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BK Polyomavirus–Specific CD8 T-Cell Expansion In Vitro Using 27mer Peptide Antigens for Developing Adoptive T-Cell Transfer and Vaccination

Maud Wilhelm,[1,a] Amandeep Kaur,[1,a] Marion Wernli,[1] and Hans H. Hirsch[1,2,3]

[1]Transplantation and Clinical Virology, Department of Biomedicine, University of Basel, Basel, Switzerland, [2]Clinical Virology, Laboratory Medicine, University Hospital Basel, Basel, Switzerland, and [3]Infectious Diseases and Hospital Epidemiology, University Hospital Basel, Basel, Switzerland

Background. BK polyomavirus (BKPyV) remains a significant cause of premature kidney transplant failure. In the absence of effective antivirals, current treatments rely on reducing immunosuppression to regain immune control over BKPyV replication. Increasing BKPyV-specific CD8 T cells correlate with clearance of BKPyV DNAemia in kidney transplant patients. We characterized a novel approach for expanding BKPyV-specific CD8 T cells in vitro using 27mer-long synthetic BKPyV peptides, different types of antigen-presenting cells, and CD4 T cells.

Methods. Langerhans cells and immature or mature monocyte-derived dendritic cells (Mo-DCs) were generated from peripheral blood mononuclear cells of healthy blood donors, pulsed with synthetic peptide pools consisting of 36 overlapping 27mers (27mP) or 180 15mers (15mP). BKPyV-specific CD8 T-cell responses were assessed by cytokine release assays using 15mP or immunodominant 9mers.

Results. BKPyV-specific CD8 T cells expanded using 27mP and required mature Mo-DCs ($P = .0312$) and CD4 T cells ($P = .0156$) for highest responses. The resulting BKPyV-specific CD8 T cells proliferated, secreted multiple cytokines including interferon γ and tumor necrosis factor α, and were functional ($CD107a^+/PD1^-$) and cytotoxic.

Conclusions. Synthetic 27mP permit expanding BKPyV-specific CD8 T-cell responses when pulsing mature Mo-DCs in presence of CD4 T cells, suggesting novel and safe approaches to vaccination and adoptive T-cell therapies for patients before and after kidney transplantation.

Keywords. BK virus; polyoma; T cells; peptides; antigen-presenting cells; vaccine.

In the current clinical pretolerance era, the success of kidney transplantation is demonstrated by allograft survival rates of up to 97% in the first year posttransplantation [1, 2] but depends on immunosuppressive therapies [3] to avert acute and chronic immunological injury resulting from T-cell–mediated and antibody-mediated rejection [4]. The unspecific nature of the standard immunosuppression protocols not only affects antidonor immunity but also impairs antiviral immune control, increasing the risk for viral complications [5], which may impact renal allograft survival rates [1, 2]. BK polyomavirus (BKPyV) plays a unique role in kidney transplantation as it continues to directly and indirectly contribute to premature allograft failure [6–9]. Prospectively collected data from the multicentric Swiss Transplant Cohort Study on 2761 patients undergoing solid organ transplantation included 1612 kidney and 73 kidney-pancreas recipients, who experienced 572 (26%) clinically relevant viral infectious disease events in the first 12 months posttransplantation [10]. BKPyV DNAemia was diagnosed in 275 of the 1685 (16%) recipients including 99 (6%) cases of presumptive and proven BKPyV nephropathy, in line with earlier studies [8, 11–16].

BKPyV is a nonenveloped double-stranded DNA virus infecting >90% of the human adult population without known illness and thereafter persists in the renourinary tract [17, 18]. Despite BKPyV-specific T cells [19–21] and neutralizing antibodies [14, 22, 23], asymptomatic low-level urinary virus shedding occurs in 10% of immunocompetent healthy individuals [24], indicating the ability of BKPyV to at least transiently escape from immune control [25, 26]. Following kidney transplantation, renourinary BKPyV replication increases in rate and magnitude, progressing to high-level viruria with decoy cell shedding and urine viral loads of >10 million copies/mL in

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[a]M. Wi. and A. K. contributed equally to this work.

Correspondence: Hans H. Hirsch, MD, MSc, Transplantation and Clinical Virology, Department of Biomedicine, University of Basel, Petersplatz 10, CH-4009 Basel, Switzerland (hans.hirsch@unibas.ch).

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20%–40%, BKPyV DNAemia in 10%–20%, and biopsy-proven nephropathy in 1%–15% [9, 11, 27, 28], and may even progress to urothelial cancer [29, 30].

Although several risk factors have been linked to BKPyV DNAemia and nephropathy, impaired BKPyV-specific antiviral immune control appears to be the common denominator [9, 22]. Conversely, reducing immunosuppression has been associated with regaining immune control over BKPyV replication while increasing the risk of allograft rejection [20, 31–35]. In an initial characterization of BKPyV-specific CD4 and CD8 T-cell responses and clearance of BKPyV replication [20], we observed that CD8 T-cell responses were higher to the nonstructural early viral gene region–encoded large tumor antigen (LTag) [20], whereas CD4 T-cell responses were more frequently directed toward the late viral gene region–encoded major viral capsid protein Vp1 and correlated with Vp1 antibody titers [36]. We have therefore focused on BKPyV-specific CD8 T cells as key cytotoxic effectors targeting virus-replicating host cells using a pool of 97 bioinformatically predicted immunodominant 9mers (9mP) [21]. Importantly, clearance of BKPyV DNAemia was associated with increased CD8 T-cell responses to the immunodominant 9mP and included confirmation of 78 different 9mers presented by several HLA types [37], including those associated with protection from BKPyV-DNAemia in kidney transplant recipients [38–40]. Here, we investigated the role of synthetic peptide pools, antigen-presenting cells (APCs), and CD4 T cells for expanding BKPyV-specific CD8 T cells in vitro as a first step to develop novel and safe approaches to vaccination and adoptive T-cell therapies for patients undergoing kidney transplantation.

MATERIALS AND METHODS

Healthy Blood Donors

Fresh buffy coats from 30 donors (Supplementary Table 1) were obtained from the blood donation center in Basel, Switzerland. Peripheral blood mononuclear cells (PBMCs) were cryopreserved as described [21]. Human leukocyte antigen (HLA) typing was performed by next-generation sequencing using TruSight-HLA version 2 sequencing Panel (Illumina, San Diego, California) and Miniseq high-output reagent kit (300 cycles) (Illumina, FC-420–1003). BKPyV-Vp1-VLP–specific immunoglobulin G antibody status was determined using a normalized enzyme-linked immunosorbent assay as described previously [41].

BKPyV EVGR-Derived Peptides and Cell Culture

All peptides were >70% pure and dissolved in dimethyl sulfoxide (DMSO; 10 mg/mL, Eurogentec, Seraing, Belgium). PBMCs were isolated on a Ficoll density gradient (Lymphoprep, Alere Technologies AS, Wädenswil, Switzerland, 1114545). $CD14^+$ monocytes were sorted using CD14 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, 130-050-201) and differentiated into immature monocyte-derived dendritic cells (imMo-DCs) in 7 days using monocyte-derived dendritic cell (Mo-DC) differentiation medium (Miltenyi-Biotec, 130-094-812). In brief, $CD14^+$ cells were plated at 1×10^6 cells/mL in 6-well plates and half of medium (1.5 mL) was changed on day 3. For maturation of Mo-DCs, RPMI medium (Sigma-Aldrich, St Louis, Missouri, R2405) supplemented with fetal calf serum (10%, Biochrom, Berlin, Germany, S0115), glutamine (2 mM, Bioconcept, Allschwil, Switzerland, 10K50-H), and tumor necrosis factor alpha (TNF-α; 100 ng/mL, Miltenyi-Biotec, 130-094-015) was used and 1×10^6 cells/mL were plated in 24-well plates during 3 additional days. Monocyte-derived Langerhans cells (Mo-LCs) were differentiated from $CD14^+$ cells in 6 days using RPMI medium supplemented with transforming growth factor β (10 ng/mL, Miltenyi Biotec, 130-095-067), granulocyte macrophage colony-stimulating factor (GM-CSF; 100 ng/mL, Miltenyi Biotec, 130-095-372), and interleukin 4 (IL-4; 10 ng/mL, PeproTech, London, United Kingdom, 200–04). On days 2 and 4, half of medium (500 µL) was changed with medium without IL-4.

Nine-Day Expansion of T Cells

APCs were pulsed with 27mP (1 µg/mL) or 15mP (0.5 µg/mL) for 4 hours at 37°C/5% carbon dioxide. After washing, APCs were added to autologous $CD14^-$ cells in a 1:10 ratio and cocultured for 9 days. On day 4, interleukin 2 (20 U/mL, PeproTech, 200–02) and interleukin 7 (5 ng/mL, PeproTech, 200–07) were added. On day 9, the cells were analyzed.

Enzyme-Linked Immunosorbent Spot Assay

BKPyV-specific T-cell responses were determined by measuring the number of IFN-γ–secreting cells using IFN-γ enzyme-linked immunosorbent spot assay (ELISpot), as described previously [20, 21, 37]. In brief, expanded T cells were rechallenged with BKPyV-specific peptides (15mP or 9mP, 0.5 µg/mL) for 18–24 hours. Cells stimulated with phytohemagglutinin-L (PHA, 1 µg/mL, Roche Diagnostics, Rotktreuz, Switzerland) were used as positive control and unstimulated cells were used as negative control. ELISpot data are averaged of duplicates after subtracting the background (negative control).

Flow Cytometry Analysis

Expanded cells were rechallenged with 15mP or 9mP (0.5 µg/mL, Eurogentec) for 6 hours in the presence of Golgi stop (Becton Dickinson [BD], Franklin Lakes, New Jersey, 554715). Cells incubated with RPMI medium alone served as negative control and cells stimulated with staphylococcus enterotoxin B (3 µg/mL, Sigma-Aldrich, S4881) served as positive control.

For extracellular staining, the following antibodies were used: CD14 (BD, 555399), CD209 (Miltenyi Biotec, 130-099-707), HLA-DR (BioLegend, San Diego, California, 307617), CD86 (BioLegend, 305426), CD83 (BioLegend, 305326), CD4 (BD, 562424), CD8 (BioLegend, 301012), CCR7 (BD, 561144), CD45RA (BD, 550855), PD1 (BD, 557946), CD69 (BD, 555530), CD107a (BD, 561348), and phycoerythrin-labeled streptamer (IBA, Hamburg, Germany). For intracellular staining, the following antibodies were used: CD207 (BD, 564727), CD1a (BD, 563939), IFN-γ (BD, 341117), and TNF-α (BD, 554512). Acquisition was done on a Fortessa cytometer (BD) and analyzed using FlowJo version 10.6.1 software.

Carboxyfluorescein Diacetate Succinimidyl Ester Proliferation Assay

$CD14^-$ cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE, 0.25 μM, Invitrogen, Carlsbad, California, 0850-84) before being expanded with autologous 27mP pulsed mature monocyte-derived dendritic cells (mMo-DCs) and analyzed by flow cytometry on day 9.

Cathepsin Inhibitor I and Lactacystin Treatment

Mature Mo-DCs were pulsed with 27mP (1 μg/mL) for 4 hours in the presence or absence of cathepsin inhibitor I (10 μM, Millipore, Burlington, Massachusetts, 219415) or lactacystin (2.5 μg/mL, Millipore, 426100). As solvent control, cells were incubated with DMSO and peptides. Cell viability was measured using trypan blue (Bio-Rad, Hercules, California, 1450013) after the treatment.

T2 Binding Assay

TAP-negative B X T hybrid cell line 174 X CEM.T2 and HLA-$A2^+$ (T2 cells, ATCC-CRL-1992) were used for HLA-peptide stabilization assay. One hundred thousand T2 cells were incubated overnight either with 9m579, 9m227, 15m577, or 27m570 peptides (10 μg/mL) in a 96-well U-bottom plate with RPMI medium at 37°C. After incubation, cells were washed with phosphate-buffered saline (PBS) and assessed for surface major histocompatibility complex class I expression by flow cytometry using HLA-A2 antibody (eBioscience, San Diego, California, 25-9876-42).

Killing Assay

Expanded BKPyV-specific T cells labeled with CFSE were used as effector cells and autologous PHA blasts stained with CellTrace Violet (Invitrogen, C34557) were used as target cells. PHA blasts were pulsed with 9mP (2 μg/mL) overnight and co-cultured with expanded T cells for 6 hours at different target:effector cell ratios. Target cells without effector cells were used as control. Autologous PBMCs stained with CellTrace Far Red (Invitrogen, C34564) were used for the normalization and acquisition of target and effector cells using flow cytometry. Specific killing was then calculated as 100 – (100 × [normalized events in sample / normalized events in control sample]).

Statistical Analysis

Data were analyzed with GraphPad Prism software version 8.3.1. Paired and nonparametric Wilcoxon tests were used. One-sided *P* values of <.05 were considered statistically significant.

RESULTS

Generation of APCs and Impact of CD4 T-Cell Depletion on BKPyV-Specific CD8 T-Cell Responses

A pool of 36 27mers (27mP) covering the entire BKPyV LTag protein was compared with a pool of 180 LTag 15mers (15mP; Figure 1A), which permitted to expand BKPyV-specific CD8 and CD4 T cells from healthy donors and kidney transplant recipients [20]. To investigate the role of different types of APCs in response to the longer 27mP, we differentiated from PBMCs of healthy blood donors, namely Mo-LCs (Figure 1B, top), imMo-DCs, and mMo-DCs (Figure 1B, bottom). The respective APCs were pulsed with 27mP or 15mP and cocultured with autologous $CD14^-$ PBMCs for 9 days followed by ELISpot or flow cytometry using 15mP or 9mP to assess the T-cell responses (Figure 1C).

First, we evaluated the role of CD4 T cells by pulsing imMo-DCs with 15mP followed by co-culture either with $CD14^-$ cells or $CD14^-$ cells after CD4 depletion. The results of the IFN-γ ELISpot assay indicated that the restimulation responses to the overlapping 15mP and the immunodominant 9mP were reduced in case of CD4 depletion (Figure 1D, left panel). The flow cytometry results indicated that this also affected BKPyV-specific CD8 T-cell responses (Figure 1D, right panel). We concluded that even in the case of the 15mP, CD4 T cells contributed to expanding BKPyV-specific CD8 T-cell responses.

BKPyV-Specific T-Cell Responses to LTag-Specific 27mP in imMo-DCs

To investigate the BKPyV-specific T-cell responses to the 27mP, imMo-DCs were pulsed either with 27mP or with the 15mP or mock-treated as controls followed by a 9-day co-culture with $CD14^-$ cells. By ELISpot assay, 15mP rechallenge induced significantly higher IFN-γ responses following both 15mP or 27mP pulsing compared to mock-treated cells (Figure 2A, left panel). By flow cytometry, a significant increase in IFN-γ–producing CD4 T cells was observed for both 15mP- or 27mP-pulsed imMo-DCs (Figure 2A, middle panel). Similarly, significant increases were observed for IFN-γ–producing CD8 T cells after 27mP expansion, although overall responses appeared weaker than the corresponding CD4 T-cell responses (Figure 2A, right panel). To more directly test the CD8 T-cell responses, we restimulated the 9-day co-cultures with the 9mP (Figure 2B). Overall, ELISpot and flow cytometry indicated significantly increased 9mP-dependent responses ($P = .0625$ and $P = .0312$, respectively) as illustrated in Figure 2C. Analysis of nonpulsed imMo-DCs indicated that the IFN-γ responses were dependent on pulsing with the respective peptide pools, as expected (Supplementary Figure 1). We concluded that BKPyV-specific

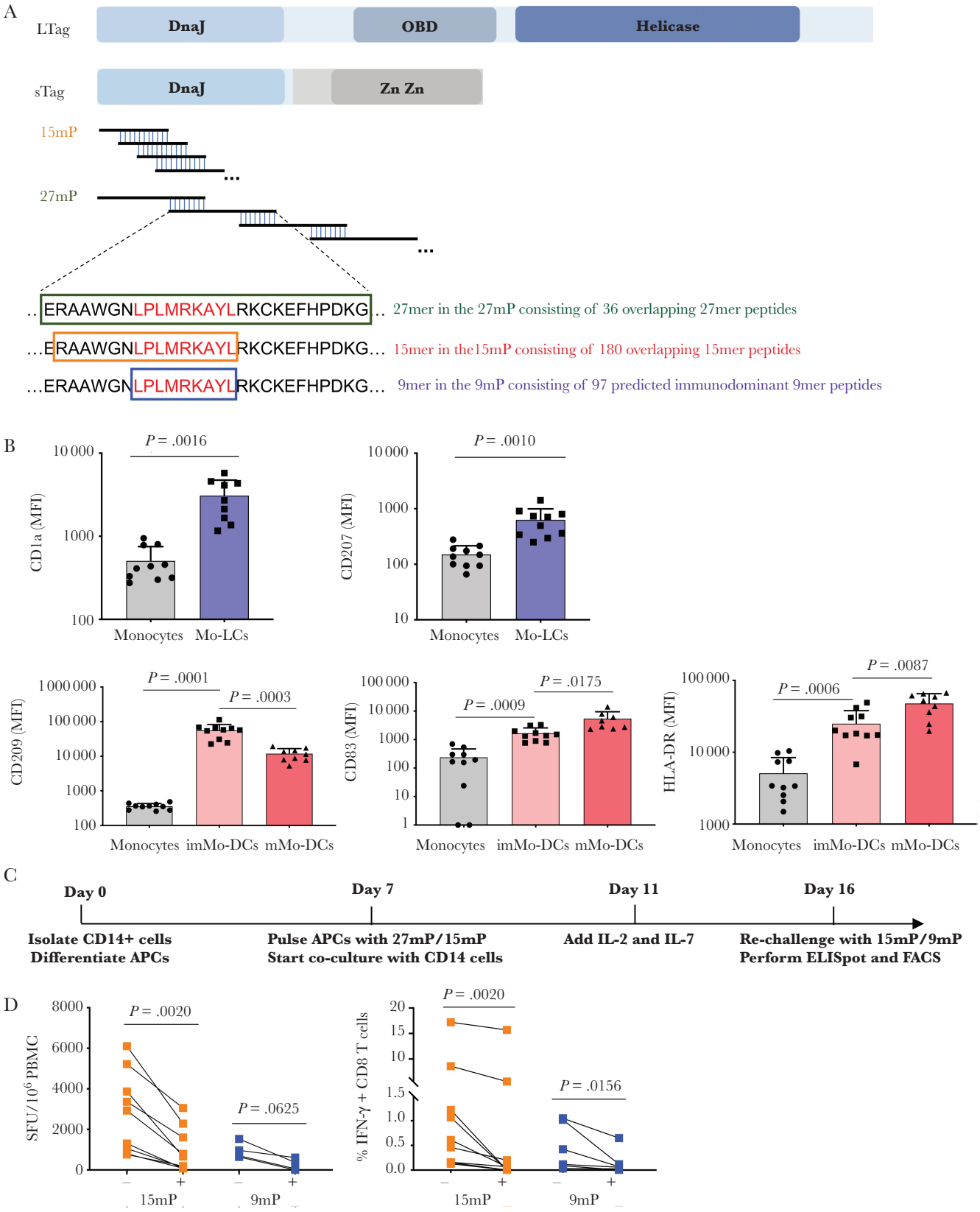

Figure 1. Modulators of BK polyomavirus (BKPyV)–specific CD8 T-cell responses. *A*, Schematic representation of large tumor antigen (LTag), small tumor antigen (sTag), and overlapping peptide pools. The 15mer pool (15mP) consisted of 180 peptides of 15 amino acids (aa) in length overlapping by 11 aa and covers the entire BKPyV LTag (Dunlop strain) as described previously [20]. The 27mer pool (27mP) consisted of 34 peptides of 27 aa, 1 peptide of 26 aa, and 1 peptide of 31 aa in length (Supplementary Table 2) overlapping by 8 aa also covering the entire BKPyV LTag (Dunlop strain). The 9mer pool (9mP) consisted of 97 bioinformatically predicted immunodominant 9mer peptides

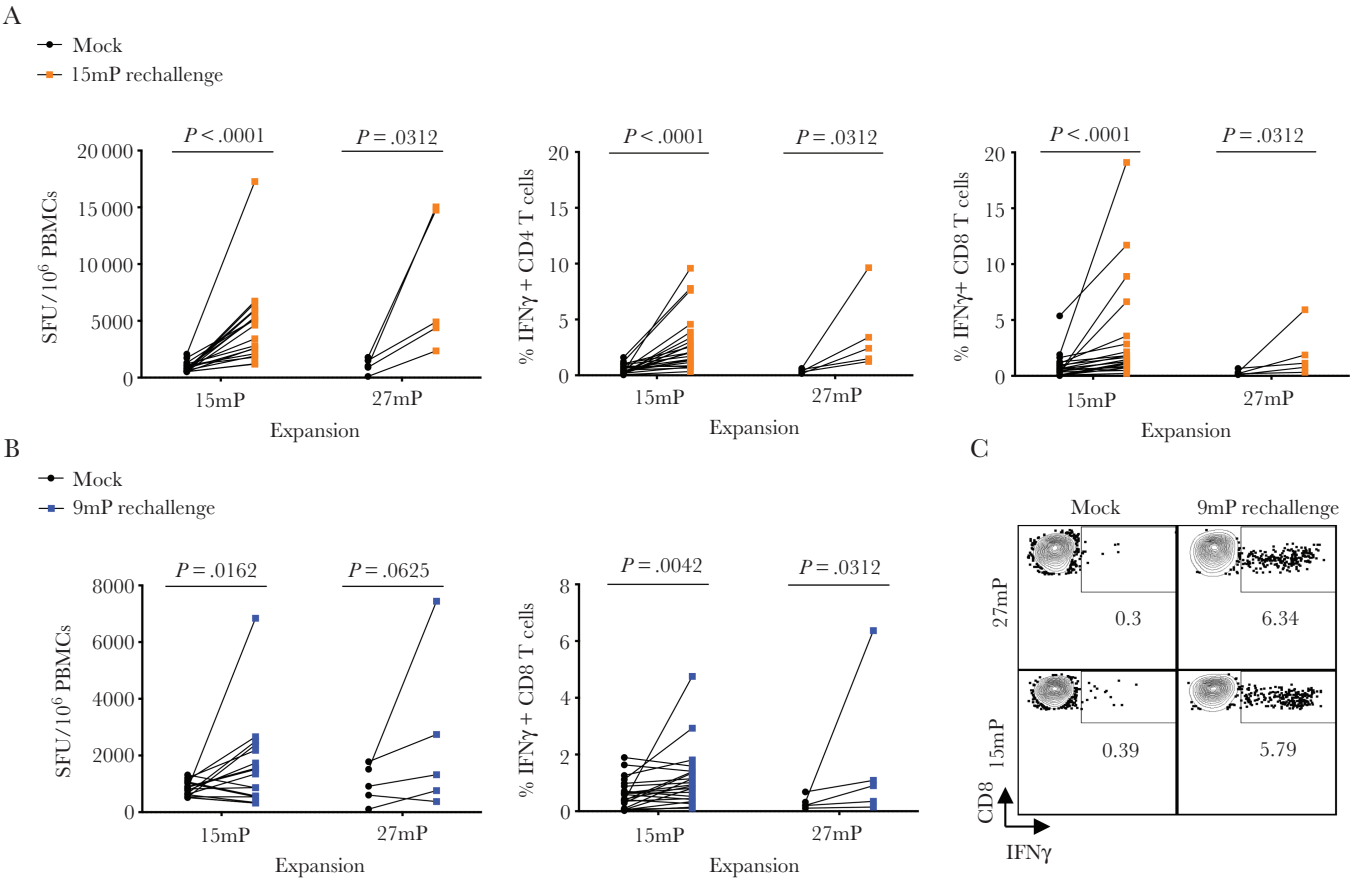

Figure 2. In vitro expansion of BK polyomavirus (BKPyV)–specific T cells induces large tumor antigen–specific 15mP and 9mP T-cell responses. Immature monocyte-derived dendritic cells were pulsed with 15mP or 27mP and co-cultured with autologous CD14$^-$ peripheral blood mononuclear cells. After the expansion, cells were rechallenged either with 15mP or 9mP. As a negative control, cells were not rechallenged (Mock). T-cell responses were measured as interferon gamma (IFN-γ) production using enzyme-linked immunosorbent spot assay (ELISpot) or flow cytometry. *A*, Comparison of the number of IFN-γ–secreting cells after 15mP rechallenge or without rechallenge (Mock) measured by ELISpot (left panel). Percentage of IFN-γ–producing CD4 (middle panel) or CD8 T cells (right panel) measured by flow cytometry. *B*, Comparison of the number of IFN-γ–secreting CD8 T cells after 9mP rechallenge or without rechallenge (Mock) measured by ELISpot (left panel). Percentage of IFN-γ–producing CD8 T cells measured by flow cytometry (right panel). *C*, Representative dot plots of BKPyV-specific CD8 T-cell responses expressed by IFN-γ production. Wilcoxon 1-tailed nonparametric test. Abbreviations: IFN-γ, interferon gamma; PBMCs, peripheral blood mononuclear cells; SFU, spot-forming units.

CD8 T-cell responses could be elicited by pulsing imMo-DCs with the novel 27mP, although overall 15mP and CD4 T-cell responses tended to be stronger.

BKPyV-Specific T-Cell Responses to LTag-Specific 27mP in Mo-LCs

To investigate whether or not Mo-LCs would shift the BKPyV-specific T-cell expansions toward CD8, we pulsed Mo-LCs with 15mP or 27mP and compared the results with imMo-DC. By ELISpot assay, 15mP rechallenge induced higher IFN-γ responses in Mo-LCs after 15mP- or 27mP-dependent expansion compared to imMo-DCs (Figure 3A, left panel), whereas the 9mP rechallenge showed little difference between both APC preparations regarding CD8 T-cell responses (Figure 3A, right panel). Flow cytometry indicated that IFN-γ–producing

derived from the BKPyV LTag as described previously [21, 37]. *B*, CD14$^+$ monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors and differentiated into different antigen-presenting cells (APCs). Top: Comparison of intracellular expression of CD1a and CD207 (Langerin) measured in monocytes (day 0) and differentiated monocyte-derived Langerhans cells (day 6). Bottom: Comparison of extracellular expression of CD209 (DC-SIGN), CD83, and human leukocyte antigen–DR in monocytes (day 0), immature monocyte-derived dendritic cells (imMo-DCs, day 7) and mature monocyte-derived dendritic cells. *C*, Experimental flow of T-cell expansion. CD14$^+$ monocytes were differentiated into different APCs, pulsed with 27mP or 15mP, and cocultured with autologous CD14$^-$ cells for 9 days. After expansion, cells were rechallenged with 15mP or 9mP and tested for interferon gamma (IFN-γ) production by enzyme-linked immunosorbent spot assay (ELISpot) and flow cytometry. *D*, Immature Mo-DCs were pulsed with 15mP and cocultured either with CD14$^-$ cells (–) or CD4-depleted CD14$^-$ cells (+) for 9 days and then rechallenged with 15mP or 9mP. BKPyV-specific CD8 T-cell responses were compared with or without CD4 help using IFN-γ ELISpot (left panel) and flow cytometry (right panel). The results are expressed as IFN-γ spot-forming units per million PBMCs in ELISpot and as percentage of IFN-γ–producing CD8 T cells in flow cytometry data. Wilcoxon 1-tailed nonparametric test. Abbreviations: APCs, antigen-presenting cells; ELISpot, enzyme-linked immunosorbent spot assay; HLA, human leukocyte antigen; FACS, fluorescence activated cell sorting; IFN-γ, interferon gamma; IL-2, interleukin 2; IL-7, interleukin 7; imMo-DCs, immature monocyte-derived dendritic cells; LTag, large tumor antigen; MFI, mean fluorescence intensity; mMo-DCs, mature monocyte-derived dendritic cells; Mo-DCs, monocyte-derived dendritic cells; Mo-LCs, monocyte-derived Langerhans cells; PBMCs, peripheral blood mononuclear cells; SFU, spot-forming units; sTag, small tumor antigen.

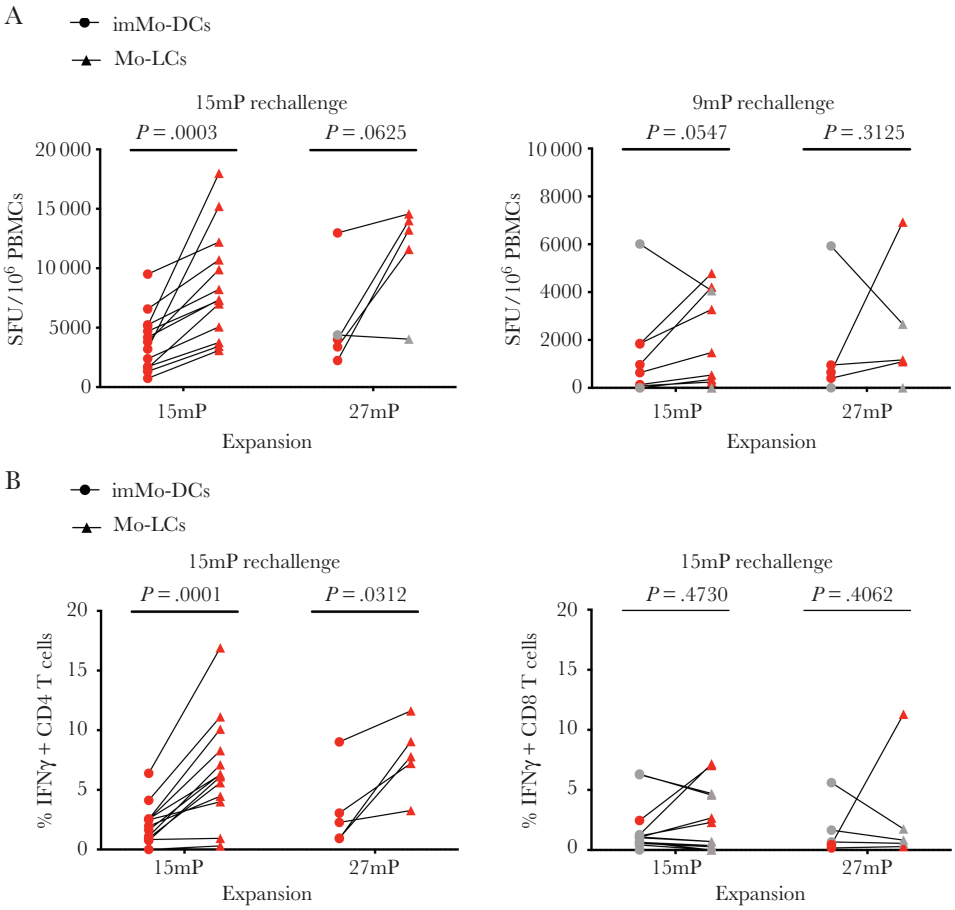

Figure 3. Monocyte-derived Langerhans cells (Mo-LCs) induce higher BK polyomavirus (BKPyV)–specific CD4 T-cell responses than immature monocyte-derived dendritic cells (imMo-DCs). ImMo-DCs (·) or Mo-LCs (Δ) were pulsed with 15mP or 27mP and co-cultured with autologous $CD14^-$ peripheral blood mononuclear cells (PBMCs). After the expansion, cells were rechallenged with 15mP or 9mP. As a negative control, cells were not rechallenged (Mock). T-cell responses were measured as interferon gamma (IFN-γ) production using enzyme-linked immunosorbent spot assay (ELISpot) or flow cytometry. Data are presented as spot-forming units/10^6 PBMCs for ELISpot and percentage of IFN-γ–producing T cells for flow cytometry after subtracting the background (Mock). Results are highlighted in red when Mo-LCs induce higher BKPyV-specific T-cell responses compared to imMo-DCs. *A*, Number of IFN-γ–secreting cells after rechallenge with 15mP (left panel) or 9mP (right panel) using imMo-DCs was compared to Mo-LCs. *B*, Percentage of IFN-γ–producing CD4 (left panel) and CD8 T cells (right panel) after rechallenge with 15mP using imMo-DCs was compared to Mo-LCs. Wilcoxon 1-tailed nonparametric test. Abbreviations: IFNγ, interferon gamma; imMo-DCs, immature monocyte-derived dendritic cells, Mo-LCs, monocyte-derived Langerhans cells; PBMCs, peripheral blood mononuclear cells; SFU, spot forming units.

CD4 T-cell responses were increased with Mo-LCs compared to imMo-DCs, whereas no differences between both APCs were seen regarding CD8 T cells (Figure 3B). We concluded that pulsing either Mo-LCs or imMo-DCs permitted the novel 27mP-dependent expansion of BKPyV-specific CD8 T cells, yet without favoring one over the other (Figure 3B).

BKPyV-Specific T-Cell Responses to LTag-Specific 27mP in mMo-DCs

To investigate whether mMo-DCs would shift the BKPyV-specific T-cell expansions toward CD8, we pulsed mMo-DCs with 15mP or 27mP and compared the results with imMo-DC. By ELISpot assay, 15mP rechallenge induced similar IFN-γ responses in mMo-DCs after 15mP- or 27mP-dependent expansion compared to imMo-DCs (Figure 4A, left panel). By flow cytometry, IFN-γ–producing CD8 T-cell responses were increased in expansion cultures using mMo-DCs compared to imMo-DCs ($P = .0312$), whereas no significant differences were observed between both APCs regarding CD4 T cells (Figure 4A, middle and right panels).

To more directly test the CD8 T-cell responses, we rechallenged expanded cells with 9mP (Figure 4B). By ELISpot, no significant difference between mMo-DCs and imMo-DCs was observed for 15mP or 27mP pulsing (Figure 4B, left panel), whereas by flow cytometry (Figure 4B, middle and right panel), an increase of 9mP-specific CD8 T-cell responses was observed for 27mP pulsing using mMo-DCs compared with imMo-DCs (Figure 4C). We concluded that mMo-DCs increased processing and presentation of 27mP, resulting in higher BKPyV-specific CD8 T-cell responses compared to imMo-DCs.

Characterization of BKPyV-LTag 27mP Presentation

To investigate whether or not CD8 T-cell stimulation by 27mers resulted from direct binding to MHC class I molecules due to the high peptide concentrations used for pulsing, a T2 assay was

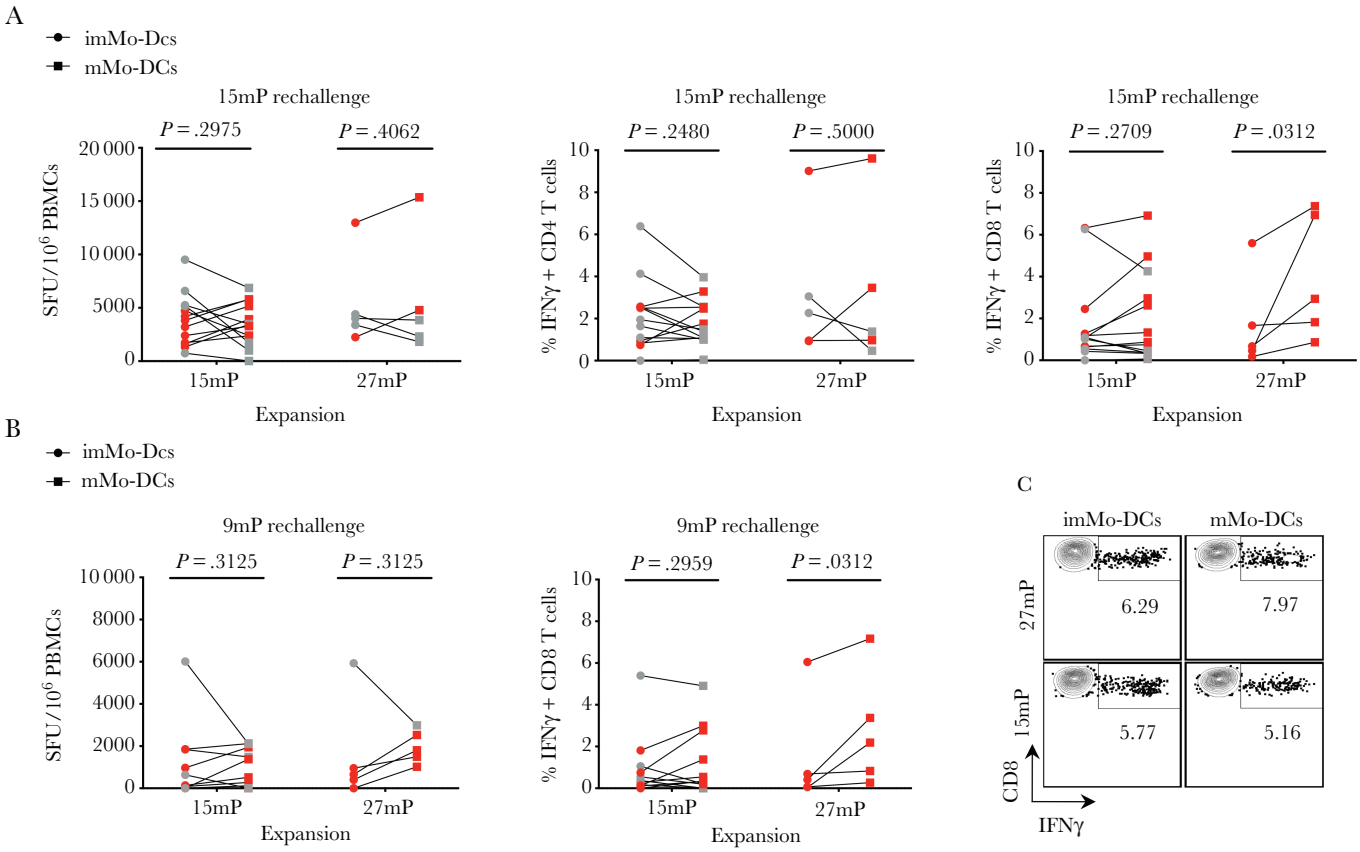

Figure 4. Maturation of monocyte-derived dendritic cells induces improved processing of BK polyomavirus (BKPyV) large tumor antigen 27mP, resulting in higher BKPyV-specific CD8 T-cell responses. Immature monocyte-derived dendritic cells (imMo-DCs, ○) or mature monocyte-derived dendritic cells (mMo-DCs;□) were pulsed with 15mP or 27mP and co-cultured with autologous $CD14^-$ peripheral blood mononuclear cells (PBMCs). After the expansion, cells were rechallenged either with 15mP or 9mP. As a negative control, cells were not rechallenged (Mock). T-cell responses were measured as interferon gamma (IFN-γ) production using enzyme-linked immunosorbent spot assay (ELISpot) or flow cytometry. Data are presented as spot-forming units (SFU)/10^6 PBMCs for ELISpot and percentage of IFN-γ–producing T cells for flow cytometry after subtracting the background (Mock). Results are highlighted in red when mMo-DCs induce higher BKPyV-specific T-cell responses compared to imMo-DCs. *A*, Overall BKPyV-specific T-cell responses after 15mP rechallenge measured by ELISpot (left panel), percentage of IFN-γ–producing CD4 (middle panel) or CD8 (right panel) T cells measured by flow cytometry. *B*, BKPyV-specific CD8 T-cell responses after 9mP rechallenge measured by ELISpot (left panel) or flow cytometry (right panel). *C*, Representative dot plots of BKPyV-specific CD8 T-cell responses after 9mP rechallenge measured by flow cytometry. Wilcoxon 1-tailed nonparametric test. Abbreviations: IFNγ, interferon gamma; imMo-DCs, immature monocyte-derived dendritic cells, mMo-DCs, mature monocyte-derived dendritic cells; PBMCs, peripheral blood mononuclear cells; SFU, spot forming units.

performed (Figure 5A). Since T2 cells lack the peptide transporter TAP required for binding and stabilization of HLA-A2 by endogenous class I epitopes, detection of HLA-A2 on the cell surface depends on stabilization by exogenous peptides. To this end, we used the previously identified HLA-A2 binding epitope 9m679 as positive control for HLA-A2 surface stabilization [21, 37] (Figure 5A). Unlike 9m679, the corresponding 27m670 harboring the 9m679 peptide sequence showed similarly low levels as no peptide, the non-HLA-A2 binding 9mer227, or the 15m573. The results indicated that 27m670 was not able to stabilize HLA-A2 on T2 cells. Thus, 27mer peptides were unlikely to elicit CD8 T-cell responses by direct binding to HLA class I molecules on the cell surface.

To investigate whether 27mer peptides require antigen processing before presentation and CD8 T-cell activation, we treated mMo-DCs with pan-cathepsin inhibitors I or the proteasome inhibitor lactacystin, which have been reported to preferentially inhibit proteolytic processing by vacuolar or cytosolic pathways of in vivo–generated dendritic cells [42], respectively. Compared to the solvent control, the cathepsin inhibitors had little effect on BKPyV-specific CD8 T-cell responses as measured by 9mP IFN-γ ELISpot or flow cytometry (Figure 5B, left panels), whereas a strong trend toward lower responses was observed by flow cytometry in the lactacystin-treated mMo-DCs (Figure 5B; right panels; $P = .0625$) as illustrated in Figure 5C. Both inhibitors did not affect cell viability at the concentrations used (Supplementary Figure 2). We concluded that 27mP did not bind directly to HLA class I, but required proteolytic processing by proteases partly inhibited by lactacystin, which are typically present in the cytosolic pathway.

Expanded BKPyV-Specific CD8 T Cells Have Functional Effector Memory Phenotype

To characterize the overall CD8 T-cell phenotype present after expansion using 27mP-pulsed mMo-DCs, we examined CCR7

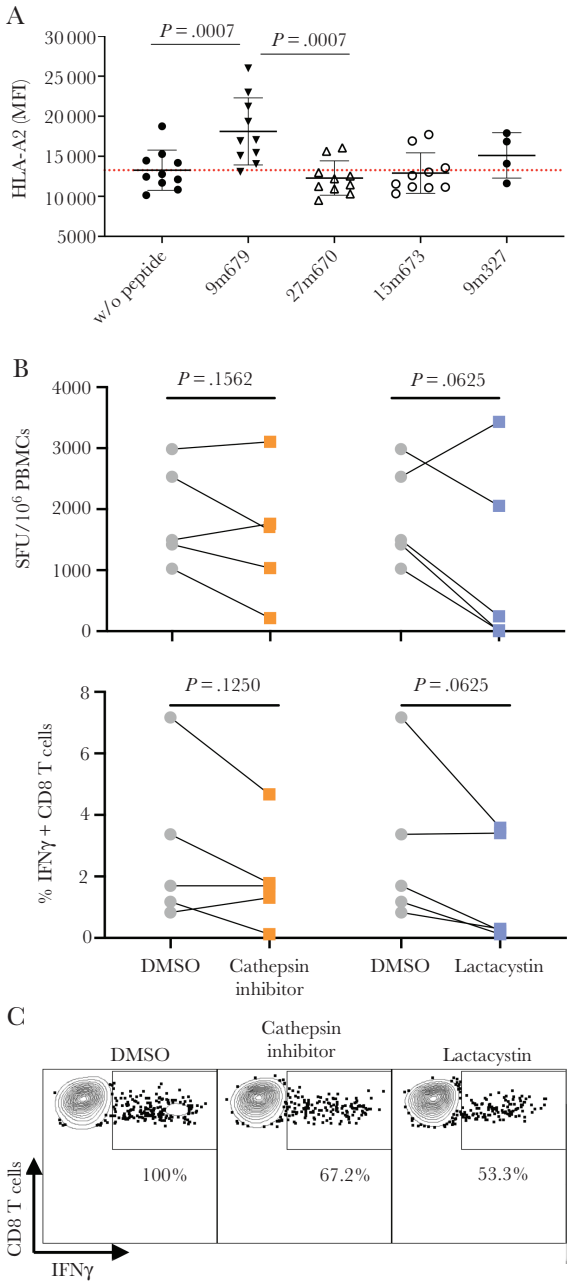

Figure 5. Processing and presentation of BK polyomavirus (BKPyV) large tumor antigen 27mP by mature monocyte-derived dendritic cells (mMo-DCs). *A*, T2 binding assay: 1 × 10^5 T2 cells were incubated with 9m679 (specific 9mer for human leukocyte antigen [HLA] A2), 9m327, 15m673, 27m670, or no peptide and analyzed for HLA-A2 expression by flow cytometry. *B*, mMo-DCs were pulsed with 27mP for 4 hours in the absence (dimethyl sulfoxide [DMSO]) or presence of cathepsin inhibitor or lactacystin. 27mP-pulsed mMo-DCs were co-cultured with autologous $CD14^-$ peripheral blood mononuclear cells for 9 days and expanded T cells were rechallenged with 9mP. 9mP-specific CD8 T-cell responses measured by interferon gamma (IFN-γ) enzyme-linked immunosorbent spot assay (left panel) or flow cytometry (right panel) in the absence (DMSO) or presence of cathepsin inhibitor or lactacystin were compared. Data are presented after subtracting the background (Mock) in respective conditions. *C*, Representative dot plots of 9mP-specific CD8 T-cell responses presented as IFN-γ production using flow cytometry. BKPyV-specific CD8 T-cell response in the condition with DMSO was set up to 100% and compared to the conditions where cathepsin inhibitor or lactacystin was used. Wilcoxon 1-tailed nonparametric test. Abbreviations: DMSO, dimethyl sulfoxide; HLA, human leukocyte antigen; IFN-γ, interferon gamma; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; SFU, spot-forming units.

and CD45RA expression on day 0 and day 9 (Figure 6A, top row). The data show that CD8 T effector memory cells increased ($CCR7^-/CD45RA^-$; 32.2% [day 0]; 65.0% [day 9]), whereas terminally differentiated effector memory CD8 T cells were reduced ($CCR7^-/CD45RA^+$; 62.3% [day 0]; 28.5% [day 9]). After 9mP rechallenge on day 9, 64.4% of IFN-γ–producing CD8 T cells were found to have effector memory phenotype (Figure 6A, bottom). Comparing CFSE-labeled $CD14^-$ PBMCs on day 0 and on day 9 indicated that CD8 T cells had been proliferating during the 9-day co-culture (Figure 6B). Stimulation with the immunodominant 9mP revealed that IFN-γ was mostly produced by the proliferating CD8 T-cell population (Figure 6B), which was also polyfunctional as indicated by the simultaneous production of TNF-α (Figure 6C). The exhaustion marker PD1 was found on approximately 3% of CD8 T cells by day 9 and comparable to baseline (Figure 6D). Conversely, >90% of IFN-γ–producing CD8 T cells after 9mP rechallenge were PD1-negative. To independently evaluate cell activation, 27mP-expanded BKPyV-specific CD8 T cells were examined for expression of PD1 and CD69, showing that IFN-γ–producing CD8 T cells after 9mP rechallenge on day 9 are $PD1^-/CD69^+$ (Supplementary Figure 3). We concluded that BKPyV-specific effector memory CD8 T cells proliferated, were not exhausted, and could be activated to secrete multiple cytokines.

Expanded BKPyV-Specific CD8 T Cells Are Cytotoxic in an HLA-Restricted Manner

To assess the specificity of BKPyV-specific CD8 T cells, 27mP-dependent expansion was performed with PBMC preparations from an HLA-B8 positive donor, for which the HLA-B*08:02-positive 9m127 was available. Streptamer staining identified a population of HLA-B*08:02-positive 9m127-specific CD8 T cells, representing 1.64% of the overall CD8 population after expansion, and which secreted IFN-γ after 9mP rechallenge (Figure 7A). Staining for CD107a antibody demonstrated degranulation of the HLA-B8 9m127-binding CD8 T cells (Figure 7B). Moreover, the HLA-B8 9m127-binding CD8 cells were able to kill 20% of target cells at a target:effector ratio of 1:20 (Figure 7C). We concluded that 27mP-pulsed mMo-DCs can be used to promote the proliferation of polyfunctional cytotoxic effector memory BKPyV-specific CD8 T cells.

DISCUSSION

Given the lack of effective and safe antiviral therapies, BKPyV remains one of the most challenging causes of premature graft failure after kidney transplantation [9, 22]. In view of 50 000 kidney transplantations each year in North America and Europe alone, at least 5000 patients are at risk, according to larger studies [10, 12, 15, 43]. The affected patients are faced with the limited option of reducing immunosuppression, the competing risk of rejection [6, 7, 22] and dire perspectives on waiting lists for retransplantation [44, 45]. However, we and others previously

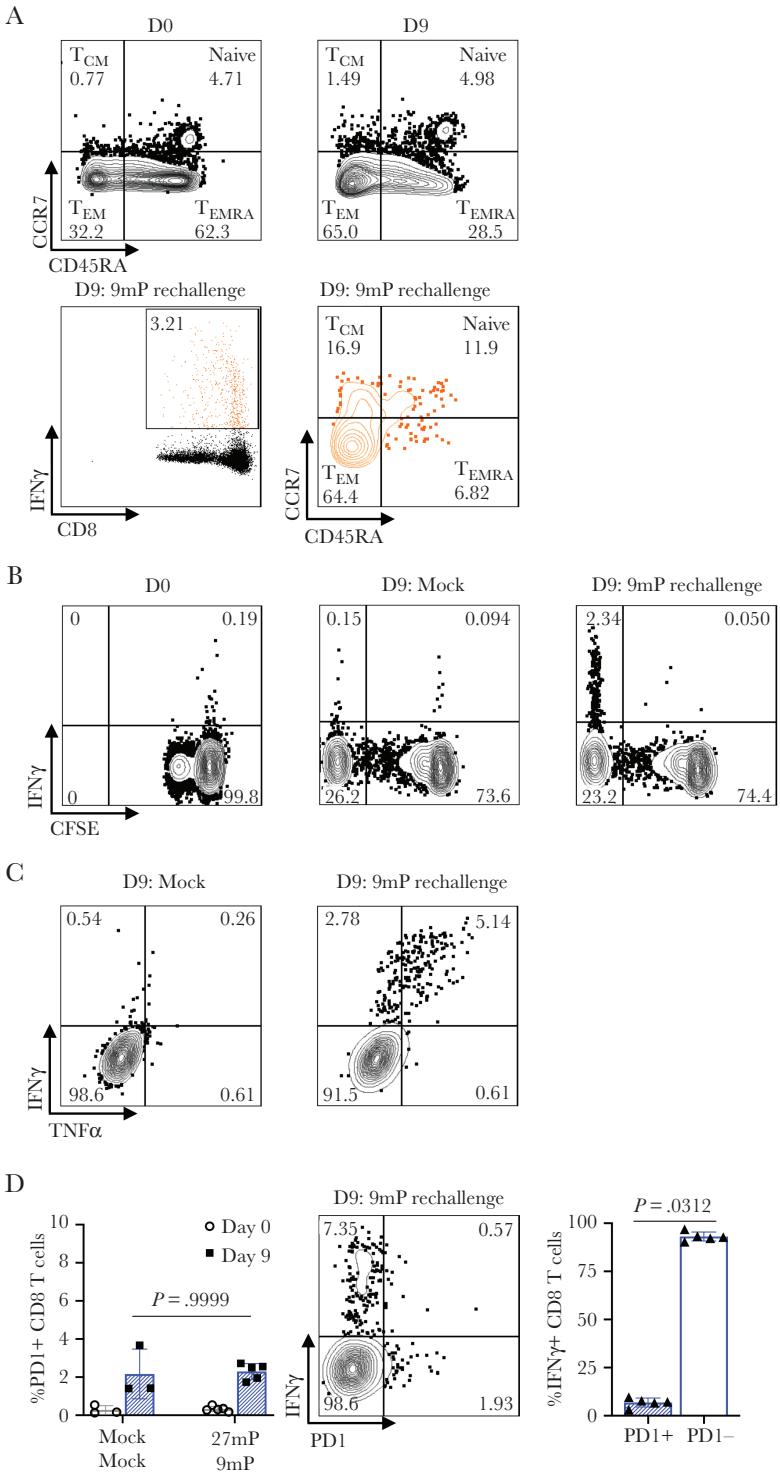

Figure 6. Characterization of expanded BK polyomavirus (BKPyV)–specific CD8 T cells. Mature monocyte-derived dendritic cells were pulsed with 27mP and co-cultured with autologous CD14$^-$ peripheral blood mononuclear cells for 9 days. Expanded T cells were rechallenged with 9mP on day 9, and their phenotyping and function were evaluated using flow cytometry. *A*, Phenotyping of CD8 T cells by CCR7 and CD45RA staining on day 0 (top left panel) and day 9 after 27mP expansion (top right panel). Interferon gamma (IFN-γ) responses of day 9–expanded BKPyV-specific CD8 T cells were measured after 9mP rechallenge on day 9 (bottom left panel, square indicates selection gate) and phenotyping using CCR7 and CD45RA staining (bottom right panel). *B*, CD8 T-cell proliferation during in vitro expansion. CD14$^-$ cells were stained at day 0 (left panel) with carboxyfluorescein diacetate succinimidyl ester dye that dilutes on cell division. Proliferation and IFN-γ production of CD8 T cells on day 0 and day 9 without rechallenge (Mock, middle panel) or after 9mP rechallenge (right panel). *C*, Function of expanded CD8 T cells was measured as IFN-γ and tumor necrosis factor–α production after 27mP expansion without rechallenge (Mock) or 9mP rechallenge. *D*, Surface expression of PD-1 measured on CD8 T cells at day 0 and on expanded cells at day 9 (left panel). Representative dot plot (middle panel) and cumulative data (right panel) of IFN-γ and PD-1 expression on expanded CD8 T cells after 27mP expansion and 9mP rechallenge on day 9. Abbreviations: D0, day 0; D9, day 9; CFSE, carboxyfluorescein diacetate succinimidyl ester; IFN-γ, interferon gamma; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{EMRA}, terminally differentiated effector memory T cells; TNF-α, tumor necrosis factor alpha.

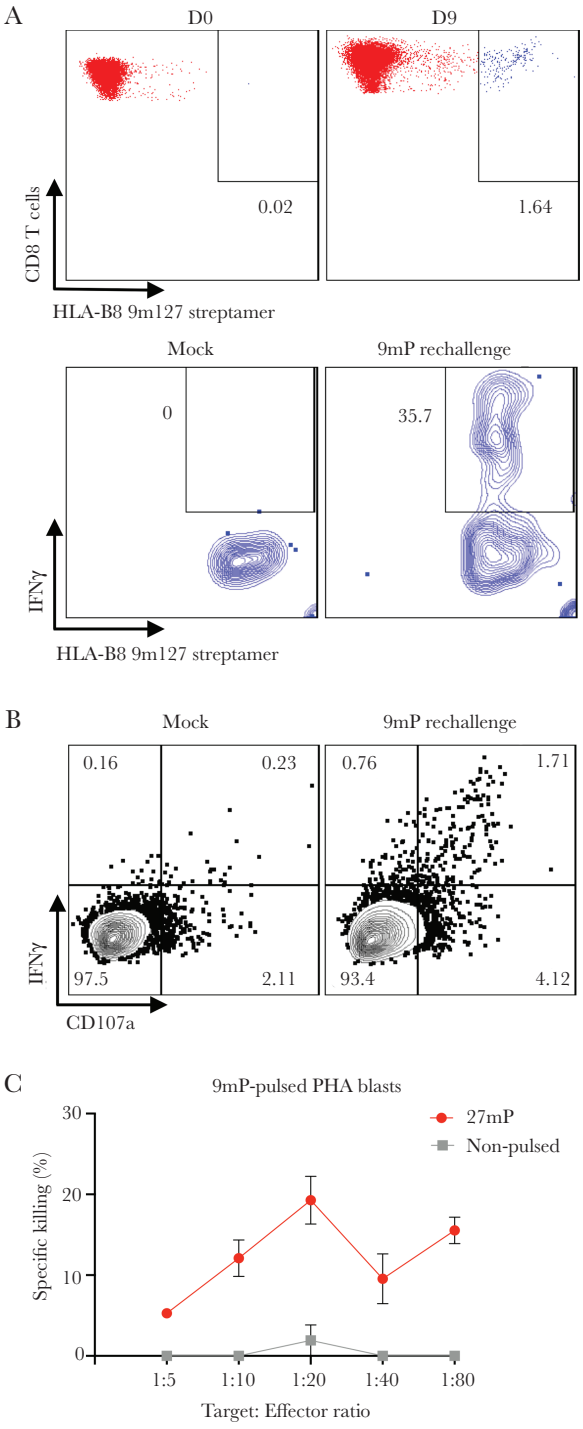

Figure 7. BK polyomavirus (BKPyV)–specific CD8 T cells are cytotoxic. Mature monocyte-derived dendritic cells of a human leukocyte antigen (HLA) B8–positive donor were pulsed with 27mP and co-cultured with autologous CD14$^-$ peripheral blood mononuclear cells for 9 days. Expanded T cells were rechallenged with 9mP or not (Mock) on day 9 and functional assays were performed. *A*, HLA streptamer staining of CD8 T cells was performed using phycoerythrin-labeled Strep-Tactin with HLA-B*0802 molecules bearing 9m127 peptide on day 0 and day 9 after 27mP expansion (top panel). Interferon gamma (IFN-γ) production by 9m127-specific CD8 T cells without rechallenge (Mock) or after 9mP rechallenge (bottom panel). *B*, BKPyV-specific degranulation of CD8 T cells is shown using IFN-γ and CD107a staining on day 9. *C*, 9mP-specific cytotoxic activity of expanded T cells. Autologous phytohemagglutinin-L blasts stained with CellTrace Violet and pulsed with 9mP were used as target cells and incubated for 6 hours with expanded T cells (effectors) using 27mP as antigen (red) or no antigen (nonpulsed, gray) stained with carboxyfluorescein diacetate succinimidyl ester. Percentage of specific target cell killing at the different target:effector cells ratios is shown. Abbreviations: D0, day 0; D9, day 9; HLA, human leukocyte antigen; IFN-γ, interferon gamma; PHA, phytohemagglutinin-L.

demonstrated that reducing immunosuppression is associated with increasing BKPyV-specific T-cell responses [20, 33, 46] and clearance of BKPyV DNAemia, whereby CD8 T cells directed against immunodominant epitopes present in the viral nonstructural proteins may play a particular role for protection [21, 37, 38]. Thus, increasing BKPyV-specific cellular immunity through adoptive T-cell transfer or vaccination may have the potential to reduce the risk of BKPyV-associated complications. In the present study, we therefore explored the potential of a novel 27mP for expanding BKPyV-specific CD8 T cells in vitro. Our study provides 3 main results: First, the 27mP can be used to expand BKPyV-specific CD8 T cells within 9 days of co-culture using PBMC-derived imMo-DCs, mMo-DCs, or Mo-LCs as APCs. Second, mMo-DCs appear to be most suitable due to consistently higher yields compared to imMo-DCs or Mo-LCs. Third, the resulting BKPyV-specific CD8 T cells have a functional 9mer epitope-restricted effector memory phenotype and are cytotoxic.

Our results are strengthened by the independent use of 2 different assays for characterizing the 9-day expansion cultures, namely ELISpot and flow cytometry, to measure the BKPyV-specific T-cell responses as well as 2 different BKPyV antigen preparations, namely 15mP and 9mP. Conversely, no expansion responses were obtained after 9-day co-culture without prior pulsing of the APCs with the corresponding 27mP or 15mP. Our attempts to promote CD8 T-cell responses by depleting CD4$^+$ cells from the co-culture resulted in a significantly reduced yield, suggesting the need for CD4 T-cell help during the expansion. Although CD4 T-cell help could be important for several reasons including promoting a Th-1 cytokine milieu, the more direct contribution such as activating the cross-presentation of the large 27mer peptides to HLA class I on the APCs seems also likely. Consistent with the latter notion is the inability of 27mer peptides to increase HLA-A2 binding on T2 cells as well as the strong trend of the proteasome inhibitor lactacystin to reduce CD8 T-cell expansion.

Unlike the frequently used 15mP consisting of 180 peptides overlapping by 11 amino acids, our novel 27mP consisted of 36 longer peptides overlapping by 8 amino acids covering the entire LTag and the common amino-terminal part of small tumor antigen of BKPyV. Thereby, production and use of immunogenic epitopes becomes less complex, facilitating well-controlled further developments of adoptive T-cell therapy or vaccination approaches. Both objectives are of significant interest given the unmet clinical need of BKPyV-associated diseases in transplant recipients [29]. While adoptive T-cell therapy is well underway

in dedicated settings with respect to production, the clinical implementation, follow-up, and vaccination approaches are little explored to reduce the risk of BKPyV replication.

Our study has several limitations. We used PBMCs of healthy blood donors, who are not immunosuppressed. The dependence on CD4 T cells is notable and may limit the responses in kidney transplant recipients, who are at increased risk of infectious complication [47, 48]. The efficacy of BKPyV vaccination and adoptive T-cell transfer posttransplant is also limited by the reduced signal-1 response in the presence of calcineurin inhibitors [49] and would require a window of transiently reduced or modified immunosuppression with the inherent risks [22]. Also, dendritic cell counts in the blood of kidney recipients developing nephropathy may be low [50], although myeloid dendritic cells may be increased in BKPyV-associated nephropathy [51]. Therefore, BKPyV peptide vaccination strategies may target patients pretransplantation for better responses, which can be safely boosted posttransplantation, and which may include adjuvants such as MF59 used in inactivated influenza vaccines. Finally, although BKPyV is employing local strategies of immune escape [26], the clearance of BKPyV replication subsequent to reducing immunosuppression argues that cellular immune control is eventually sufficient, if immune effectors are high [22]. Through peptide vaccination delivered systemically by subcutaneous injection, we would aim at increasing the number of available immune effectors prior to extensive reactivation as well as rendering the response to reducing immunosuppression more effective.

In conclusion, synthetic 27mP permit expanding BKPyV-specific CD8 T-cell responses when pulsing mature Mo-DCs in presence of CD4 T cells. The results suggest facilitated approaches to vaccination and adoptive T-cell therapies for patients before and after kidney transplantation. As direct next steps, preclinical models including HLA-humanized mice could be envisaged followed by phase 1 clinical trials.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. M. Wi. and A. K. designed and performed experiments, analyzed the data, and wrote the first draft of the manuscript. M. We. helped to perform the experiments and contributed in the discussion for the analysis. H. H. H. designed the study, interpreted the data, and wrote the final manuscript. All authors read and approved the final manuscript.

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Potential conflicts of interest. All authors: No reported conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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