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Research paper

Viral and tumor antigen-specific CD8 T-cell responses in Merkel cell carcinoma

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ABSTRACT

Merkel cell carcinoma (MCC) is a rare and aggressive cutaneous cancer, which is immunogenic, regardless of the presence of MCPyV (80% of cases). The identification of MCC-specific epitopes recognized by CD8 T cells is crucial to expand the arsenal of immunotherapeutic treatments. Until now, most efforts focused on the identification of virus-specific epitopes, whereas immune responses directed against shared cellular tumor-specific antigens have not been evidenced. In this study, we measured T-cell responses against viral (n = 3) and tumor antigens (n = 47) from TILs derived from 21 MCC tumors. Virus-specific CD8 T-cell responses dominated MCC-specific immune responses, and we identified two new HLA-peptide complexes derived from the LT antigen, located in a region encompassing 3 previously identified epitopes. Finally, we show that MAGE-A3 antigen, frequently expressed by MCC tumors, was recognized by CD8 TILs from a virus-negative MCC tumor and thus could be a target for immunotherapy in this setting.

1. Introduction

Merkel Cell Carcinoma (MCC) is a rare neuroendocrine skin cancer accounting for less than 1% of non-melanoma skin cancers [1]. An increasing number of incident cases is observed since 2000, with 0.7 cases/100,000 person-years in 2013 in the United States [2] and risk factors include age, sun exposure and immunodeficiency [3]. In 2008, Feng et al. discovered the sequence of a new polyomavirus integrated into the genome of 80% of MCCs, suggesting a virus-driven oncogenesis [4]. Indeed the Merkel cell polyomavirus (MCPyV), a small virus with a circular, double-stranded DNA, encodes regulatory viral oncoproteins, the T-antigens [5]. Since its discovery, the MCPyV genome has also been detected in healthy individuals, reflecting a lifelong skin infection [6,7]. However, in MCC patients, MCPyV acquires oncogenic potential after integration of its genome associated with mutations/deletions truncating the C-terminal part of the Large T antigen resulting in a loss of the helicase domain [8]. As a consequence, T-antigens are persistently expressed in the host cells [8–10] and promote oncogenesis by altering the regulation of cell cycle, apoptosis and other cellular pathways involved in cell transformation. On the other hand, approximately 20% of MCC do not harbor MCPyV and are mediated by a non-viral, UV-driven oncogenesis leading to mutations of numerous key oncogenes [11].

MCC is an aggressive tumor, with 5-year survival rates of 51%, 35%, et 14% for localized, nodal and metastatic stages, respectively [12]. Despite the poor prognosis in advanced stages, recent findings underlined the crucial role of cellular immunity in MCC and paved the way for immune-based strategies in MCC patients [13], especially immune checkpoint inhibitors targeting the PD-1/PD-L1 axis which are now considered standard of care in patients with advanced stages [14–16]. Indeed, MCC cases with dense intratumor immune infiltrates, especially cases enriched in CD8 T cells, display improved outcome [17–19].

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Therefore, MCC tumors appear as immunogenic and patients displaying appropriate T-cell responses might be able to control cancer growth. Because most MCC cases are virus-positive, viral antigens are hypothesized to be the dominant trigger of specific immune responses. Until now, 7 MCPyV-specific CD4 [20,21] and 17 MCPyV-specific CD8 T-cell epitopes [20,22] directed against T-antigens were identified in blood and tumors from patients with MCPyV-positive MCCs, although the processing and natural presentation of each of these peptides has not been formally documented for all of these potential epitopes [19]. These findings set the stage for adoptive transfer strategies of MCPyV-specific T cells in MCPyV-positive metastatic MCC patients [23]. However, although associated with improved prognosis [19], intra-tumoral virus-specific T cells do not seem to impact responses to immune checkpoint inhibitors, and globally, there is no current evidence that MCPyV-positive MCC cases show increased immunogenicity or are better candidates for immunotherapies than MCPyV-negative cases [24]. Despite not harboring virus-derived antigens, MCPyV-negative tumors have a high mutational burden, which is associated with increased probability of presenting neoantigens derived from mutated peptides, targetable by the immune system [11]. Nevertheless, the proportion of these predicted neoepitopes actually presented by tumor cells has not been evaluated so far, and although of great interest for personalized immune-based cancer treatment, neoantigens are the result of sporadic mutations, and are thus mostly patient-specific, preventing a systematic use in clinical trials. The presence of immunogenic tumor epitopes, deriving from shared and tumor-specific antigens has never been explored in MCC. The identification of T-cell effectors specific for shared tumor epitopes would be of great interest for immunotherapy of both MCPyV-positive and -negative tumors. In this study, we investigated the presence of tumor-antigen specific CD8 T cells, within tumor-infiltrated lymphocytes (TIL) populations derived from MCC tumors, in comparison with the presence of CD8 T cells specific for virus epitopes. We studied the reactivity of 21 TIL populations to COS-7 cells co-transfected with combinations of cDNA coding for 44 tumor antigens, 3 mutated forms of P53 antigen, reported as potentially associated with progression of a subset of MCCs [25], and 3 viral proteins together with the class I HLA alleles expressed by each patient, according to a method validated before for TILs derived from melanoma tumors [26].

The objectives of this study were to evaluate the relative contribution of virus and tumor-antigen-specific CD8 T cells to MCC immune responses and to characterize new epitopes that would diversify immunotherapy targets in this pathology.

2. Material and methods

2.1. Patients and ethics

Twenty-one MCC patients undergoing surgery for either primary tumor or metastases were recruited prospectively from November 2014 until July 2016 in the Dermatology Departments from 9 French Hospitals (Tours, Angers, Le Mans, Nantes, Orléans, Poitiers, Rennes, Boulogne-Billancourt, Besançon). Cases of MCC were included if histological data confirmed the diagnosis of MCC based on the morphology of the tumor and positive immunostaining for cytokeratin 20 and/or the neuroendocrine markers synaptophysin and chromogranin A. Follow-up was performed as recommended in the National French Guidelines [27] by clinical examination every 3–6 months and imaging [lymph node ultrasonography and/or computed tomography (CT) scan] every 6–12 months. The clinical variables collected included age, sex, type of MCC tumor specimen (primary or metastases). None of the 21 MCC patients was immunosuppressed, as defined by solid organ transplantation, current hematological or solid malignancies or immunosuppressive treatment [28]. The study was approved by the ethics committee of Tours, France (no. ID RCB 2009-A01056-51) and all participants gave written consent.

2.2. Detection of MCPyV markers in tumors and serum of MCC patients

MCPyV status was determined by quantitative PCR on tumor DNA using specific primers of MCPyV LT1 sequences (positive threshold $\Delta CT = 1.2$), as previously described [29]. In addition, serum T-Antigen antibodies were detected by an indirect ELISA in blood samples, as previously described [30]. Briefly, antibody titers were determined by the last three-fold sera dilution (from 1/33 to 1/218700) for which ELISA was positive (cut-off value, optical density 0.2) and considered positive when above dilution 1/100. Clinical characteristics and MCPyV status are summarized in Table S1.

2.3. Tumor Infiltrating Lymphocytes and T cell clones

Tumor Infiltrating Lymphocytes were isolated by culturing mechanically disaggregated tumor fragments in 24-well tissue culture plates with RPMI 1640 (Sigma-Aldrich) containing 8% human serum (local production), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) and 150 U/ml rIL-2 (Eurocetus, Rueil-Malmaison, France) for 10–14 days. These populations were then expanded by a single round of stimulation with PHA-L (Sigma-Aldrich) in the presence of irradiated feeder cells (allogeneic PBMCs and B-EBV B cells), as described [31–33], and followed by positive selection of CD8 T cells on magnetic columns using a CD8 T-cell enrichment kit according to the manufacturer's recommendations (Miltenyi Biotec). For each patient, HLA class I genotypes (A, B, C loci) were determined from 10×10^6 expanded TILs using next generation sequencing (HLA laboratory, EFS, Nantes, France). The CD8 T-cell clones MK1.71b and MK2.J10 were respectively derived from TILs of MK1 and MK2 MCC patients, by limiting dilution and expanded as described above.

2.4. Cell lines

The MCPyV-positive MCC cell lines MKL-1 [34] and MS-1 [35] were purchased from Sigma-Aldrich. The MCPyV-positive MCC cell lines MKL-2 [36], WaGa [37] and PeTa [38] were kindly gifted by D. Schrama and R. Houben (University Hospital Würzburg, Würzburg, Germany). Mouse fibrosarcoma WEHI 164 clone 13, used for TNF production assay, and COS-7 cells were obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). The B-EBV-transformed cell line LAZ 338 was a gift from T. Hercend (Vertex Pharmaceutical, Abingdon, UK), other B-EBV cell lines were a gift from B. Clémenceau (UMR1232, Nantes, France). MCC, WEHI and B-EBV cell lines were cultured in RPMI 1640 medium containing 10% of fetal bovine serum (FBS, Gibco-BRL, France), 100U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (Sigma-Aldrich, France). COS-7 cells were cultured in DMEM medium supplemented with 10% FBS, antibiotics and L-glutamine.

2.5. Tumor-associated antigens (TAA) and HLA cDNAs

Most cDNA coding for TAA (n = 44) were obtained from T. Boon (LICR, Brussels, Belgium). NA88-A, NA17-A and *meloe* were cloned in our laboratory [39–41]. Her-2/neu was a gift from Kostas Kosmatopoulos (UMR484 INSERM, Villejuif, France). EpCAM and Rb pCMV6-XL4 plasmids were purchased from Origene, France. The MCPyV major capsid protein VP1 plasmid was purchased from Addgene, France. The MCPyV LT and ST antigens were cloned in the laboratory of Antoine Touzé (UMR 1282, Tours, France). The cDNAs coding for HLA molecules were obtained from T. Boon (LICR, Brussels, Belgium) and from E. Houssaint (UMR463 INSERM, Nantes, France). All tested antigens and HLA molecules are listed respectively in supplemental Tables S2 and S3. In order to introduce P278S, R248W and Y220C mutations into the wild type P53 antigen, we conducted PCR site-directed mutagenesis on P53 pcDNA vector with PfuUltra high-fidelity DNA Polymerase (Agilent

Technologies), with primers listed in Table S4. PCR site-directed mutagenesis was also used to introduce premature STOP codons in the LT and MAGE-A3 pcDNA vector, to generate truncated fragments of these antigens, with primers listed in Table S4. The presence of mutations on P53 antigen and of STOP codons on LT and MAGE-A3 antigens were confirmed by DNA sequencing.

2.6. Transfection of COS-7 cells and stimulation assay

The COS-7 cells were transfected with cDNA coding for the HLA alleles expressed by each patient and with the cDNA coding for each of tumor-associated or viral antigen, as previously described [26]. Briefly, 1.6×10^4 COS-7 cells were transiently co-transfected with 100 ng of plasmid coding for an HLA and 100 ng of plasmid coding for an antigen, using the DEAE-dextran-chloroquine method. Transfected COS-7 cells were then used to stimulate TILs or TIL-derived clones. In brief, TILs (5×10^5) or T-cell clones (1×10^4) were added to transfected COS-7 cells 48 h after transfection in duplicate. Culture supernatants were harvested 6 h later and tested for TNF content. TNF determination was done by a biological assay measuring the cytotoxicity of culture supernatant on the highly sensitive WEHI 164 clone 13 in a MTT colorimetric assay, as previously described [42].

2.7. Transfection of MCC cell lines

MCC cell lines were transfected, with cDNA coding for HLA-A*1101 or HLA-B*1801 molecules, using Lipofectamine LTX Reagent (ThermoFisher Scientific, France). Briefly, 1 μg of plasmid coding for each HLA molecules were incubated with 2 μl of Lipofectamine LTX Reagent for 20 min in Optimem medium, then, the mixture was added to 5×10^5 MCC cell lines. 48 h after transfection, transfected MCC cell lines were used for T-cell stimulation assay.

2.8. T-cell stimulation assay

Peptides were purchased from ProteoGenix (Schiltigheim, France). Purity (> 70%) was controlled by reversed-phase HPLC. Peptides were lyophilized, dissolved in DMSO at 10 mg/ml, and stored at −80 °C. To identify the recognized minimal peptide, 2×10^5 HLA-matched B-EBV cells were pulsed with a range of each candidate peptide in 96 U well plates for 1 h. 1×10^5 of T cells were then added in the presence of brefeldin A, and co-cultured with peptide-loaded B-EBV cells during 5 h. For the reactivity against MCC cell lines, TILs or T-cell clones (1×10^5) were cultured with MCC cell lines (2×10^5) in the presence of Brefeldin A for 5 h. For both experiments, at the end of the culture period, cells were harvested and stained with CD8-APC specific mAb (Biolegend), then permeabilized with saponin and stained with TNF-α specific mAb (Biolegend). Data were acquired with FacsCalibur cytometer (Becton Dickinson) and analyzed with CellQuest software. Data are reported as percentage of CD8 T cells producing TNF-α within TIL populations.

2.9. RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted from MCC tumors or MCC cell lines using NucleoSpin RNA II kit (Macherey-Nagel, France). 1 μg of total RNA was retrotranscribed using SuperScript III reverse transcriptase and oligodT (Thermo Fisher Scientific, 18080–044 and 18418–020). MAGE A3-specific primers (sense 5′-TGGAGGACCAGAGGCCCCC-3′, antisense 5′-GGACGATTATCAGGAGGCCTGC-3′) were purchased from Eurofins Genomics (France). PCR amplification was performed on 20 ng of the cDNA with PCR buffer, 1.5 mM MgCl2 (Life Technologies, France), 0.8 mM dNTP mix, 1 μM primers, and 0.1U of Platinum™ Taq DNA Polymerase (Invitrogen, France) in a final water volume of 25 μl. Thermal cycling was one step at 95 °C for 10 min, followed by 40 cycles (95 °C for 30 s and 72 °C for 60 s). A total of 10 μl of PCR products was size fractionated on a 1% agarose gel. Expected length was 725 bp for the amplified fragment. Positive control was constituted by cDNA extracted from melanoma M113 cell line, MAGE-A3 positive.

3. Results

3.1. Viral status of MCC patients

We prospectively collected MCC tumor specimens from 21 patients undergoing surgery for either primary or metastatic disease. MCC diagnosis was histologically confirmed and MCPyV status was determined using quantitative PCR on tumor DNA using specific primers of MCPyV LT1 sequences, as previously described [29]. In addition, serum T-Antigen antibodies were detected by an indirect ELISA in blood samples, as previously described [30]. Among the 21 cases, 18 were MCPyV-positive and 3 were MCPyV-negative, according to qPCR and serum T-antigens (Table S1).

3.2. MCC intra-tumoral CD8 T-cell responses are dominated by viral-specific responses

The reactivity of CD8 T cells isolated from expanded 21 TIL populations was tested against COS-7 cells co-transfected with combinations of cDNA coding for a given tumor-associated (n = 47) or viral (n = 3) antigens and each HLA allele of each patient (Tables S2 and S3).

As shown in Fig. 1A, 6 out of 21 Merkel TIL populations (29%) exhibited T-cell specific responses, of whom 5 were directed against the MCPyV LT-antigen. These viral-specific T-cell responses were directed against the truncated LT antigen, in 4 different HLA contexts: HLA-A*1101, B*0801, B*1801 and CW*0602. Among the 47 tumor-associated antigens (TAA) tested, only one tumor antigen-specific response was evidenced in the HLA-B*0801 context, directed against the cancer germline antigen MAGE-A3, from TIL derived from a MCPyV-negative tumor (MK3, Table S1). Fig. 1B illustrates TNF production by specific polyclonal CD8 TIL lines in response to their cognate HLA-Antigen complex. As shown, the amounts of secreted TNF are variable from one TIL line to another, suggesting heterogeneous proportions or reactivity of specific CD8 T cells within these polyclonal TIL populations.

3.3. Identification of two new LT antigen-derived epitopes

To identify new viral epitopes, potentially useful for immune-based therapies, we focused on HLA-A and B restrictions because HLA-C molecules are often expressed at low levels at the cell surface, which prevents the use of peptides restricted by HLA-C for vaccination purposes [43,44]. Among the three HLA-A/B molecules identified, we selected MK1 and MK2 TIL populations respectively restricted by HLA-B*1801 and A*1101 (frequently expressed in Caucasian population (around 10%)), and exhibiting a strong TNF response. We thus cloned these two TIL populations by limiting dilution, and derived CD8 T-cell clones recognizing LT antigen in HLA-A*1101 and HLA-B*1801 contexts (respectively MK2.J10 and MK1.71b T-cell clones). To pinpoint the cDNA regions coding for each epitope, we generated shortened fragments of the truncated LT antigen, that were co-transfected with either HLA-A*1101 or HLA-B*1801 in COS-7 cells. Their recognition by the two derived T-cell clones was tested. As shown in Fig. 2A, the HLA-A*1101 restricted epitope is located in the region spanning between 270 and 564 bp that corresponds to a protein region of 39 amino acids. The HLA-B*1801 restricted epitope is located in the region spanning between 213 and 270 bp, matching that corresponds to a protein region of 20 amino acids illustrated on the Fig. 2B.

Using *in silico* HLA-binding prediction algorithms, we looked for peptide candidates exhibiting good HLA-A*1101 and -B*1801 binding scores, especially those matching the known HLA-binding motifs, i.e. V, I, F or Y at position 2, K or R at position 9 for HLA-A*1101 and E at position 2 and F or Y at position 9 for HLA-B*1801. Recognition of the

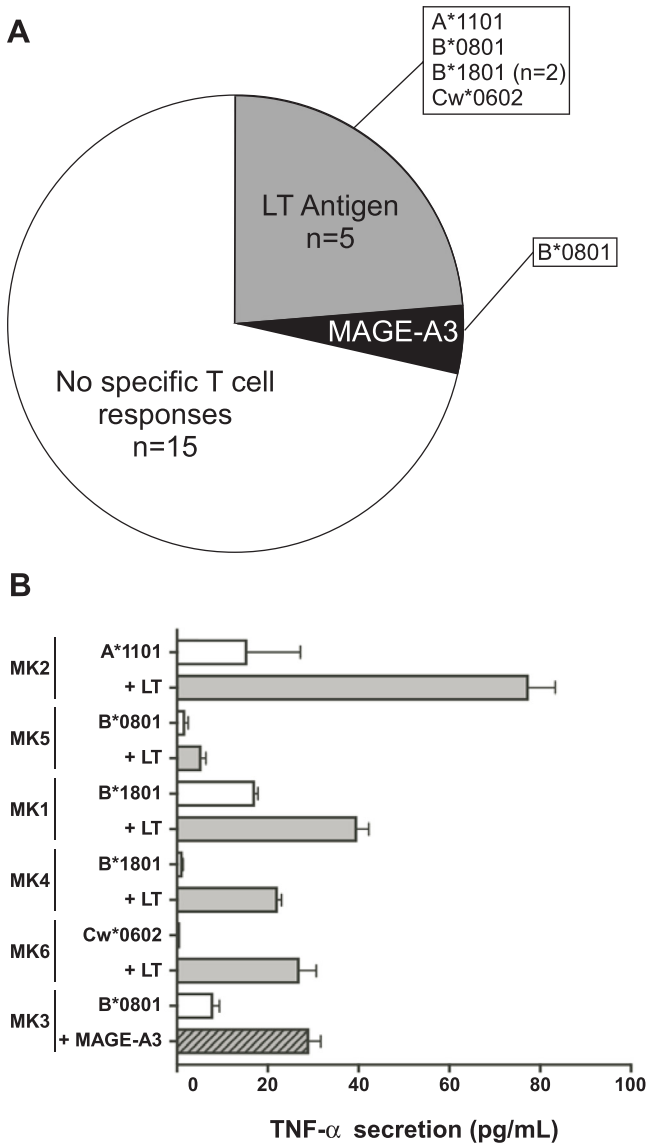

Fig. 1. (A) Relative frequencies of MCC TIL populations recognizing viral and tumor antigens. (B) TNF-α production by the 5 positive MCC TIL populations in response to COS-7 cells co-transfected with a given HLA and LT (grey bars) or MAGE-A3 (hatched bar) antigens. Empty bars correspond to TNF-α production in response to COS-7 cells transfected with the HLA alone.

selected peptides by the T-cell clones was then tested in TNF-α production assays.

In the HLA-A*1101 context, a LT-deriver nonamer peptide (LT_{81-89} EAPIYGTTK), located at the beginning of the recognized region had been predicted to bind with medium affinity to the A*1101 (and also A*0301) molecules [22]. This peptide was not recognized by the MK2.J10 T-cell clone (Fig. 2C), that recognized a nonapeptide (RK-9_{95-103}) and the corresponding decapeptide (RA-10_{95-104}). Peptide titration analysis showed that the best fitting peptide was the nonamer RSGG-FSFGK$_{95-103}$, with a half-maximal lysis at 4.10^{-12}M (Fig. 2C). This peptide, with a K residue at the C-term position, favoring HLA-A*1101 anchoring, had also been predicted to be a strong binder to HLA-A3 molecules, although no specific T-cell responses had been reported so far in these two restrictions [22].

The MK1.71b T-cell clone recognized the nonapeptide DY-9_{77-85} (DEVDEAPIY) in the HLA-B*1801 context, with a half-maximal lysis of 4.10^{-11}M (Fig. 2D). The overlapping peptides FI-9_{76-84} (FDEVDEAPI) and EG-9_{78-86} (EVDEAPIYG) were not recognized by this T-cell clone. We further showed that this peptide is also recognized by the other positive B*1801 Merkel TIL population MK4, with 16% of CD8 specific T cells, as illustrated on Fig. 2E.

3.4. MCPyV-derived peptides recognized by TIL clones from MCC patients are efficiently presented by Merkel cell lines

To assess the potential interest of the epitopes recognized by these T-cell clones as targets for immunotherapy, we analyzed their presentation by a panel of five MCPyV-positive Merkel cell lines (WAGA, MS1, MKL-1, MKL-2, PETA). None of these cell lines express the HLA B*1801 molecule and only MKL-1 expresses HLA-A*1101. Thus, these cells were transfected (or not) to express the appropriate HLA allele, and treated with IFN-γ for 48 h to enhance HLA expression. As shown, transfected MCC cell lines induced TNF-α production by MK2.J10 and MK1.71b (Fig. 3A and B, lower panels). It is noteworthy that the MKL1 cell line, which naturally expresses the HLA-A*1101 molecule, was spontaneously recognized by MK2.J10, after IFN-γ treatment (Fig. 3A, upper panel). From these data, we conclude that the identified peptides, presented in HLA-A*1101 and HLA-B*1801 contexts, were naturally processed by MCC cell lines.

3.5. MAGE-A3 tumor-specific antigen is recognized by TIL derived from MCC patients

As shown in Fig. 1, among the 21 TIL populations tested against 47 TAA, we observed one specific TIL population recognizing MAGE-A3, a cancer/germline associated antigen in the HLA-B*0801 context. Interestingly, this TIL population originates from a virus-negative tumor (MK3, Table S1). We investigated the expression of this antigen by RT-PCR on MCC cell lines and on tumors. All MCC cell lines and 14/16 MCC tumors (among them the MK3 tumor, lane 4) were positive for MAGE-A3 (Fig. 4A). We then confirmed the recognition of COS-7 cells co-transfected with HLA-B*0801 and MAGE-A3 by the specific TIL population, through intracellular TNF labeling, from which we were able to estimate the percentage of MAGE-A3 specific T cells in this population (1%, Fig. 4B). We further restricted the minimal region coding for the potential peptide to a fragment spanning 636 bp and 705 bp, corresponding to a long-peptide of 30 aa (204–234) (Fig. 4C). We tested various overlapping 15-mer and 9-mer peptides according to HLA-binding prediction algorithms (Table S5). None of these peptides were recognized by the specific TIL population, after loading on a HLA-B*0801 presenting B-cell line, suggesting a post-translational modification (such as peptide splicing as described before for a gp100-derived epitope [45]). This particularity prevented us from going further in the precise identification of the minimal epitope. Nonetheless, this is the first evidence of a T-cell response directed against a common tumor-associated antigen in MCC.

4. Discussion

We performed a systematic screening of the viral and tumor-associated antigens recognized by 21 Merkel cell carcinoma TIL populations using COS-7 cells co-transfected with one of the tumor (n = 47) or viral (n = 3) antigens, and individual HLA class I alleles expressed by the TIL populations. We previously validated this method through the comprehensive analysis of the specificity of melanoma-derived CD8 T-cell populations [26].

Six out of 21 TIL populations contained CD8 T cells that recognized antigens: 5 specific for LT antigen and one for MAGE-A3. It is interesting to note that no specific T-cell responses were detected against VP1 and ST antigens in our study whereas in a previous study, epitopes from these proteins were shown to elicit specific CD8 T-cell responses from MCC patients' PBMC in various HLA contexts; however processing and presentation of these potential T-cell epitopes was not assessed [22]. Our results suggest that LT antigen may be more ST antigen.

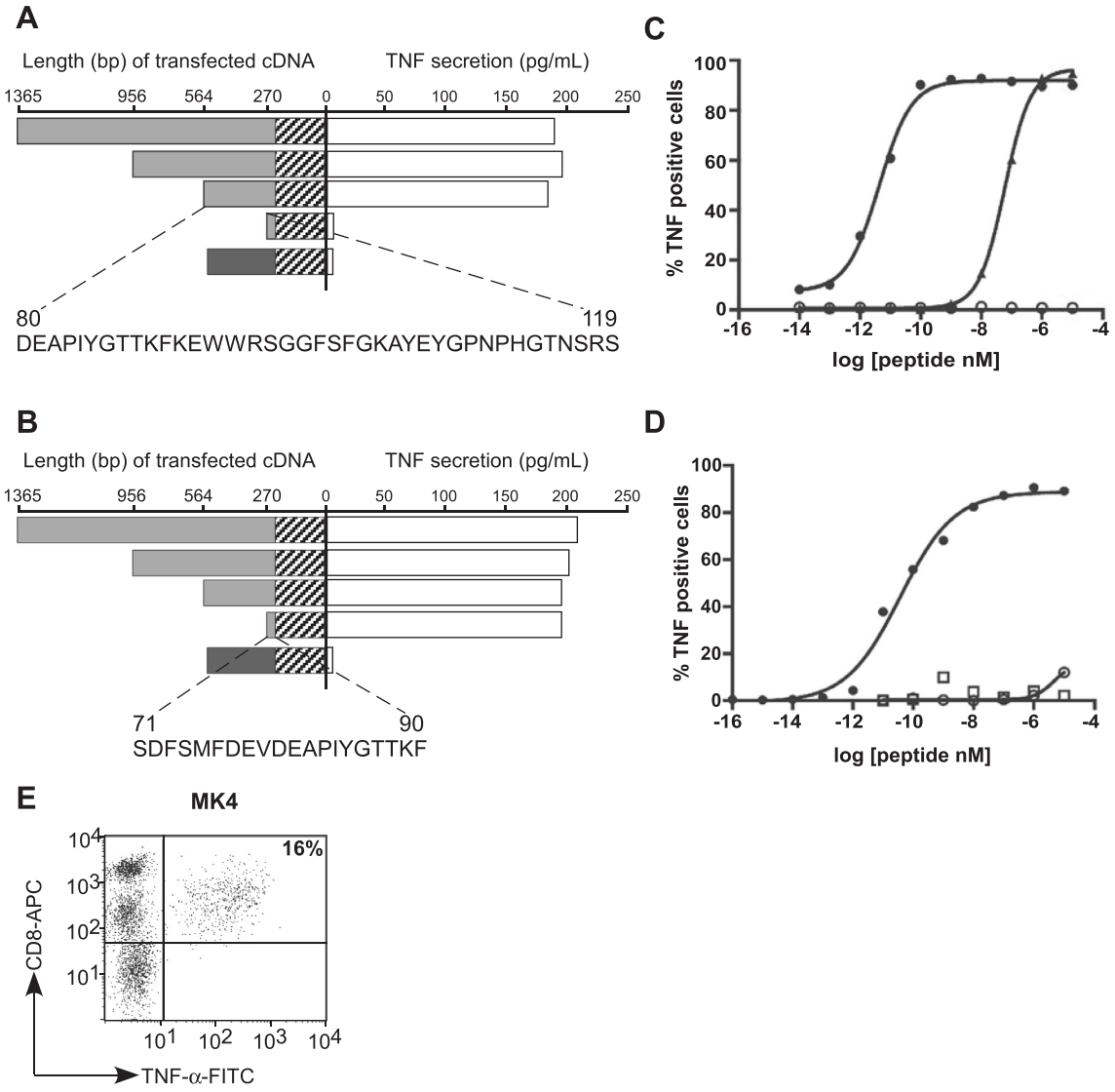

Fig. 2. TNF-α secretion (empty bars) by MK2.J10 (A) and MK1.71b (B) T-cell clones in response to COS-7 cells co-transfected respectively with HLA-A*1101 and B*1801, and full-length LT and ST antigens or truncated constructs of the LT antigen. Hatched segments correspond to the region shared by ST and LT antigens. Grey segments correspond to the LT antigen specific sequence and black segment to ST specific sequence. The minimal recognized peptidic region is indicated on each figure. (C) TNF-α production by MK2.J10 T-cell clone in response to HLA-A*1101 B-EBV cell line pulsed with a range of the peptides $LT_{81\text{-}89}$ (empty circles), $LT_{95\text{-}103}$ (black circles) and $LT_{95\text{-}104}$ (black triangles). (D) TNF-α production by MK1.71b T-cell clone in response to HLA-B*1801 B-EBV cell line pulsed with a range of the peptides $LT_{76\text{-}84}$ (empty circles), $LT_{77\text{-}85}$ (black circles) and $LT_{78\text{-}86}$ (empty squares). (E) TNF-α production by B*1801 MK4 TIL population, detected by intracellular labeling, after 5 h of incubation with $LT_{77\text{-}85}$ peptide.

Overall, antigen-specific T-cell responses were restricted by only 4 HLA contexts among 27 tested (Table S3). This diversity is much lower than that previously observed in melanoma, with antigen-specific responses detected in 14 different HLA contexts [26]. This could be potentially due to the reported transcriptional loss of HLA expression by MCC tumors due to specific T-cell pressure [46]. Furthermore, among the 18 virus-positive tumors, LT-specific TIL responses were observed in 4 different HLA contexts: HLA-*1101 (1/2), B*0801 (1/4) HLA-B*1801 (2/2) and HLA-Cw*0602 (1/4) (Fig. 1, and Tables S2 and S3).

Notably, it is intriguing that no responses were observed in the HLA-A2 context, which is expressed by 12 out 21 MCC patients. In a previous study, 12 potential viral peptides were shown to elicit CD8 T-cell responses in the HLA-A2 context, from patient blood, by tetramer labeling. Nonetheless, only two of these peptides, derived from the LT antigen have been proven to be effectively processed, presented and recognized by two MCC-derived HLA-A*0201 TIL populations among 26 HLA-A2 TIL populations tested. [22,47]. This low frequency (2/26) could explain the absence of detection of HLA-A2 specific TIL responses in our study, performed only on 12 HLA-A2 TIL populations.

This also raises the question of the activation status of MCC-derived TILs. Indeed, our screening method relies on TNF production by specific T cells, not only their presence as non-activated T cells (that can be detected by tetramer labeling). It is well known that T cells can become dysfunctional upon persisting stimulation, for example by virus-infected cells [48]. The sustained expression of viral T-antigens in MCC, therefore may similarly induce a state of exhaustion in virus-specific T cells, leading to a low cytokine production, as previously shown for MCC-derived TILs [49]. Thus specific T-cell responses detected in our study may represent the fraction of not yet exhausted T cells.

The interesting advantage of the COS-7 screening method is that the full-length antigen-coding cDNA is transfected into COS-7 cells, and thus the presentation of the appropriate HLA/peptide complex involves the natural processing of the epitope. Therefore, the identification of immunogenic peptides does not rely exclusively on epitope prediction algorithms, potentially leading to non naturally processed peptides, and generally biased towards the selection of strong binder peptides.

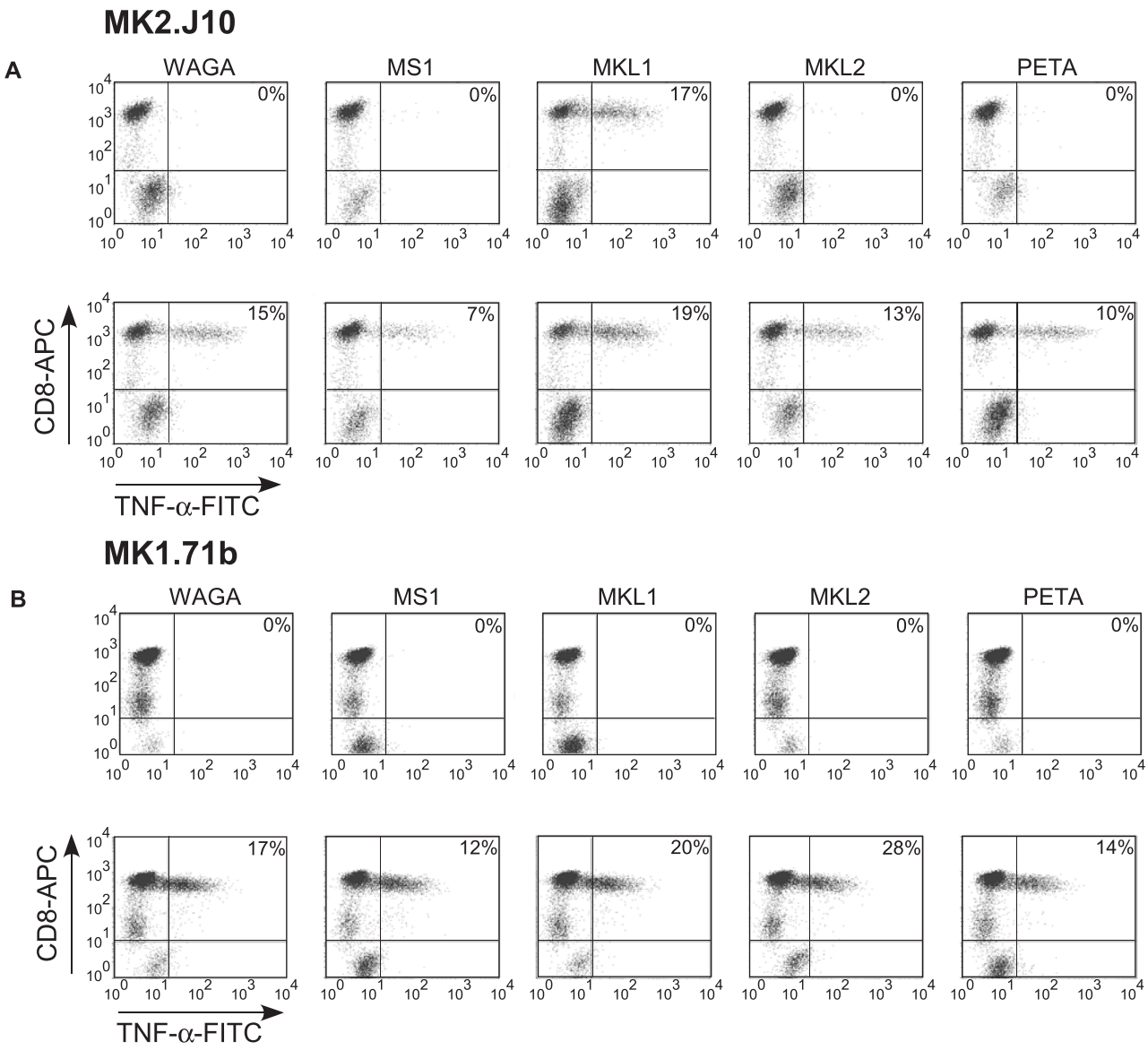

Fig. 3. TNF-α production by MK2.J10 (A) and MK1.71b (B) in response to untransfected (upper dot plots) and to HLA-transfected (lower dot plots) MCC lines. The percentage in the upper right quadrant indicates the fraction of CD8 T-cell clone producing TNF-α in response to MCC lines.

Based on these results, we fully characterized two new LT-derived epitopes, naturally presented by MCC cell lines and recognized in HLA-A*1101 and B*1801 contexts, expressed by around 10% of the Caucasian population. Interestingly, the peptide presented in the HLA-A*1101 context ($LT_{95\text{-}103}$ RSGGFSFGK) is largely overlapping with the peptide $LT_{92\text{-}101}$, recognized in the HLA-A*2401 context by MCC-specific CD8 T cells [20]. Located in the same region of the LT antigen, the other newly identified epitope ($LT_{77\text{-}85}$ DEVDEAPIY) recognized in the B*1801 context is also overlapping with two epitopes previously identified from patient blood ($LT_{74\text{-}84}$ SMFDEVDEAPI (A2 context) and $LT_{82\text{-}90}$ APIYGTTKF (B7 context)) [22]. These two new peptides enrich the small group of peptides whose processing and natural presentation has been validated.

Overall, this suggests that the region between 74 and 103 aa from the LT antigen is particularly rich in immunogenic epitopes and that vaccination approaches with synthetic long peptides covering this region could be a relevant approach for immunotherapy purposes.

To date, only MCPyV-directed T-cell responses have been evidenced in MCC patients [20,22] whereas no data are available regarding the presence and the relative frequencies of non-virus-directed T-cell responses. We tested the recognition of 47 shared TAA (among them 3 mutated forms of P53 antigen) by CD8 T cells derived from 21 MCC tumors. Only one response was detected against MAGE-A3 antigen from the unique virus-negative HLA*B*0801 TIL population (among 5 tested). This is the first evidence of the recognition of a cellular antigen in Merkel carcinoma. This antigen was frequently detected by qPCR in MCC tumors (14/16), however its use as an alternative target for immunotherapy purposes for MCPyV-negative MCC tumors appears unlikely at the moment, because of the low frequency of specific responses detected and restricted only by HLA-B*0801 molecule so far. Unfortunately, although we succeeded in narrowing down the recognized fragment to 31 aa, we could not identify the minimal epitope. As we tested all the possible overlapping peptides covering this region, we hypothesized that the recognized peptide could derived from proteasome-induced splicing events, as previously described for a gp100-derived epitope [45].

The low frequency of cellular antigen-specific responses reported in this study does not exclude the presence of CD8 T cells specific for mutated antigens, not evaluated in our study (with the exception of 3 mutated P53 isoforms, previously frequently described in MCC tumors

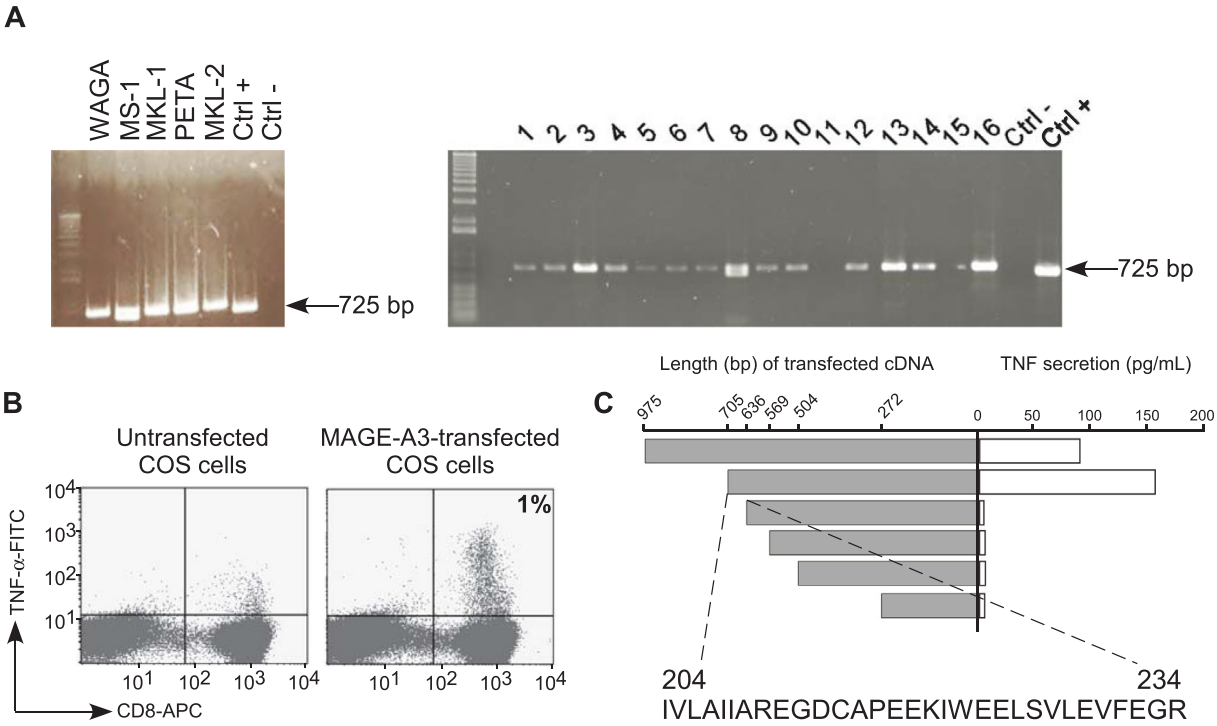

Fig. 4. (A) MAGE-A3 expression in 5 MCC lines (left) and 16 MCC tumors (right) measured by RT-PCR, Lane 4 = MK3 tumor, Ctrl + = MAGE-A3 positive melanoma cell line. (B) TNF-α production, detected by intracellular labeling, by MK3 TIL population in response to COS-7 cells transfected either with HLA-B*0801 alone (left) or HLA-B*0810 and MAGE-A3 antigen (right). The percentage indicates the fraction of CD8 T cells producing TNF-α. (C) TNF-α secretion (empty bars) by MK3 TIL population in response to COS-7 cells co-transfected with HLAB*0801 and full-length or truncated constructs of the MAGE-A3 antigen. The minimal recognized peptidic region (204–234) is indicated on the figure.

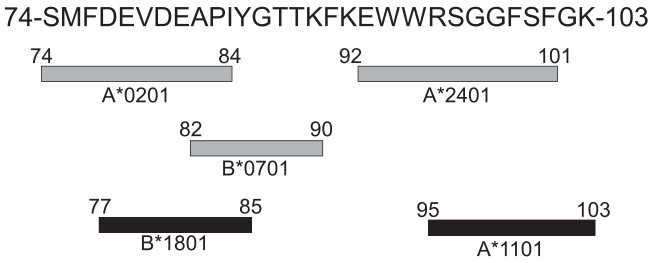

Fig. 5. Summary of immunogenic epitopes identified from the large T antigen in the protein region 74–103, with the two newly identified epitopes (black boxes).

(Table S4)) [11]. Indeed, mutations have been described in oncogenes such as *HRAS*, *PRUNE2* and *NOTCH* in MCPyV-negative MCC (globally characterized by a high overall mutation load, compared to virus-positive ones), that could potentially lead to the expression of immunogenic epitopes [50].

In summary, our study shows that viral-specific CD8 T-cell responses are dominant within MCC-derived TIL populations, compared to TAA-specific responses, and that the use of a screening method that detects functional T cells could lead more directly to the characterization of relevant epitopes for immunotherapy. In terms of therapeutic application, the use of a synthetic long peptide (SLP) covering a region of 29 aa (74–103) of the LT antigen, could be an interesting approach for vaccination purposes, as it contains 5 peptides recognized by CD8 T cells in diverse and frequently expressed HLA contexts (Fig. 5). For MCPyV negative MCC tumors, immunotherapy approaches targeting MAGE-A3 antigen should be further explored.

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Declaration of Competing Interest

The authors declare no conflict of interest of this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103961.

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