5 minutes, and washed and mixed 1:1 with mock-transfected control K562 cells that had been labeled with 5 µmol/L Far-Red (FR) (Invitrogen; cat# C34553). The cell mixture (1:1) was loaded into a 96-well plate with 1,000 target cells per well followed by addition of effector T cells. The background ratio between CFSE- and FR-stained cells was obtained from wells with no effector cells. The ratio between CFSE and FR in wells containing effector cells was obtained from wells with no effector cells. The background ratio between CFSE- and FR-stained cells was obtained from wells with no effector cells. The ratio between CFSE and FR in wells containing effector cells was normalized to the ratio in wells without effector cells. The experiment was performed once in duplicates, and cultures were incubated for 48 hours before analysis. To investigate cytoxicity against MCC cell lines (Waga, MKL-2, and MCC-13), a chromium release assay was performed. The MCPyV-positive MCC cell line Waga was stimulated with INF-γ (Imukin; cat# 001748A; 2,000 U/mL for 72 hours) for expression of HLA class I. No stimulation of the MCPyV-positive MCC cell line MKL-2 and the MCPyV-negative MCC cell line MCC-13 was necessary (Supplementary Fig. S4). Cells (10^5) were stained with 40 µL 5mC31Cr (Perkin Elmer; cat#NE200050MC) for 2 hours and mixed every 15 minutes. T cells specific for A2-LTA/STAKLL (21% CD8 of total cells, 92% specific T cells of CD8) were added to the labeled cells in an actual ratio of 1:1 and incubated for 4 hours. One-hundred microliters of the supernatant was counted in a g-counter (Perkin Elmer precisely, Wizard, 1470 Automatic Gamma Counter). The experiment was performed once in duplicate. MCC cell lines were generated as primary cell lines from patients with a confirmed MCC tumor after surgical removal. Cell lines were analyzed for LTA and STA expression, mycoplasma negativity, HLA expression, and INF-γ-induced expression of HLA.

Statistical analysis
Statistical analysis is performed as described in the result section. Asterisks in the figures indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$).

Results
Generating peptide libraries of HLA ligands derived from LTA, STA, and VP1
To create a library of potential T-cell epitopes derived from LTA, STA, and VP1, we first performed an in silico selection of MHC class I binding peptides based on prediction databases, SYFPEITHI (www.syfpeithi.de; ref. 27) and netMHC (www.cbs.dtu.dk/services/NetMHC; ref. 28). The selected LTA sequence corresponds to a truncated version of LTA lacking the helicase binding domain. Nine-, 10- and 11-mer peptides potentially binding to HLA-A1, -A2, -A3, -A11, and -B7 with prediction values $\geq$1,000 in netMHC were selected for further analyses, i.e., a total of 398 peptides (Supplementary Table S2). All 398 candidate peptides were synthesized and functionally evaluated using an MHC ELISA assay (22, 25). For each peptide, the affinity to the cognate HLA molecule was calculated relative to established T-cell epitopes binding with high affinity to the respective HLA class I molecules. On the basis of an arbitrary-chosen threshold of 60% stabilization efficiency relative to the high-affinity control peptide derived from cytomegalovirus, influenza, or Epstein–Barr virus, we identified medium to high-affinity MHC-binding peptides.

<table>
<thead>
<tr>
<th>Table 1. Selection of MHC-binding MCPyV peptides</th>
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<tr>
<td><strong>HLA control peptide</strong></td>
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<td>HLA-A1</td>
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<tr>
<td>HLA-A2</td>
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<tr>
<td>HLA-A11</td>
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<tr>
<td>HLA-B7</td>
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</table>

Abbreviations: CMV, cytomegalovirus; EBV, Epstein–Barr virus; FLU, influenza.
derived from the MCPyV-encoded proteins (Table 1 and Supplementary Fig. S1). On the basis of these results, 196 selected peptide ligands were subsequently used to screen PBLs obtained from patients with MCC and healthy donors for reactive T cells. Some peptides could bind multiple HLA types and thus we tested 237 different peptide–MHC complexes: 9 for HLA-A1, 59 for HLA-A2, 82 for HLA-A3, 53 for HLA-A11, and 34 for HLA-B7 (Supplementary Table S3).

Multiple MCPyV-derived peptides are recognized by T cells

As an initial step, we prepared phycoerythrin-labeled peptide–MHC multimers for specific enrichment of reactive T cells. T-cell enrichments were performed on PBLs obtained from 38 patients with MCC and 30 healthy donors by capturing phycoerythrin-labeled MHC multimer binding T cells with antiphycocerythin magnetic beads. Previous analyses have established that up to 400 different T-cell specificities can be enriched simultaneously by this procedure (20, 29). After enrichment for MCPyV protein reactivity, T cells were cultured for 3 weeks in the presence of CD3/CD28-coated expander beads, IL-2, and IL-15 before further analysis by combinatorial encoding with peptide–MHC multimers and flow cytometry (21, 23). This method enables the simultaneous detection of 27 different T-cell populations per sample (20). Each specific response was verified by either a second experiment using differently labeled combinatorially encoded peptide–MHC multimers or a secondary enrichment (Fig. 1A).

We detected T-cell responses against LTA-, STA-, and VP1-derived epitopes for all MHC restriction elements included. In total, 53 MCPyV-directed T-cell responses were identified and confirmed in 27 individuals, recognizing 35 different peptide sequences (Table 2). For each specificity, representative examples of peptide–MHC-reactive T-cell populations are depicted in Supplementary Fig. S2. Notably, for several MCPyV-derived epitopes, we detected peptide–MHC-reactive T cells in multiple individuals, including A3-VP1TVL (code shown: HLA restriction, viral protein with subscript of first three amino acids), A3/A11-VP1QMW, -VP1SLF, -VP1LQM, -VP1SLD, A2-LTASMF, A2-LTAQLL, and B7-LTAAPI, indicating that these could be immune-dominant epitopes. MCPyV-specific T-cell responses were present in both patients with MCC and healthy donors, but were more frequently detected in the patient group (21 of 38 patients versus 6 of 30 healthy donors, \( P = 0.006, \) Fisher exact test) and patients further displayed a broader response in terms of number of recognized epitopes (\( P = 0.0007, \) unpaired \( t \) test; Fig. 1B). Indeed, T-cell responses against epitopes of LTA and STA were exclusively detected in patients with MCC (13 of 38) in contrast to none of the healthy donors (\( P < 0.0001; \) Fisher exact test; Fig. 1C). VP1 responses were detected in both groups, but with a tendency of less frequent detection among healthy donors (\( P = 0.08; \) Fisher exact test; Fig. 1C). There was no correlation between LTA- and STA-specific T-cell responses in patients with MCC with the viral status of the tumors (Supplementary Fig. S3). Because LTA- or STA-specific T-cell responses were not present in any of the healthy individuals and MCC samples

![Figure 1. T-cell responses against MCPyV in patients with MCC and healthy donors. A, after T-cell enrichment, specific responses were detected using flow cytometry and combinatorially encoded MHC multimers followed by verification with either a second enrichment (top) or a second MHC multimer detection using a new color code (bottom). Representative examples from 2 patients with MCC showing LTA/STA-directed T-cell responses: MCC#7 B7-LTA/STA\textsubscript{APN} (top) and MCC#8 A2-LTA/STA\textsubscript{ALL} (bottom). B, the number of MCPyV-specific T-cell responses per individual in patients with MCC or healthy donors; every dot represents one individual. Asterisks indicate significant levels, two-tailed unpaired \( t \) test. C, the number of patients with MCC or healthy donors where LTA/STA- or VP1-specific T-cell responses were found. Asterisks indicate significant levels, Fisher exact test.](clincancerres.aacrjournals.org)
may have been classified as MCPyV-negative due to the detection threshold of the method used (5) or may have lost the virus (30), our observation indicates that those patients with MCC with detectable LTA- or STA-specific T-cell responses were exposed to relevant amounts of LTA and/or STA protein, albeit the presence of MCPyV in the tumor could not be confirmed. Data on MCPyV copy numbers were available from 20 patients, but no correlation was found with the number of LTA/STA responses (Pearson correlation \(P = 0.83; R^2 = 0.0025\)). Also, the number of total MCPyV responses and LTA/STA responses were independent of stage of disease on the time of sampling.

**MCPyV-specific T cells have cytotoxic potential**

The next series of experiments addressed the functional activity of LTA- or STA-specific T cells and the clinical relevance of such responses in patients with MCC. Specific CD8 T-cell lines were established for three LTA-derived peptides: A2-LTAKLL, A11-LTAAAF, and B7-LTAAPN.

We scrutinized both the processing of LTA to the relevant peptide epitopes and the effector function of the respective T cells. Table 2 shows the peptides eliciting CD8 T-cell responses in patients with MCC and healthy donors.

<table>
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<th>Antigen</th>
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<th>Healthy donors</th>
<th>HLA restriction</th>
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<td>20</td>
<td>11</td>
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<td></td>
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<tr>
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<td>KTLLETDYCLL</td>
<td>171</td>
<td>11</td>
<td>1/26</td>
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<td></td>
<td>TLETDYCL</td>
<td>172</td>
<td>9</td>
<td>1/26</td>
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<tr>
<td></td>
<td>QLRDSKACI</td>
<td>113</td>
<td>10</td>
<td>1/26</td>
<td>A2</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** Peptides in bold: T-cell epitopes in which processing and presentation are verified (see Fig. 3). MCC and healthy donor column: number of times a response was detected/numbers of investigated HLA-matched individuals.

*aDetected in the same individual.*

NA: Not available. B7 tissue-type was not available for the patients with MCC; they were all tested for B7 responses.

**T-cell Epitopes in MCPyV-Encoded Proteins**

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cells by means of genetically modified K562 cells expressing HLA-A2, -A11, or -B7 transfected with LTA mRNA (Supplementary Fig. S4A), which were used as target cells in cytotoxicity assays. Cytotoxicity was determined using a flow cytometry–based assay comparing the ratio of differentially labeled target cells with nontarget cells after coculture with and without effector T cells. These assays clearly demonstrated that every LTA-reactive T-cell line specifically killed the LTA-transfected but not the mock-transfected K562 cells (Fig. 2A), thus demonstrating LTA processing and presentation in context of MHC class I. However, the clinically relevant question is whether the LTA-specific T cells exert cytotoxicity against MCC. Consequently, we next used HLA-A2–positive MCPyV-positive MCC tumor cell lines (i.e., WaGa and MKL-2) as target cells for the A2-LTAKLL–specific CD8 T-cell line; as control, we used an HLA-matched MCPyV-negative MCC cell line (MCC-13; Fig. 2B). MCC tumors and MCC cell lines are known to downregulate MHC class I expression, which can be reinduced by interferons. Consequently, reinduction or amplification of MHC

Figure 2. Processing and presentation of MCPyV LTA allow T-cell–mediated killing by MCPyV–specific T cells. A, T-cell–mediated killing of HLA-expressing LTA-transfected K562 cells (Supplementary Fig. S4A) detected by a flow cytometry–based cytotoxicity assay. The Y-axis shows percentage-specific lysis at different effector:target ratios with T cells recognizing A2-LTA/STAKLL (left), B7-LTA/STAAPN (middle), and A11-LTS/STAAAF (right). Reactive T-cell populations were enriched to purities ranging from 92% to 95% of CD8 T cells. Circles, K562 HLA+ LTA were stained with CSFE and K562 HLA were stained with FR. Squares, unspecific lysis of HLA mismatched K562 cells stained with CSFE and a second HLA mismatched K562 was stained with FR for identifying the background lysis. Specific lysis was measured and calculated as described in Material and Methods. Each point represents the mean of two replicates and error bars the SEM, n = 1. The asterisk indicates significant levels. One-tailed unpaired t test. B, chromium release assay was performed using the 51Cr-labeled target cells: MCPyV+/HLA-A2+ MCC cell line WaGa preincubated with INF-γ (Supplementary Fig. S4B), MCPyV+/HLA-A2+ MCC cell line MKL-2, and the MCPyV-negative A2+ MCC cell line MCC-13. Target cells were incubated at effector/target cell ratio of 1:1 in duplicates for 4 hours before 51Cr release was measured. Bars represent the mean of two replicates and error bars the SEM, n = 1. Asterisks indicate significant levels, two-tailed unpaired t test. C, dot plot showing A2-LTAKLL- or A2-STAKTL–specific CD8 T cells as detected in TIL cultures from a total of four patients with MCC with MHC multimers (APC/PE-CF594 for A2-LTA/STAKLL and Qdot605/PE-CF594 for A2-STAKTL). The frequency of LTA/STA-specific T cells in CD8 T cells is given.
T-cell Epitopes in MCPyV-Encoded Proteins

Figure 3. Sequence homology of MCPyV-derived CD8 T-cell epitopes within different strains of MCPyV and among other human polyomaviruses. A, circle diagram illustrating the variation within the T-cell epitope sequences of LTA from 88 different strains of MCPyV. The sequences (17%) carried an SNP at amino acid position 20, located in both the A2-LTAKLL and B7-LTAAPN epitope, causing the indicated changes in the amino acid sequence. B, alignment of the MCPyV LTA amino acid sequence (top) with the corresponding amino acid sequences from the 10 other known human polyomaviruses, BKPyV, JCPyV, KIPyV, WUPyV, HPV16, HPV17, HPV9, MVPyV, and STLPyV. Gray boxes, the position of the MCPyV-derived A2-, A11-, and B7-restricted epitopes. Amino acids from the other human polyomaviruses that differ from the MCPyV sequence are highlighted in bold. Below, in each epitope, the anchor residues are in bold and the auxiliary anchor residues are underlined.

class I expression was attained by culture in low doses of interferon before the assay (Supplementary Fig. S4B). These experiments revealed that A2-LTAKLL-specific CD8 T cells efficiently killed the virus-positive MCC cell lines (Waga vs. MCC13, P < 0.001, MRL-2 vs. MCC13, non significant, two-tailed unpaired t test). To date, it is not entirely clear if T-cell responses to solid tumors detected in peripheral blood do indeed reflect ongoing responses in the tumor microenvironment, as there are several immune escape mechanisms operating in the latter (31). Hence, to further substantiate the clinical relevance of such LTA/STA-specific T cells, we analyzed if these would be present among TILs. Notably, when screening TILs from 19 patients with MCC, by combinatorially encoded peptide–MHC multimers using the panel of 35 MCPyV-derived peptides previously found to enable T-cell recognition in peripheral blood (Table 2), we detected A2-LTAKLL-specific CD8 T cells in TILs from 2 patients and A2-STAKLL-specific CD8 T cells from another 2 patients. We found no VP1-directed T-cell responses in TILs. PBL had also been analyzed from 1 of these patients where no specific T cells were identified (Fig. 2C). In conclusion, these observations underscore the clinical relevance of the identified T-cell epitopes for MCC.

T-cell epitopes are shared among different MCPyV strains

To investigate viral strain specificity, we tested if the respective epitopes are encoded by the described MCPyV sequences. Alignment of all 88 MCPyV LTA sequences deposited at the National Center for Biotechnology Information nucleotide database revealed that the three LTA epitopes identified are encoded by most MCPyV strains. The variation within the T-cell epitope sequences is relatively low; only 17% carry a single-nucleotide polymorphism (SNP) at amino acid position 20 located in both the A2-LTAKLL and B7-LTAAPN epitopes, which results in a change from alanine to serine in 14%, to threonine in 2%, and to phenylalanine in 1%. Further, 1% carried a SNP at amino acid position 26 located only in the B7-LTAAPN epitope; this SNP causes a change from asparagine to glutamine. In the region of MCPyV compared with the additional 10 currently described human polyomaviridae (33,34). In the region of the A2-, A11-, and B7-restricted LTA/STA-derived epitopes, we found a minimum of five differences in the amino acid sequence of the T-cell receptor recognition site. Thus, although anchor residues are in some cases shared, this difference makes cross-recognition between the different polyomaviruses highly unlikely (Fig. 3B; ref. 32).

Discussion

Recent clinical achievements in immunotherapy for the treatment of solid tumors confirmed the relevance of the immune system in general and the role of cytotoxic T cells in particular for control of cancer (35–38). Although the impact of cytotoxic T-cell responses for disease control is variable among different cancers, multiple lines of evidence suggest that cellular immune function is unusually important for MCC: (i) intratumoral CD8 lymphocyte infiltration
is significantly associated with improved survival (4). (ii) spontaneous regression, particularly after cessation of immune suppression, is observed even in metastatic MCC (2), and (iii) immune suppression is a strong risk factor for MCC and an independent predictor of diminished survival (3, 39). Indeed, when comparing the relevance of the immune system for disease control between cutaneous melanoma (i.e., the tumor entity for which the best evidence for the impact of therapeutic immune modulation has been reported) and MCC, it should be noted that the ratio of melanoma to MCC in the immune competent host is 60:1, whereas only 6:1 in the immune comprised. The association of MCC with MCPyV explains the explicit immunogenicity of this tumor. Indeed, MCPyV-encoded proteins are likely targets for cytotoxic immune responses as they are both foreign to the host and their expression is necessary to maintain the oncogenic phenotype (10–13).

To date, only a single MCPyV-derived MHC class I-restricted epitope has been described (17), thus impeding specific monitoring of T-cell responses to MCC. Consequently, the purpose of this study was to identify and validate a sufficient number of MCPyV-derived T-cell epitopes for future characterization of spontaneous, modulated, or induced immune responses to MCC. This goal was achieved by means of a high-throughput flow cytometry–based platform whereby CD8 T-cell responses against 35 MCPyV-derived peptide epitopes with a broad range of MHC class I–restriction elements were identified in peripheral blood of patients with MCC. T-cell cultures specific for three peptides derived from the region shared between MCPyV LTA and STA killed HLA-matched LTA-expressing K562 as well as derived peptide epitopes with a broad range of MHC class I–restriction elements were identified in peripheral blood of patients with MCC. T-cell cultures specific for three peptides derived from the region shared between MCPyV LTA and STA killed HLA-matched LTA-expressing K562 as well as MCC cell lines. This observation demonstrates that the respective peptides are indeed presented and processed. Furthermore, we could demonstrate the presence of A2- LTA(341–350) and A2-STA(137–146)–specific CD8 T cells among TILs, indicating the presence of such responses in the tumor microenvironment. T-cell responses in TILs were detected without any prior enrichment strategy of MCPyV-reactive T cells, which may account for the difference in response frequency observed (4 in 19 TIL samples versus 21 in 38 PBL samples, P < 0.05, Fisher exact test). However, it may also reflect the lack of VP1-specific responses in the TILs, possibly due to lack of intratumoral expression of this protein (14), as VP1 responses constitute a large fraction of the T-cell responses observed in the PBL samples. When considering only LTA/STA-directed responses, no significant difference is observed between the two sources of cells (4 in 19 TIL samples versus 13 in 38 PBL samples, P > 0.05, Fisher exact test).

Interestingly, although T cells recognizing the MCPyV-encoded VP1 were present in both patients with MCC and healthy donors, T-cell responses to the oncopgenic proteins LTA and STA were exclusively detected in patients with MCC. This notion is in accordance with recent observations about humoral immune responses, i.e., antibodies to MCPyV capsid antigens are frequently present in the general population, whereas anti-LTA and anti-STA responses are largely restricted to patients with MCC (16). Notably, for patients who have no evidence of disease after therapy (n = 9), LTA/STA T-cell reactivity was apparent in 2 patients on average 3 years after removal of the primary lesion, suggesting the generation of a memory T-cell response.

We observed a tendency toward a lower VP1-directed T-cell response frequency in the group of healthy donors compared with MCC, which may be explained by the fact that our healthy donor cohort was not selected of MCPyV positivity.

MCPyV is currently accepted to be a critical causative agent at least for MCPyV-positive MCC cases. Speculations about the origin of MCPyV-negative MCC cases have been proposed (30, 40). However, advances in detection methods for MCPyV imply that the number of "true" MCPyV-negative MCC is smaller than initially assumed, e.g., using improved PCR-based methods, the MCPyV genome was detected in 100% of the MCC tumors investigated (5). The observation that LTA- and STA-specific T-cell responses are detected exclusively in patients with MCC, irrespective of their virus status, supports the hypothesis that most, if not all patients with MCC have indeed been subjected to LTA/ STA protein expression during the course of their disease. A recent report describes CD4 T-cell recognition of LTA from the IC polyomavirus in healthy donors, with mapping of epitopes in the region of LTA that is truncated in MCC, and thus not included in the present study (41). It is possible that this region of MCPyV LTA may likewise be immunogenic in MCPyV-positive healthy donors.

For 16 patients with MCC, we investigated the presence of MCPyV-specific responses in PBLs directly ex vivo, revealing no MCPyV-specific responses. Because MCPyV-specific responses were detected in most of the samples following T-cell enrichment, these responses are below the detection limit for MHC multimer analyses (0.005% of CD8 T cells). It should be noted that this observation also raises the concern that T cells from the naïve repertoire can also be selected, expanded, and subsequently detected after the peptide–MHC-based enrichment (29). However, the complete lack of responses against LTA/STA in healthy donors in conjunction to the readily detected VP1 response indicates that this is not the case in the current setting.

Evasion of immune responses is one of the hallmarks of cancer (42) and tumor cells exploit numerous of these mechanisms to create a tumor-promoting immune-suppressive microenvironment (4). Indeed, it was recently reported that MCC tumors use a spectrum of immune-evasion strategies to locally suppress T-cell responses by inducing high concentrations of both CD4+ and CD8 Tregs and the expression of PD-L1 and PD-L2 within the tumor microenvironment (43). This immune suppression seems not to be confined to the tumor microenvironment, but extends to antigen-specific T cells in circulation, as these cells display an exhausted phenotypic profile (44). However, to date immunologic studies in MCC were hindered by the limited number of known MCPyV-specific T-cell epitopes. Our present report provides the tools for further in-depth analysis of such mechanisms, covering a broad range of possible T-cell specificities. This is particularly
important with the advent of efficient immune-modulatory therapies such as immune checkpoint blocking antibodies or antibody-targeted cytokine therapy as it allows tracking and monitoring of T-cell responses (45–47). Moreover, the T-cell epitopes described here are of viral origin and—in the case of MCPyV LTA and STA—exclusively recognized in MCC; therefore, they represent an ideal target for immunotherapy. Because these antigens are foreign to the patient’s immune system, it is highly feasible to generate high-affinity T-cell populations that recognize these antigens and subsequently use them in adoptive cell therapies along with immune-modulating therapies.

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

Authors’ Contributions
Conception and design: R. Lynga, P. Nghiem, O. Met, J.C. Becker, S.R. Hadrup
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Lynga, N.W. Pedersen, C.A. Thrue, D. Brans, O. Met, P. Nghiem, J.C. Becker, S.R. Hadrup
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Lynga, C.A. Thrue, O. Met, J.C. Becker, S.R. Hadrup

References

Writing, review, and/or revision of the manuscript: R. Lynga, D. Schrama, O. Met, P. Nghiem, J.C. Becker, S.R. Hadrup
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Schrama, S.R. Hadrup
Study supervision: J.C. Becker, S.R. Hadrup

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T-cell Responses to Oncogenic Merkel Cell Polyomavirus Proteins Distinguish Patients with Merkel Cell Carcinoma from Healthy Donors

Rikke Lyngaa, Natasja Wulff Pedersen, David Schrama, et al.