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BK Polyomavirus-Specific 9mer CD8 T Cell Responses Correlate With Clearance of BK Viremia in Kidney Transplant Recipients: First Report From the Swiss Transplant Cohort Study

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BK polyomavirus (BKPyV) causes premature kidney transplant (KT) failure in 1–15% of patients. Because antivirals are lacking, most programs screen for BKPyV-viremia and, if positive, reduce immunosuppression. To evaluate the relationship of viremia and BKPyV-specific immunity, we examined prospectively cryopreserved plasma and peripheral blood mononuclear cells at the time of transplantation (T0) and at 6 mo (T6) and 12 mo (T12) after transplant from 28 viremic KT patients and 68 nonviremic controls matched for the transplantation period. BKPyV IgG seroprevalence was comparable between cases (89.3%) and controls (91.2%; p = 0.8635), but cases had lower antibody levels (p = 0.022) at T0. Antibody levels increased at T6 and T12 but were not correlated with viremia clearance. BKPyV-specific T cell responses to pools of overlapping 15mers (15mer peptide pool [15mP]) or immunodominant CD8 9mers (9mer peptide pool [9mP]) from the early viral gene region were not different between cases and controls at T0; however, clearance of viremia was associated with stronger 9mP responses at T6 (p = 0.042) and T12 (p = 0.048), whereas 15mP responses were not informative (T6 p = 0.359; T12 p = 0.856). BKPyV-specific T cells could be expanded *in vitro* from all patients after transplant, permitting identification of 78 immunodominant 9mer epitopes including 50 new ones across different HLA class I. Thus, 9mP-responses may be a novel marker of reconstituting CD8 T cell function that warrants further study as a complement of plasma BKPyV loads for guiding immunosuppression reduction.

Abbreviations: 15mP, 15mer peptide pool; 9mP, 9mer peptide pool; ATG, anti–thymocyte globulin; BKPyV, BK polyomavirus; ELISpot, enzyme-linked immunospot; EVGR, early viral gene region; IFN-γ, interferon γ; IQR, interquartile range; IVIG, intravenous immunoglobulin; JCPyV, JC polyomavirus; KT, kidney transplant; LTag, large T antigen; MMF, mycophenolate mofetil; MPS, enteric-coated mycophenolate sodium; PBMC, peripheral blood mononuclear cell; SFU, spot-forming unit; sTag, small T antigen; STCS, Swiss Transplant Cohort Study; T, time; Vp1, viral capsid protein 1

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Introduction

In the past decade, BK polyomavirus (BKPyV)–associated nephropathy has emerged as a significant complication in kidney transplant (KT), causing premature graft failure in 1–15% of patients (1,2). Notably, BKPyV seems rather benign in immunocompetent people, infecting >90% of the general population during early childhood without ill effects and persisting asymptomatically thereafter in the renourinary tract with low-level urinary shedding (3,4). Following KT, however, 20–40% of patients develop high-level viruria, and 10–20% progress to BKPyV viremia, which closely mirrors the onset and resolution of histologically proven nephropathy (5–9). Several risk factors for BKPyV replication have been reported in KT patients including older age and male gender of recipients, high donor and low recipient antibody levels, higher number of HLA mismatches, higher corticosteroid exposure, and acute rejection episodes, as well as tacrolimus-based immunosuppressive regimens (2,10–13). However, their relative importance for BKPyV replication is still difficult to predict in the individual constellation of recipient immunity, donor graft properties, and immunosuppression (14). In clinical practice, BKPyV viremia is regarded as a net result of the extremely rapid viral replication (15) and functional BKPyV-specific immune responses (16–18). In the absence of antiviral drugs, most transplant programs screen KT patients for BKPyV viremia and, if positive, preemptively reduce immunosuppression. Although interventional clinical trials are lacking, this approach is widely recommended (14,19,20) based on viremia clearance rates of 80–100% in dedicated prospective cohort studies (8,17,21,22); however, allograft rejection is seen in 0–14% of patients (8,17,20,22,23). Consequently, improved strategies and markers are warranted that complement viremia dynamics for better guidance of immunosuppression reduction.

Cellular immunity has been suggested to be crucial for the control of BKPyV replication and has been explored in KT patients using overlapping 15mer peptide pools (15mPs) for stimulating interferon γ (IFN-γ) release assays (16–18). BKPyV-specific T cell responses in blood, however, are generally much lower than, for instance, cytomegalovirus-specific T cells (24,25) but significantly increase in KT patients clearing BKPyV viremia (16,17,26,27). Although BKPyV-specific CD8 T cells are thought to be central to cytotoxic control of virus-infected host cells (28–30), previous studies focused on overlapping 15mer pools, which elicit functional responses in CD4 and to a lesser extent in CD8 T cells (16,17,28). In fact, BKPyV-specific CD8 T cells and their epitopes are little studied and, if so, are typically restricted to HLA-A*0201 (31–33). We focused on immunodominant 9mer epitopes encoded in the BKPyV early viral gene region (EVGR) encoding the viral small T antigen (sTag) and large T antigen (LTag) (34,35) that are able to elicit defined CD8 T cell responses across a wide range of different HLA types in healthy individuals and pediatric KT patients (36). The 9mer-specific CD8 T cells had been further characterized using HLA multimers, carboxyfluorescein diacetate succinimidyl ester proliferation, CD107a degranulation, and chromium 51–release killing assays (36). We examined BKPyV-specific antibody as well as cellular immune responses using overlapping 15mer and immunodominant 9mer pools in a case-control study of 96 KT patients consisting of 28 viremic cases and 68 nonviremic controls participating in the Swiss Transplant Cohort Study (STCS).

Patients and Methods

Study design

This study has been conducted within the framework of the multicenter STCS (project number FUP056), supported by the Swiss National Science Foundation, the Swiss University Hospitals (G15), and Swiss transplant centers (Basel, Bern, St. Gallen, Lausanne, Geneva, and Zurich). Signed written informed consent and sample collection forms were obtained from all patients included, as approved by the local institutional review board and the STCS committee. Prospective biobanking of participating patients' peripheral blood mononuclear cells (PBMCs) and plasma was conducted at the time of transplantation (T0) as well as 6 mo (T6) and 12 mo (T12) after transplantation by the participating study centers. Antibody and T cell responses were determined at all three time points in all BKPyV-viremic KT patients and compared with two nonviremic control patients using a case-control cohort approach.

Patient identification and study population

Adult KT patients (aged ≥18 years) were identified in the STCS database as of July 1, 2014, if they had received a first kidney transplant at the University Hospital of Basel between May 1, 2008, and December 31, 2013, to permit follow-up of at least 18 mo. All patients gave informed consent to participate in the STCS and had PBMCs and plasma samples cryopreserved at the time of transplantation (T0). Patients were excluded if they underwent retransplantation or additional nonkidney solid organ transplantation or had primary nonfunction of the renal allograft. Screening for BK virus replication was done according to a standard protocol described previously (8), consisting of urine cytology for decoy cells every 2 weeks until month 3 and then at 6 and 12 mo and yearly thereafter. KT patients with positive decoy cells were tested for plasma BKPyV loads by quantitative real-time polymerase chain reaction at the next visit, as described (8). KT patients were allocated to cases if at least one plasma BKPyV load ≥500 copies/mL was detected within the first 12 mo after transplant. Of a total of 217 KT patients in the database, 33 were identified as BKPyV viremic. Controls were defined as nonviremic if plasma BKPyV load was undetectable within the first 18 mo after transplant. Per one viremic patient, two nonviremic patients transplanted within the same time period (2008–2009, 2010–2011, 2012) were randomly identified as controls (n = 66) using frequency matching. Patient chart and laboratory review resulted in exclusion of five cases because of a missing sample (n = 1) and incorrect data (n = 4), two of which had to be reclassified as controls. Thus, the final study group consisted of 96 KT patients, 28 as BKPyV viremic cases and 68 as nonviremic controls. The patient characteristics regarding age, gender, donor and recipient HLA, immunosuppressive regimen, and some viremia history were obtained from the STCS database (Table 1). Additional information such as immunosuppression dosing, trough levels, and serum creatinine concentration were obtained from the local patient flowchart.

Table 1: Characteristics of viremic (case) and nonviremic (control) patients matched for transplantation period

Characteristic	Cases	Control	p-value
Patients, n	28	68	
Age, median (IQR)	57 (40, 66)	55 (43, 66)	0.907
Gender, female	6 (21.43)	19 (27.9)	0.685
Living donation	14 (50)	27 (39.7)	0.283
Blood group compatible	24 (85.7)	64 (88.2)	1.000
Delayed graft function	4 (14.3)	8 (11.8)	1.000
Sum HLA mismatch 0	2 (7.1)	2 (2.9)	0.735
Sum HLA mismatch 1	4 (14.3)	5 (7.6)	
Sum HLA mismatch 2	1 (3.6)	6 (8.8)	
Sum HLA mismatch 3	7 (25)	14 (20.6)	
Sum HLA mismatch 4	5 (17.9)	15 (22.1)	
Sum HLA mismatch 5	6 (21.4)	14 (20.6)	
Sum HLA mismatch 6	3 (10.7)	12 (17.7)	
Rituximab	4 (10.7)	4 (5.9)	0.671
IVIG	4 (10.7)	4 (5.9)	0.671
ATG	3 (10.7)	6 (8.8)	1.000
Basiliximab	24 (85.7)	61 (89.7)	1.000
MMF or MPS	28 (100)	68 (100)	
Tacrolimus	28 (100)	68 (100)	

Data are shown as n (%) except as noted. ATG, anti–thymocyte globulin; IQR, interquartile range; IVIG, intravenous immunoglobulin; MMF, mycophenolate mofetil; MPS, enteric-coated mycophenolate sodium.

Virus serology and DNA load

The BKPyV loads were determined as described previously (5), with the modifications outlined (37). BKPyV and JC polyomavirus (JCPyV) IgG antibody levels were determined in plasma samples obtained at T0, T6, and T12 by a normalized ELISA using viral capsid protein 1 (Vp1) virus-like particles, as described (38).

Direct ex vivo *T cell responses to BKPyV*

BKPyV-specific T cell responses against the EVGR epitopes were measured using viable cells per well obtained after thawing prospectively cryopreserved PBMCs by IFN-γ enzyme-linked immunospot (ELISpot) assay (16,36). Briefly, 288 PBMC samples were thawed, and viable cells (mean viability at 6 h was 87% ± 19%) were counted and seeded, as described below (36). Peptide pools of 180 15mers overlapping by 11 amino acids spanning the entire BKPyV EVGR (15mP) (16) or of 97 predicted immunodominant 9mers (9mer peptide pool [9mP]) at 1 μg/ mL (Eurogentec, Belgium) (36) or medium only for a negative control were used to assess he IFN-γ production in duplicates of 250 000 PBMCs per microtiter well. Phytohemagglutinin-L (2 mg/ mL; Hoffmann-La Roche Ltd., Basel, Switzerland) was used as the positive control on 50 000 cells per well, yielding between 300 and 500 spot forming units (SFUs) per well, to avoid spot saturation per well. All tests were performed in duplicates SFUs were enumerated by an ImmunoSpot analyzer (CTL Europe GmbH, Bonn, Germany) and averaged. Spot counts of the negative control were multiplied by two to define the cutoff, and ELISpot results above the cutoff were considered positive and expressed per million PBMCs to display specific responses.

In vitro *expansion of virus-specific T cells*

Virus-specific T cells were expanded by seeding 3 million viable PBMCs into 1.5 mL medium in 24-well plates pulsed with BKPyV EVGR 15mP (200 ng/ mL). Cells were incubated at 37°C in 5% CO_2 in RPMI supplemented with 5% human serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Sigma-Aldrich) for 14 days. At day 7, half of the medium was replaced and cultures were restimulated with the 15mP. Recombinant IL-2 (20 U/mL; PeproTech, Rocky Hill, NJ) and IL-7 (5 ng/ mL; PeproTech) was provided at days 3 and 10. At day 14 after expansion, the cells were washed, and an IFN-γ ELISpot was performed using 15mP and 9mP, as described. If sufficient cells were available, cells were available, restimulation was performed with 9mer-subpools subpools for 9mer epitope response mapping by cross-identification in checkerboards, whereby each of the 97 9mers were present in two different unique subpools, as described in detail by Cioni et al in figure 3B (36).

Statistical analysis

The chi-square test was used for categorical variables, and the Kruskall–Wallis, Wilcoxon, and Mann–Whitney *U* tests and analysis of variance were used for multiple comparisons, as indicated. Two-sided p-values of <0.05 were considered statistically significant.

Results

In total, 96 adult KT patients were identified who participated in the STCS and were transplanted in Basel before December 31, 2013, permitting follow-up of at least 18 mo. The study encompassed 28 viremic cases and 68 nonviremic control patients matched for the same transplant period (Table 1). Approximately half of the patients had received living donor grafts, and 12% were ABO-incompatible. Three or more HLA mismatches were present in 75% of the transplants. Basiliximab was administered for induction, but approximately one-fifth of the patients required depleting antibodies such as anti–thymocyte globulin or rituximab. All patients received maintenance immunosuppression with tacrolimus–mycophenolate combinations. Although controls were matched solely for the time of transplantation, there were no significant differences between viremic cases and nonviremic controls for any of the baseline parameters (Table 1). Notably, BKPyV viremia onset and duration among cases varied during the first year after transplantation such that different groups could be distinguished relative to the time points of plasma and PBMC cryopreservation at T6 and T12 (Figure 1).

BKPyV-specific IgG was determined at T0 using a normalized ELISA, as detailed recently (38). The seroprevalence of cases and controls was 89.3% and 91.2%, respectively, and was not significantly different ($p = 0.8635$). BKPyV IgG levels at T0, however, were lower in patients who later developed viremia compared with the non-viremic patients ($p = 0.0220$) (Figure 2A). JCPyV seroprevalence was not significantly different between cases and controls (89.3% vs. 89.7%; $p = 0.9411$), and no significant difference in JCPyV IgG levels could be observed (Figure 2B).

The lower BKPyV antibody levels among cases at T0 were not caused by inability to mount stronger IgG responses, since significant increases could be observed

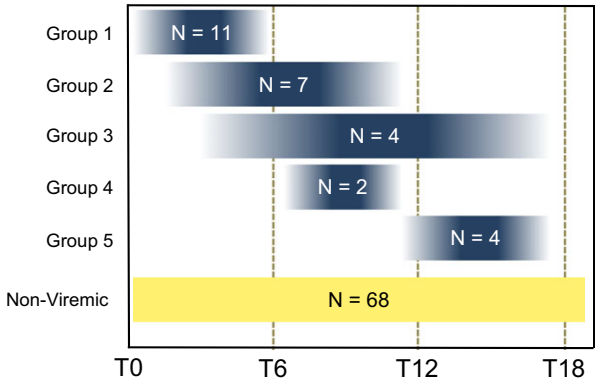


Figure 1: Onset and duration of BK polyomavirus viremia after kidney transplantation. Onset, duration, and number of patients are shown in blue bars, nonviremic control patients are shown in the yellow bar. T0, T6, T12, and T18 indicate time of transplantation and 6, 12, and 18 mo after transplantation, respectively.

at T6 and T12 after transplant despite maintenance immuosuppression ($p < 0.0001$) (Figure 2C), as reported previously (27,39). By comparison, the average BKPyV IgG levels did not change in nonviremic KT patients from T0 to T6 and T12 ($p = 0.7884$) (Figure 2C). Thus, BKPyV-specific antibodies indicated not only past exposure to BKPyV but also significant recent exposure after transplant, as seen in BKPyV viremic patients.

To investigate the role of antibodies and clearance of BKPyV viremia, we compared BKPyV IgG levels in cases having cleared viremia with those having ongoing viremia at T6 ($n = 11$ in group 1 vs. patients with ongoing viremia groups 2 and 3, $n = 11$; see Figure 1), and at T12 (groups 1, 2, and 4 vs. groups 3 and 5; see Figure 1). However, no significant differences in antibody levels were detected (Figure 2C), indicating that antibody expansion was not significantly associated with viremia clearance.

To investigate the BKPyV-specific cellular immunity, cryopreserved PBMCs from all patients were tested by IFN-γ ELISpot assay after stimulation with standard overlapping 15mPs covering the entire BKPyV LTag (16) or with a 9mP containing 97 immunodominant 9mer EVGR peptides (36). Because comparative data on 15mer- versus 9mer-stimulated IFN-γ ELISpot assay are not available, we noted that the overall responses to 9mP were of equal magnitude to 15mP responses, indicating that the shorter length and lower complexity of the 9mP did not result in a principally reduced response (data not shown).

At T0, no significant differences in between KT patients becoming viremic and their matched nonviremic controls could be detected by either 15mP or 9mP stimulation ($p = 0.1496$ and $p = 0.5703$, respectively). In viremic patients, however, a significant expansion of 9mP responses was observed at T6 and T12 ($p = 0.0005$) (Figure 3A blue dots, left panel), which was not observed in the 15mP responses ($p = 0.2760$) (Figure 3A blue dots, left panel). In nonviremic KT patients, no significant changes in 15mP or 9mP T cell responses were seen ($p = 0.4992$ and $p = 0.906$, respectively) (Figure 3A yellow dots, right panel). The data indicated that 9mP responses showed significant expansion dynamics in viremic patients that were not detectable in nonviremic controls. In contrast, 15mP responses did not reveal similar timing or magnitude.

To investigate the role of T cell responses as indicators of clearance of BKPyV viremia, we compared 9mP and 15mP responses in cases having cleared viremia with those having ongoing viremia. At T6, 9mP responses were significantly higher in patients having cleared viremia (group 1) compared with patients with ongoing viremia (groups 2 and 3) (Figure 3B), whereas the corresponding 15mP responses showed no significant differences. At T12, patients having cleared viremia (groups 1, 2, and 4), had significantly higher 9mP responses than patients with ongoing viremia (groups 3 and 5) (Figure 3C). In contrast, no significant difference was seen in 15mP responses between patients having cleared viremia and those with ongoing viremia.

Although the small sample size precluded more extensive analyses, a receiver operating characteristic curve analysis of 9mP responses predicting clearance of viremia yielded an area under the curve of 0.712 ($p = 0.013$), whereas 15mP responses were not significant at 0.606 ($p = 0.214$) (Figure S1).

Because low or missing ELISpot responses could be due to immunosuppressive drugs or complete lack of BKPyV-specific T cells (24), we investigated the *in vitro* expansion of PBMCs following 15mP stimulation and testing for 15mP and 9mP IFN-γ ELISpots, as described previously (16). With few exceptions, a substantial increase in respective IFN-γ responses was seen in both viremic and nonviremic patients at almost all time points ($p < 0.0001$) (Figure 4A). The results indicated that BKPyV-specific CD8 T cells were present in most patients at the respective time points and suggested that *in vivo* expansion could be readily expected in patients if immunosuppression was relaxed.

To investigate the specificity of the expanded 9mP responses, the 97 immunodominant 9mers were assessed in a checkerboard analysis. Sufficient cell numbers were available for a complete checkerboard of 147 (51%) of the 288 time points (T0, T6, and T12) of the 96 KT patients (62 from the 28 viremic KT patients and 85 from 66 nonviremic controls), yielding 136 (92.5%) positive 9mer responses. A total 78 different 9mer responses were identified, confirming 28 9mers

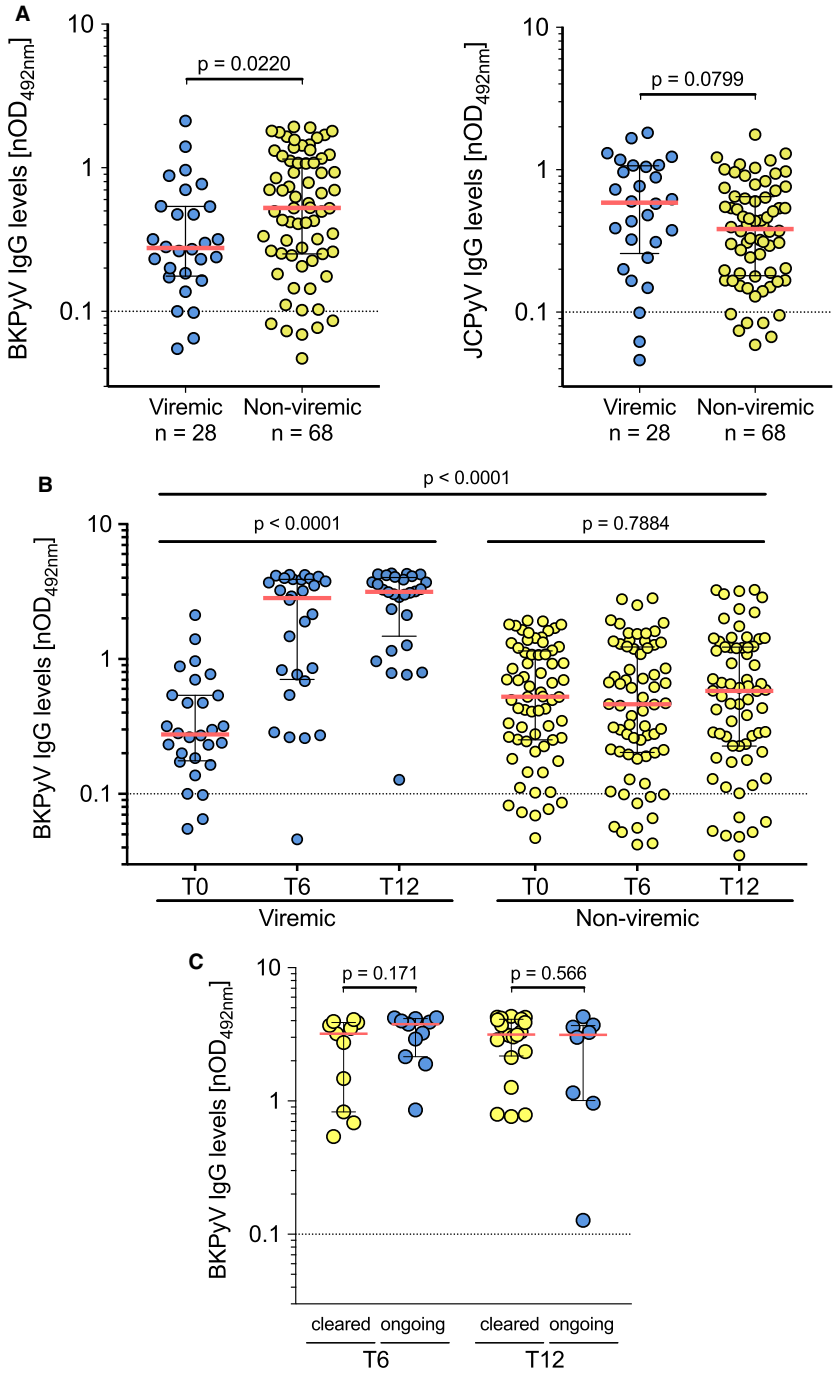


Figure 2: BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) antibody response. Prospectively cryopreserved plasma samples from 28 cases and 68 control kidney transplant (KT) recipients were available at time of transplantation (T0) and at 6 mo (T6) and 12 mo (T12) after transplant and were tested for IgG antibodies by a normalized viral capsid protein 1–based virus-like particle-based ELISA (as described in Patients and Methods). (A) BKPyV- and JCPyV-specific IgG levels at T0 in patients later developing viremia and nonviremic patients (median with interquartile range is shown; Mann–Whitney test, two-tailed). (B) Evolution of BKPyV antibody levels after transplantation (analysis of variance; Kruskal–Wallis, two-tailed). (C) BKPyV-specific antibody levels and clearance of viremia. BKPyV IgG levels were compared in KT patients having cleared viremia were compared with patients with ongoing viremia at T6 (group 1 vs. groups 2 and 3) and T12 (groups 1, 2, and 4 vs. groups 3 and 5); Mann–Whitney test, two-tailed.

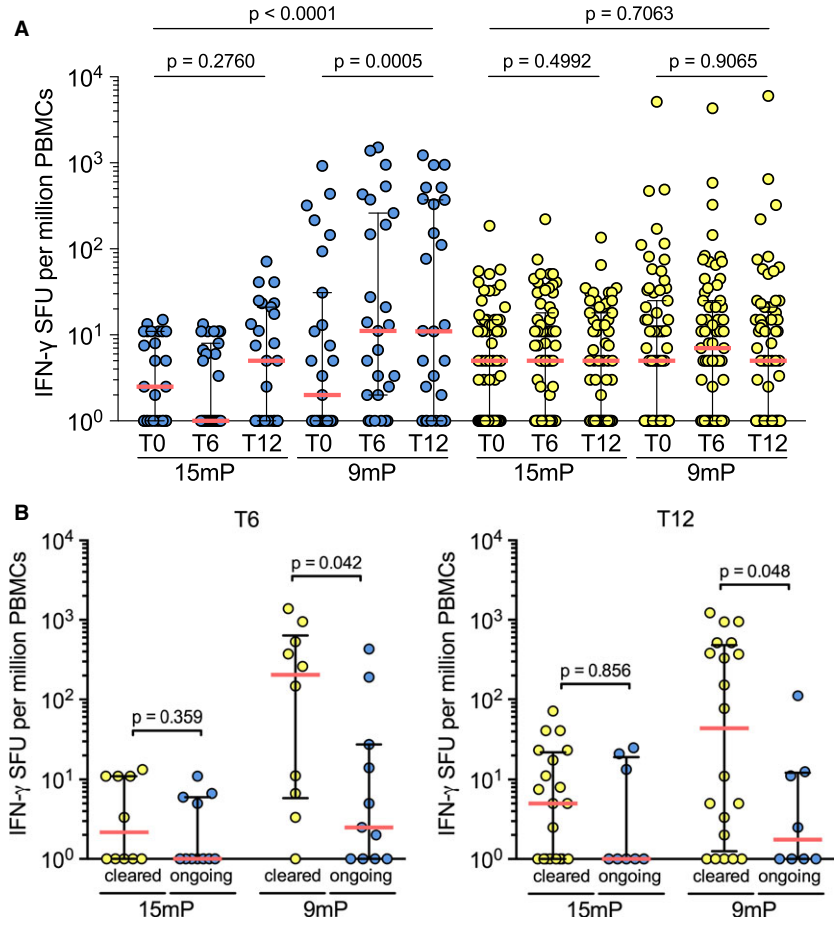
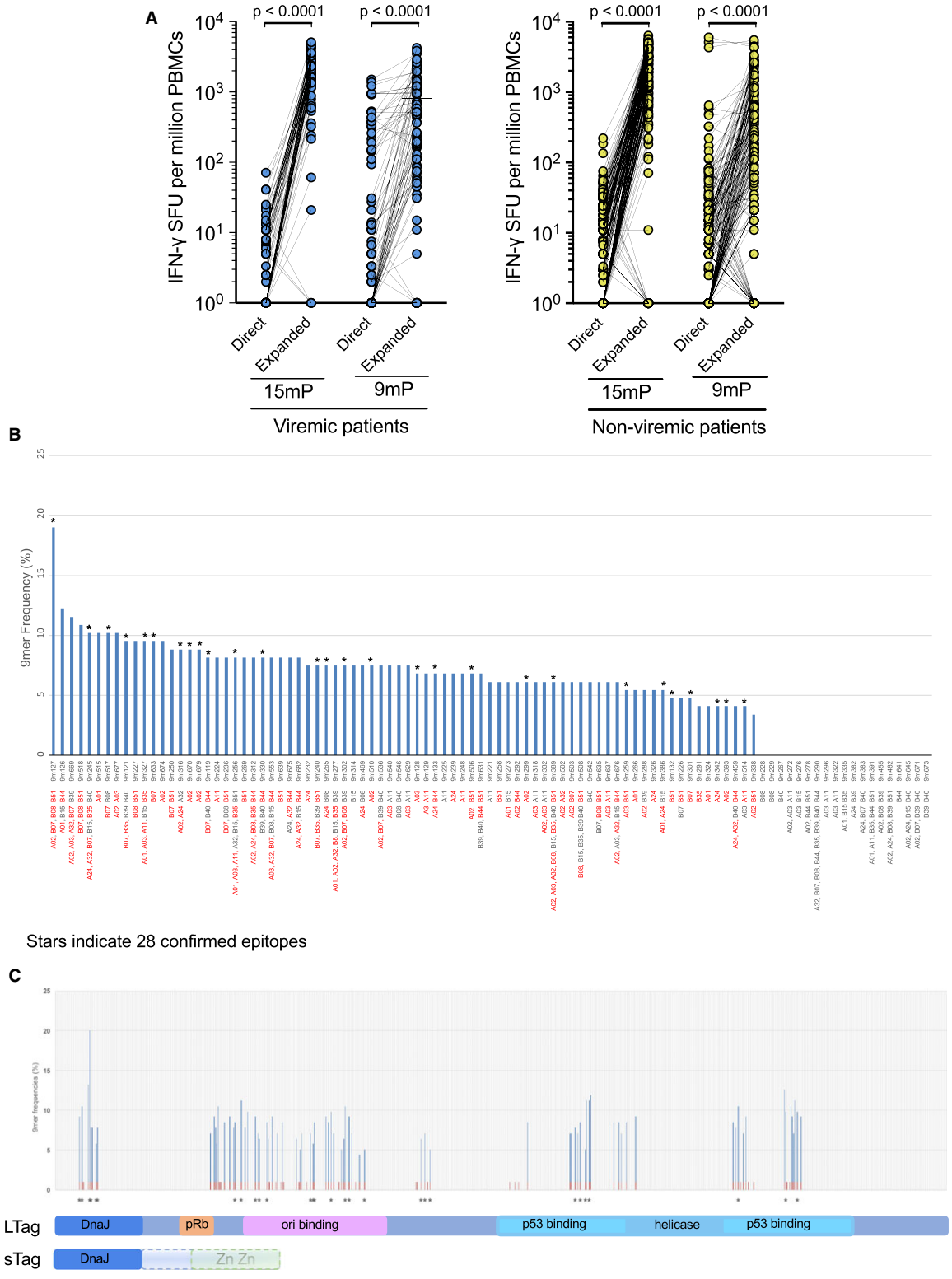


Figure 3: Direct BK polyomavirus (BKPyV) early viral gene region–specific 15mP and 9mP T cell responses in kidney transplant (KT) patients. Peripheral blood mononuclear cells (PBMCs) obtained at the indicated time points were stimulated with 15mer peptide pools (15mPs) or with 9mer peptide pool (9mPs) and tested for interferon γ (IFN-γ) release by enzyme-linked immunospot (ELISpot) assay (see Patients and Methods section). The results are expressed as IFN-γ spot-forming units (SFU) per million PBMCs. Bars indicate median and interquartile range. (A) Overall 15mP- and 9mP-stimulated T cell responses after transplantation. Responses detected in cases (blue dots) and control KT patients (analysis of variance, Friedman; two-sided). (B) BKPyV-specific T cell responses and clearance of viremia at T6 and T12. The 15mP- and 9mP-induced ELISpot responses of KT patients having cleared viremia (yellow dots) were compared with those in patients with ongoing viremia (blue dots). (Left) T6 (group 1 vs. groups 2 and 3, respectively; see Fig. 1). (Right) T12 (groups 1, 2, and 4 vs. groups 3 and 5, respectively; see Fig. 1). Mann–Whitney test, two-tailed. T0, T6, and T12 indicate time of transplantation and 6 and 12 mo after transplantation, respectively.

Figure 4: *In vitro* expansion of BK polyomavirus–specific T cells from viremic and nonviremic patients. Peripheral blood mononuclear cell (PBMC) samples from kidney transplant (KT) patients were stimulated with 15mer peptide pools (15mPs) and expanded *in vitro* for 2 weeks before restimulation and testing for interferon γ (IFN-γ) release by enzyme-linked immunospot assay using15mP and 9mer peptide pools (9mPs), as indicated (see also Patients and Methods). (A) Comparison of direct and *in vitro*-expanded 15mP and 9mP T cell responses (Wilcoxon test, two-sided). (B) Frequency of specific 9mer responses in expanded T cells from 97 adult KT patients. Epitope response mapping was performed in 147 expansions taken as 100%. Hierarchy of 9mer responses (blue bars) and the respective HLA type found (in red) or predicted or not yet encountered in patients (in gray). *9mer response identified previously (36). (C) Distribution of 9mer responses along the coding regions of the early viral gene region (EVGR). Red lines indicate location of predicted immunodominant 9mer epitopes in the EVGR coding region, blue bars indicate frequency of identified 9mer epitope responses. *9mer responses identified previously (36). Schematic drawing of the respective domains in large T antigen (LTag) and small T antigen (sTag; according to DeCaprio and Garcea [35]) are indicated below the diagram to visualize the approximate location of the different 9mer epitopes (DnaJ homology region, retinoblastoma protein binding domain [pRb], origin of DNA replication binding domain [ori], p53 binding domain). Of note, the amino-terminal DnaJ homology domain of sTag is identical to LTag and covered by both the 15mP and the 9mP, whereas the carboxy-terminal sTag-unique Zinc domain generated by alternative splicing is not covered by either pool.



identified previously in healthy donors and pediatric KT patients (Figure 4B, marked with asterisks) (36) and 50 novel ones. The average frequency of the 9mers ranged from 4% to 12% of the 147 checkerboards tested, with the exception of the outstanding 9m127, reported previously (Figure 4B). In most instances, the KT patients

carried the HLA class I type predicted to present the corresponding 9mer, but a few 9mers were not confirmed (Figure 4B). Schematic alignment of the EVGR open reading frame encoding the LTag and the common domain of the sTag (Figure 4C) revealed that most 9mer responses were located in clusters, as expected from the prediction for immunodominant responses (36). Interestingly, several 9mers not eliciting responses were also located in clusters residing in parts of the p53-binding domains of the LTag (Figure 4C).

Discussion

The persisting challenge of BKPyV in KT patients is currently addressed by regular posttransplant screening for BKPyV viremia and preemptive reduction of immunosuppression (14,19,20). Despite preferential manifestation during the first year after transplant, actual onset and duration of BKPyV viremia is highly variable in individual patients (5) (Figure 1). To better understand the virus–immune dynamics in KT patients, we conducted a case-control study of 96 adult KT patients participating in the STCS, from whom prospectively collected data and biobanking at T0, T6, and T12 were available (40). Our results demonstrate, for the first time, that a pool of immunodominant 9mers from the BKPyV EVGR elicits IFN-γ ELISpot responses in adult KT patients and provides functional information that differs from 15mP responses in both dynamics and magnitude. Indeed, the 9mP responses showed significant increases in BKPyV viremic patients at T6 and T12 that were not seen in nonviremic control patients. Importantly, higher 9mP CD8 T cell responses were associated with clearance of viremia at both T6 and T12.

We also investigated BKPyV-specific antibody responses and found significant increases at T6 and T12 in viremic cases that were not observed in nonviremic controls; however, unlike the 9mP CD8 T cell responses, the BKPyV-specific antibody levels were not correlated with clearance of viremia. These observations suggest that BKPyV-specific antibodies are mainly a marker of past and recent viral exposure, as has been reported in great detail previously (27,39,41). This interpretation is also supported in a prospective study of pediatric KT patients, demonstrating that increasing BKPyV antibody levels occurred early in patients with rising viremia before immunosuppression was reduced and prior to mounting of significant T cell immune responses (17). The notion that T cell effector functions are key to the control of BKPyV replication has been reviewed recently (28) and is well illustrated by cases with recurrence of initially cleared BKPyV viremia in which the reconstituted IFN-γ BKPyV-specific T cell responses disappeared despite persistently increased high antibody levels (see also Figure 1 in Comoli et al [41]). Given the conserved nature of the LTag between BKPyV and JCPyV, it is important to note that the JCPyV IgG levels were not different between patients groups at T0, suggesting that cross-reacting JCPyV-specific T cell immunity (16,36) was not a major confounder in our study.

Recently, Schmidt et al (42) observed that higher BKPyV IgG levels were associated with stronger BKPyV-specific CD4 T cell activity in 15 postviremic adult KT patients compared with 24 nonviremic adult KT patients, using overlapping 15mPs covering the LTag and the Vp1 epitopes. Our present study of 96 KT patients focused on overlapping 15mers covering EVGR-encoded LTag and sTag epitopes, thereby deliberately avoiding the prominent CD4 T cell responses to the Vp1 capsid epitopes. Indeed, in a study of 42 adult KT patients tested before and after clearing BKPyV viremia, Binggeli et al (16) demonstrated that Vp1-specific IFN-γ responses were significantly stronger than the LTag-induced responses and contained a larger proportion of CD4 T cells, whereas the opposite was seen for LTag-specific responses having a higher CD8 T cell proportion (16). This observation was independently confirmed in pediatric and adult KT patients (17,18). Recently, CD8 T cell responses to LTag or Vp1 have received more attention in exploratory concept studies (32,33), but translation and clinical utility still need to be addressed. The difference between 9mer and 15mer responses in KT patients also need further study, but conceptually and shown by using MHC class I streptamers (36), 9mers are presented in an MHC class I context and recognized by CD8 T cells. Instead, 15mers bind to MHC class II–detecting CD4 T cells and, to a lesser extent, to MHC class I–stimulating CD8 T cells (16,17). Another potential factor is the change in 9mer responses over time and relative to viremia; these changes need further study to clarify the expansion and contraction dynamics of CD8 T cells in the peripheral blood of KT patients.

We also showed that T cell responses to BKPyV EVGR 9mP and 15mP are expandable *in vitro* in most viremic and nonviremic patients at the different time points, even though not detectable by direct testing in some instances. The expanded T cells may be important for the development of novel strategies aiming to boost BKPyV-specific T cell responses in KT patients and suggest the possibility of exploring autologous adoptive T cell therapy with respect to both CD4 and CD8 T cell effectors. However, the "net state of immunosuppression" will remain a major obstacle even after adoptive T cell transfer, due to the need for a window period of reduced immunosuppression with its inherent risks.

To characterize the specificity of the expanded T cells, we tested the 97 individual 9mers of the 9mP in a checkerboard analysis. Complete results were obtained in 136 of 147 expansions (92.5%) with sufficient cell numbers after 2 weeks to identify 78 individual 9mers, including 50 novel ones. With few exceptions, the responses were found in patients having the HLA type predicted to present these

immunodominant BKPyV 9mers. Interestingly, 17 of the predicted 9mers had not been identified repeatedly, even though the HLA types are not particularly rare in our population. However, several of the nonresponding peptides clustered in domains of the p53-binding region of the BKPyV LTag. This observation is intriguing and suggests the testable hypothesis that certain epitopes predicted to be immunodominant may be underrecognized because of tolerizing clonal deletion resulting from high similarity/identity with the hosts' self antigens. Conversely, this may also help to identify 9mer epitopes that are associated with better viremia clearance.

Even though this study is one the largest of KT patients combining BKPyV loads and specific humoral and cellular immunity in a proper case-control design of prospectively collected cohort information and specimens (40), the availability of 3 time points (T0, T6, and T12) must be acknowledged as a limitation for fine mapping. Moreover, some patients seemed to have delayed viremia clearance, suggesting that other, as yet undefined factors play a role. We conclude, however, that the data are encouraging and warrant follow-up with fine mapping of the type and role of antibody and T cell responses at critical time points of reduced immunosuppression and may permit a randomized controlled interventional trial for ultimate clinical evidence. Such a trial should address the testable hypothesis that a strong 9mer response in a patient clearing or having cleared viremia does not need further reduction in immunosuppression. Another important question would be whether or not immunosuppression could be safely reincreased in such patients. Although the present ELISpot format is convenient for the clinical situation, more sophisticated assays would be needed to research details on the role of characterizing T cell cytokine profiles, polyfunctionality, and activation/differentiation markers (28). Regardless, our results on immunodominant 9mP responses raise significant interest in CD8 T cell dynamics and complement other recent studies limited to HLA-A0201 restricted responses (32,33).

In conclusion, we provide evidence that 9mP responses may be a novel dynamic marker of reconstituting CD8 T cell function across a range of common HLA types that is directly testable in peripheral blood and thus warrants further studies exploring whether 9mP responses permit complementation of plasma BKPyV load results in guiding immunosuppression reduction.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Specificity and sensitivity of 15mer- and 9mer peptide pool–elicited BK polyomavirus–specific T cell responses for clearance of viremia. Receiver operating characteristic curve analysis.